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Mechanisms of Transmissible Gastroenteritis Coronavirus Neutralization

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Transmissible gastroenteritis virus (TGEV) was neutralized more than 10^9-fold with antibodies of a single specificity [monoclonal antibodies (MAbs)]. Most of the virus was neutralized in the first 2–3 min of a reversible reaction, which was followed by a second phase with a decreased neutralization rate and, in some cases, by a persistent fraction, which was a function of the MAb and of the antibody-to-virus ratio. Neutralization of TGEV is a specific event that requires the location of the epitope involved in the neutralization in the appropriate structural context, which is present in the wild-type virus but not in certain MAb escaping mutants. In neutralization of TGEV by binary combinations of MAbs specific for the same or for different antigenic sites, either no cooperation or a synergistic effect, respectively, was observed. Mechanisms of TGEV neutralization by MAbs were characterized at high, intermediate, and low antibody-to-virus ratios. Under these conditions, mainly three steps of the replication cycle were inhibited: binding of virus to the cell, internalization, and a step that takes place after internalization. In addition, virus aggregation could be responsible for the neutralization of 10 to 20% of virus infectivity.

INTRODUCTION

Porcine transmissible gastroenteritis (TGE) is an enteric disease of swine associated with a high mortality in neonates, caused by a coronavirus that consists of a single-stranded positive-sense RNA molecule of 26–30 kb and three structural proteins S, N, and M, with 1447, 382, and 262 amino acids, respectively (Siddell *et al.*, 1983; Sturman and Holmes, 1983; Saif and Bohl, 1986; Kapke and Brian, 1986; Laude *et al.*, 1987; Raschaert and Laude, 1987). In coronaviruses the S protein is responsible for the induction of neutralizing antibodies (Garwes *et al.*, 1976, 1978; Sturman and Holmes, 1983). Studies on the antigenic structure of prototype coronaviruses (mouse hepatitis virus, infectious bronchitis virus, and TGEV) have shown that at least two critical sites are involved in their neutralization (Talbot *et al.*, 1984; Wege *et al.*, 1984; Mockett *et al.*, 1984; Fleming *et al.*, 1986; Delmas *et al.*, 1986). In TGEV only the S glycoprotein has been involved in virus neutralization in the absence of complement. In this protein, a minimum of seven antigenic sites have been defined; site A is the major inducer of neutralizing antibodies, although other sites (B and D) (Correa *et al.*, 1988), less immunogenic, are also involved in TGEV neutralization (Enjuanes *et al.*, 1990; Delmas *et al.*, 1986; Correa *et al.*, 1990; Posthumus *et al.*, 1990). In addition, antibody-mediated complement-dependent virus neutralization has been reported with M-specific monoclonal antibodies (MAbs) (Woods *et al.*, 1987; Laude *et al.*, 1986).

Virus neutralization can be achieved by inhibition of any step of virus replication (Dimmock, 1984, 1987). In TGEV, very little is known about the mechanisms of neutralization. One report (Nguyen *et al.*, 1986) has suggested that the neutralization of TGEV by secretory IgA from milk or IgG from serum is due to inhibition of the internalization of the adsorbed virus, but not to blockage of virus binding to the host cell.

In this paper, we characterize the mechanisms for neutralization of TGEV by monoclonal and polyclonal antibodies, at high, intermediate, and low antibody-to-virus ratios.

MATERIALS AND METHODS

Cells and virus. The epithelial swine testicle cell line ST developed by McClurkin and Norman (1966) was used to grow the Purdue strain of TGEV (PUR46) (Bullido *et al.*, 1988; Sánchez *et al.*, 1990), which was plaque purified (Jiménez *et al.*, 1986).

Virus neutralization and purification. Two procedures were followed in the plaque reduction assay. In one, a fixed amount of TGEV PFU were incubated with the antibody, and dilutions of the virus–antibody mixture were plaqued. In the second, 10-fold dilutions of the virus were incubated with a fixed amount of antibody, and the mixtures were applied to the cells without further dilution. Both plaque reduction assays were performed by incubating, at 37° for 30 min (unless otherwise indicated), the TGEV in 50 μl of phosphate-

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buffered saline (PBS) containing 2% fetal calf serum and 1 vol of the MAb or antiserum dilution. Portions containing 50 μl of each mixture were applied to cell monolayers grown in 24-well microplates. After 1 hr of virus adsorption, the inoculum was replaced with medium containing 2% fetal calf serum, 40 μg of DEAE-dextran/ml, and 0.1% agarose, and the cells were incubated at 37° for 2 days in a humidified CO_2 incubator. Cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet, and the plaques were counted. The extent of neutralization was expressed by the neutralization index (NI), which is the log of the ratio of the number of PFU of virus to that of control medium or antibody.

[^{3}H]Leucine- or [^{35}S]methionine-labeled TGEV was grown in Dulbecco's modified Eagle (DME) medium completely or partially depleted of leucine or methionine, respectively, in the presence of fetal calf serum dialyzed against PBS, and 20 μCi of [^{3}H]leucine (177 Ci/mmol, NET-460, New England Nuclear) or 20 μCi of [^{35}S]methionine (800 Ci/mmol, SJ204, Amersham), respectively, per milliliter.

TGEV was purified through two sucrose gradients in 1 *M* NaCl, the first one containing 0.2% Tween 20, as previously described (Correa *et al.*, 1988).

Radioimmunoassay. Binding of the antibody to the virus was determined in a radioimmunoassay (RIA) in which TGEV (0.25 μg of protein per well in 50 μl of PBS) was adsorbed to polyvinyl disposable flat-bottom plates (Titertek, Flow Laboratories) by overnight incubation at 37°. The subsequent steps of the assay were performed as described by Sanz *et al.* (1985), using a rabbit antiserum to mouse immunoglobulins to amplify the reaction and ^{125}I-labeled protein A to develop the assay. The titer in the RIA was defined as the maximum antibody dilution that bound threefold the background radioactivity.

Polyclonal antiserum. TGEV-specific swine antiserum was induced by immunizing 6-week-old minipigs (haplotype cc) (Sachs *et al.*, 1976) with three doses of 35 μg of purified TGEV, in complete Freund's adjuvant, incomplete adjuvant, and saline solution. The serum had a titer by RIA of 78,125 and its NI was >7.1.

Monoclonal antibodies and "mar" mutants. MAbs and MAb-resistant (*mar*) mutants were produced and characterized as described previously (Jiménez *et al.*, 1986; Correa *et al.*, 1988). The properties of the MAbs used in this study are summarized in Table 1. The *mar* mutants isolated were neutralized less than $10^{0.5}$-fold by the homologous MAb, which neutralized the original virus more than $10^{4.8}$-fold.

Kinetics of the neutralization. Hybridoma culture supernatants were titrated in the RIA and diluted to the same titer (2000). Samples (200 μl) of each hybridoma

TABLE 1

CHARACTERISTICS OF THE TGEV-SPECIFIC MABS USED IN THIS STUDY

MAb	Specificity[a]	Isotype
6A.C3	S Ac	γ_1 k
1B.H6	a	γ_{2a} k
1G.A7	a	γ_1 k
1D.E7	b	γ_1 k
1D.B3	a	γ_{2a} k
1B.B1	a	γ_1 k
1C.C12	a	γ_{2a} λ_1
1B.C1	a	γ_1 k
1G.A6	a	γ_1 λ_1
1A.F10	c	γ_{2a} k
1H.D2	b	γ_1 k
1E.F9	a	γ_1 k
1B.B5	c	γ_{2b} k
1D.E8	b	γ_1 k
1E.H8	a	γ_1 k
1H.C2	ND[b]	γ_1 k
1B.H11	S B	γ_1 k
1D.B12		γ_1 k
8F.B3		ND
5B.H1	S C	γ_{2a} k
6A.A6		γ_1 k
1D.G3	S D	γ_1 k
8D.H8		ND
1H.B1	S ND	μ k
1B.G4		μ k
1C.H6		γ_1 k
1H.G5		γ_1 k
3B.D3	M	γ_1 k
3D.H10	N	γ_1 k

[a] The first and second capital letters indicate the virus protein and the antigenic site, respectively, and the small letter indicates the subsite (Correa *et al.*, 1988).

[b] Not determined.

were equilibrated at 37° and a volume of the virus (5×10^7 PFU) was added to each MAb. At the indicated times, 20-μl samples were diluted 100-fold in PBS with 2% fetal calf serum at 4° and immediately titrated on ST cells.

Binding of TGE virus to ST cells. ST cells were grown to confluence in flat-bottom 96-well microplates (Falcon Plastics). All remaining steps were performed at 4°. Nonspecific binding sites were blocked by incubating each well for 15 min in the presence of 200 μl of PBS containing 5 mg of bovine serum albumin (BSA) per milliliter. The blocking solution was removed, and 100 μl of ^{3}H-labeled purified TGEV (1×10^4 to 2×10^4 cpm, 1.9×10^4 cpm/μg of protein, 1×10^8 PFU/μg) was added per well in DME medium (with no bicarbonate), supplemented with 2 m*M* glutamine, 50 μg of gentami-

cin/ml, 50 m*M* Hepes, and 10% fetal calf serum, pH 7 (binding medium). The microplates were incubated for 120 min or the indicated times. Unbound virus was collected and the wells were washed three times with 200 μl of 2% fetal calf serum in PBS. Cell-bound radioactivity was solubilized with 100 μl of 1% Nonidet P-40 in PBS per well, samples of this material and of the supernatants were precipitated with 5% trichloroacetic acid, and the radioactivity was evaluated by liquid scintillation techniques.

To determine the inhibition of binding of virus to ST cells induced by MAbs, the assay was performed as described, except that samples of 50 μl with the indicated amounts of each antibody were incubated at 4° for 15 min with 1 vol of the labeled virus. Then, the mixture was incorporated into the wells. The percentage of specific binding, that is, the binding of labeled virus to the cells that was inhibited by unlabeled virus, obtained in the presence of an inhibitor MAb was calculated with the formula

$$\% \text{ binding} = \frac{\text{cpm}_c - \text{cpm}_o}{\text{cpm}_t - \text{cpm}_o} \times 100$$

cpm_c and cpm_t are the radioactivity associated with the cells in the presence and the absence of inhibitor MAb, respectively; and cpm_o is the radioactivity associated with the cells in the presence of a 40-fold excess of unlabeled virus. The radioactivity bound, within the different batches of virus, ranged from 10 to 40% of the total labeled virus added to the cells. This amount was taken as the reference against which to calculate the binding inhibition by different antibodies or competitor virus.

Internalization of TGEV in ST cells. Internalization of TGEV (or its binding to ST cells, when it was correlated with internalization) was studied on ST cell monolayers grown on 24-well microtest plates (Costar). Mixtures of ^{35}S-labeled purified virus (1.5×10^4 cpm, 1.2×10^4 cpm/μg, 1.4×10^8 PFU/μg) and antibody dilutions in 100 μl of binding medium were incubated at 4° for 15 min, added to each well, and incubated at 37° for 60 min (unless otherwise indicated), on a rocking platform, in a CO_2 incubator. The plates were cooled on ice, the supernatants were collected, the cells were washed once with binding medium, and the supernatants were combined. To determine the amount of cell-associated virus, the monolayers were rinsed twice more with binding medium. Cell-associated radioactivity was determined by solubilizing the monolayers with 1% Nonidet P-40 in PBS. TCA-insoluble radioactivity was determined in samples from the supernatants as well as from the cell monolayers.

To determine the internalized virus, after it was bound to the cells, the monolayers were washed once with 40 m*M* citric acid, 10 m*M* KCl, 135 m*M* NaCl, pH 3, at 4° for 1 min and immediately washed a second time with binding medium (Highlander *et al.*, 1987), and the radioactivity was determined as indicated above.

RESULTS

TGEV neutralization by MAbs. The majority of TGEV was neutralized (Fig. 1) by individual murine MAbs in the first 2–3 min of the reaction, following an exponential decline in surviving virus, which was followed by a second-phase reaction with a decreased rate of neutralization, leaving in some cases a persistent fraction that remained constant after 2 hr of incubation (results not shown). The extent of neutralization in the first minutes of the reaction was similar to that obtained (Fig. 1) when the virus adsorption period was reduced to 5 min. Extent of virus neutralization was a function of the MAb (Fig. 1A) and, for each MAb, it was related to its concentration (Fig. 1B). Culture supernatants containing MAb 1E.F9 neutralized virus infectivity 100-fold, while 10^{-1} or 10^{-3} dilutions of this MAb reduced infectivity 10-fold or virtually not at all, respectively. Neutralization of TGEV by MAbs was reversed between $10^{2.7}$- and $10^{6.1}$-fold (Table 2) by diluting the virus–antibody complexes and incubating to allow their dissociation, which indicates that TGEV neutralization is a reversible process.

Characterization of TGEV and of 11 *mar* mutants derived from it, by RIA and neutralization using MAbs (Fig. 2), showed that some MAbs (for example, MAb 1B.C1) that bound and neutralized the original strain of TGEV also bound, but did not neutralize, the *mar* mutants, indicating that neutralization requires more than simple binding of the antibody to the critical epitope.

To determine if MAbs specific for the different antigenic subsites involved in TGEV neutralization cooperated in this process, inactivation by all possible binary combinations of saturating amounts of six MAbs, which bind to three antigenic subsites (subsite Aa: MAbs 1G.A6, 1C.C12, and 1B.C1; subsite Ab: MAb 1D.E8; subsite Ac: MAbs 1A.F10 and 6A.C3) was studied. The neutralization by any pair of MAbs tested resulted in a nonsignificant cooperation. In contrast, when site A-specific MAbs (1G.A7, 1B.C1, 1D.E8, 1A.F10, and 6A.C3) were combined with MAb 5G1 specific for a site different from site A (R. Woods, R. Wesley, and L. Enjuanes, unpublished results), a synergistic effect was observed, which produced an increase of 2 in the NI above the NI obtained with the best neutralizing MAb individually used (results not shown).

Relationship between TGEV neutralization and inhibition of virus binding to cells. Virus binding to ST cells

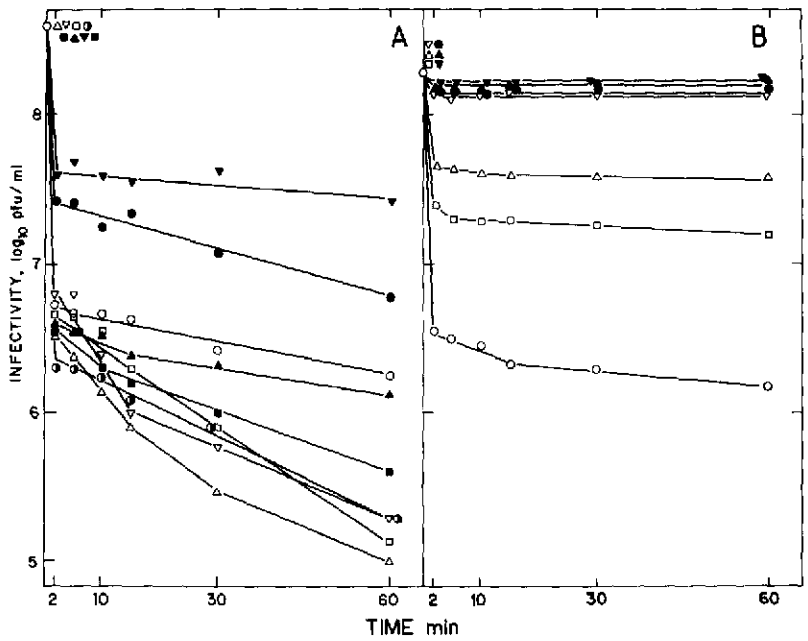

FIG. 1. Kinetics of TGEV neutralization by MAbs. Neutralization of TGEV by hybridoma supernatants diluted to have a titer in RIA of 2000 (A), containing the following MAbs: ▼, 1H.C2; ●, 1E.F9; ○, 1B.B1; ▲, 1G.A7; △, 6A.C3; □, 1C.C12; ◑, 1G.A6; ▽, 1B.H6; ■, 1B.C1. Kinetics of TGEV neutralization by dilutions of MAb 1E.F9, starting from the dilution with a titer of 2000 in RIA (B): ▼, 10^{-6}; ▲, 10^{-5}; ●, 10^{-4}; ▽, 10^{-3}; △, 10^{-2}; □, 10^{-1}; and, ○, 10^{0}.

was saturable, reaching a plateau 90–120 min after the reaction was started (Fig. 3A), when free virus was still available. The binding was specific, as it was inhibited up to 90% by a 40-fold excess of unlabeled virus, but not by similar amounts of serologically unrelated viruses, such as mouse hepatitis coronavirus (MHV) or Moloney leukemia virus (Fig. 3B). A possible relationship between inhibition of virus binding to ST cells and its neutralization was studied with 26 MAbs specific for the S protein, which were tested as *undiluted* culture supernatants. The binding of the virus to the cells (Table 3) was best inhibited by site A-specific MAbs (78–96%), followed by site D (53–55%)-, site B (9–39%)-, and site C (24–26%)-specific MAbs. As expected, the M- and N-specific MAbs did not inhibit binding. A general direct correlation between the blocking activity of a MAb and its capability to neutralize the virus was observed (Table 3). MAb 1B.G4 has low neutralizing activity (NI 0.3) and inhibited binding 65%, possibly because it is large (IgM isotype), and not as a consequence of the proximity of the epitope bound by the MAb to the determinant recognized by the cellular receptor.

TABLE 2

REVERSIBILITY OF TGEV NEUTRALIZATION BY DILUTION

MAb	Neutralization index[a]		
	Virus–Ab mixture titrated immediately	Virus–Ab mixture titrated after dilution and incubation	Decrease in neutralization index[b]
6A.C3	>8.7	2.6	>6.1
1B.C1	7.0	3.1	3.9
1G.A6	6.2	2.7	3.5
1B.B1	6.1	2.1	4.0
1C.C12	6.0	2.7	3.3
1G.A7	5.6	2.3	3.3
1B.H6	5.5	2.8	2.7
1H.C2	5.5	1.0	4.5
1E.F9	4.5	1.6	2.9

[a] The NI was determined either immediately after neutralizing the virus or after diluting 100-fold the virus–antibody mixture in PBS with 2% fetal calf serum and incubating for 3 hr at 4°.

[b] The decrease in the NI was determined by subtracting the NI of the virus–antibody mixture diluted 100-fold and incubated for 3 hr to allow dissociation of the complex, from the NI for the mixture immediately titrated.

The effect of the antibody-to-virus ratio on TGEV neutralization and on inhibition of virus-to-cell binding was studied (Figs. 4A, B). Interestingly, MAbs specific for two critical antigenic subsites of S protein did not inhibit at all the binding of TGEV to ST cells at 4°, at concentrations at which they neutralized the virus infectivity 10^5-fold, indicating that the two activities of the MAbs (binding inhibition and neutralization) can be split.

Internalization and neutralization of TGEV. The binding of ^{35}S-labeled TGEV to ST cells reached a plateau

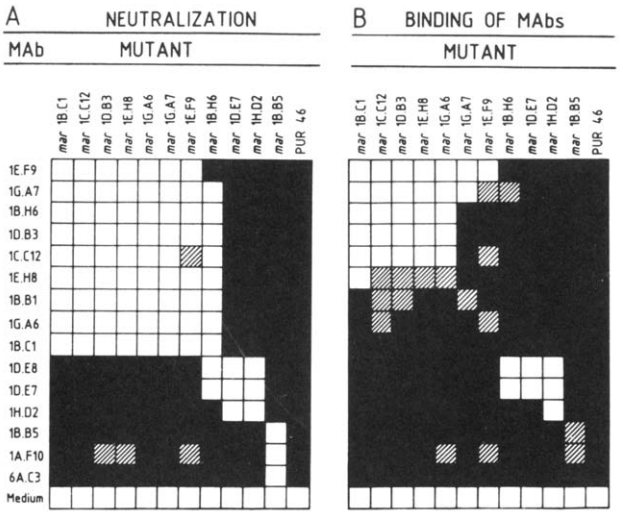

FIG. 2. Characterization of TGEV-derived "*mar*" mutants by neutralization and binding using MAbs specific for the original TGEV. The neutralization of TGEV *mar* mutants (A) was determined in a plaque reduction assay with 10^2 and 10^4 PFU of each mutant, following the standard procedure (see Materials and Methods). White, hatched, and black squares indicate NI of <1, between 1 and 2, and >2, respectively. To study the binding of virus-specific MAbs to the *mar* mutants (B), the standard RIA (see Materials and Methods) was performed. Binding to the mutants was expressed as a percentage of the binding to the original virus (PUR46 clone). White, hatched, and black squares indicate 0–20, 21–40, and 41–100% binding, respectively.

after 1–2 hr of incubation at 37° (Fig. 5A). Virus internalization proceeded with 15 to 30 min of delay and reached the maximum 2 hr later. Virus internalization was temperature dependent (Fig. 5B), being maximal at 37°. More than 90% of the virus was internalized through a specific receptor, as this process was efficiently inhibited by a 40-fold excess of unlabeled virus and by site A-specific MAbs (i.e., 1C.C12), but not by MAbs specific for other sites (i.e., 6A.A6) (Fig. 5B).

TGEV neutralization at 37° was observed (Fig. 6) at low antibody concentrations (dilutions between 1 in 64 and 1 in 256) which only slightly prevented internalization of the virus and which increased virus binding to cells. At intermediate antibody concentrations (dilution of 1 in 32), the antibody inhibited mainly the internalization, with little blocking of the binding. At high antibody concentrations (dilutions lower than 1 in 4), both internalization and binding were inhibited and extensive (between 10^2- and 10^6-fold) neutralization was observed.

MAbs specific for three subsites of S glycoprotein still neutralized 75 to 97% of the virus after it was cell associated (Table 4). Under these conditions about 10% of the virus was not exposed at the cell surface since it was not detachable with a low-pH buffer (see Materials and Methods) (Fig. 5B), which may be the cause of the incomplete neutralization of cell-associated virus.

To determine if there was a correlation between inhibition of binding and internalization, and the neutralization of TGEV by polyclonal antibodies, the effect of different antiserum concentrations on these parameters was determined. The results (Fig. 7) showed that the inhibition of internalization and that of binding were the same, and that extensive (10^2-fold) neutralization was observed at antibody dilutions (i.e., 1 in 80) for which no inhibition of either binding or internalization was observed. Furthermore, at higher dilutions (between 1 in 80 and 1 in 640), at which the antiserum produced an increase in binding and in internalization of the virus (Fig. 7), significant neutralization was observed. Although dilutions between 1 in 80 and 1 in 640 of the control serum induced a small increase in the binding of the virus, no increase in its internalization and neutralization were noted.

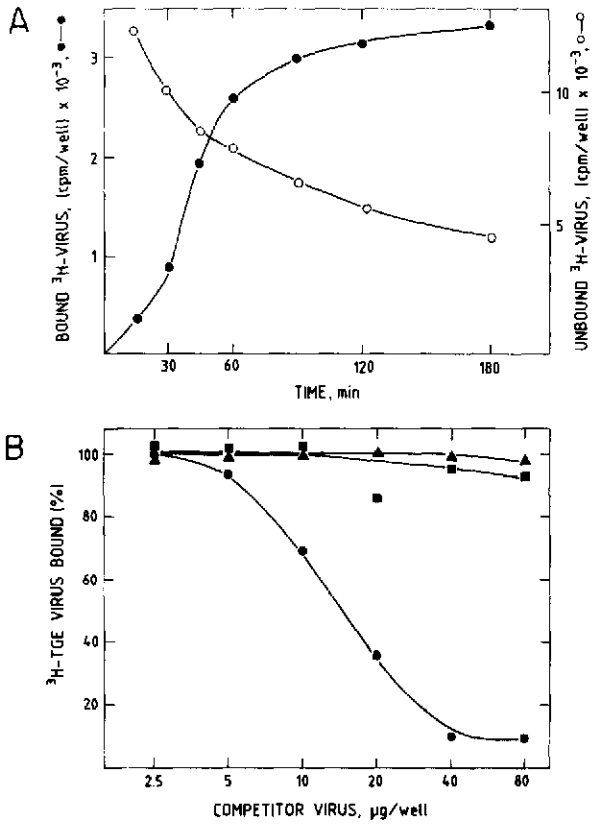

FIG. 3. Kinetics of TGEV binding to ST cells and specific inhibition of this binding. (A) Monolayers of ST cells were grown in 96-well microplates, and the binding of ^{3}H-labeled purified TGEV was determined after incubation at 4° for the indicated times (see Materials and Methods). Bound virus, ●; unbound virus, O. (B) Binding of ^{3}H-labeled virus to ST cells was performed as indicated in (A), in the presence of unlabeled purified TGEV (●), the antigenically unrelated mouse hepatitis coronavirus (▲), or the Moloney leukemia virus (■).

TABLE 3

RELATIONSHIP BETWEEN NEUTRALIZATION AND BINDING INHIBITION BY TGEV-SPECIFIC MAbs

MAb	Specificity	Binding inhibition[a] (%)	Neutralization index
6A.C3	S A	96 ± 5[b]	>8.7
1B.H6		96 ± 5	5.5
1G.A7		95 ± 2	5.4
1D.E7		94 ± 1	6.0
1D.B3		93 ± 4	5.6
1B.B1		92 ± 8	6.1
1C.C12		92 ± 7	6.1
1B.C1		91 ± 6	6.1
1G.A6		91 ± 1	6.1
1A.F10		90 ± 4	1.8
1H.D2		89 ± 5	6.0
1E.F9		88 ± 6	4.5
1B.B5		86 ± 4	2.5
1D.E8		85 ± 4	3.5
1E.H8		78 ± 17	4.0
1B.H11	S B	39 ± 11	0.3
1D.B12		37 ± 27	0.3
8F.B3		9 ± 13	0.3
5B.H1	S C	26 ± 8	0.3
6A.A6		24 ± 16	0.3
1D.G3	S D	55 ± 10	0.3
8D.H8		53 ± 2	1.0
1H.B1	S ND[c]	72 ± 1	1.6
1B.G4		65 ± 15	0.3
1C.H6		9 ± 13	0.3
1H.G5		0	0.3
3B.D3	M	4 ± 2	0.3
3D.H10	N	2 ± 5	0.3

[a] Binding of ^{3}H-labeled purified TGEV to ST cell monolayers was performed at 4° and was inhibited by hybridoma supernatants containing the indicated MAbs.

[b] ± standard deviation.

[c] ND, not determined.

DISCUSSION

It has been shown that TGEV can be neutralized by antibodies of a single specificity, in a reversible reaction, which is followed by a second phase with a decreased neutralization rate and, in some cases, by the onset of a persistent fraction. In addition, the predominant mechanisms of TGEV neutralization have been determined. Neutralization of TGEV by MAbs occurs rapidly (2–3 min). Adsorption of the virus was performed after diluting 100-fold the antibody–virus mixture, and it was shown (Table 2) that the neutralization of TGEV by the MAbs used could be reversed by incubating the diluted mixtures, and never increased. In fact, when the time of adsorption was reduced to 5 min, no significant decrease in the extent of neutralization was observed.

The critical epitopes must be in a certain structural context for an effective neutralization (Fig. 2), since although certain epitopes were present in some *mar* mutants, as demonstrated by the binding of MAbs to these viruses (Fig. 2B), they were not neutralized (Fig. 2A), in contrast to the neutralization of the wild-type virus. Probably the structure of the antigenic subsite is changed in the mutant. A similar phenomenon has been described for poliovirus, in which the loss of the neutralization function of an epitope does not necessarily result in loss of its antibody-binding capacity. Such epitopes binding MAbs that neutralize the wt virus, but which do not neutralize the *mar* mutants, exist naturally on Mahoney and Sabin 1 viruses (Blondel *et al.*, 1986).

The neutralization of TGEV by the MAbs was reversed by dilution (Table 2). In contrast, the neutralization of poliovirus is apparently an irreversible process which is not reversed by dilution (Icenogle *et al.*, 1983). These results probably reflect the avidity of the antibodies used in each case, as the neutralization reaction is reversible in most viral systems (Mandel, 1979).

Very little cooperation in TGEV neutralization was observed between site A-specific MAbs, even when MAbs reacting to different antigenic subsites (Correa *et al.*, 1988) were used. This result seems logical, as the binding of a site A-specific MAb prevented the binding of a second one specific for the same site. In contrast, a synergistic effect was observed between MAbs binding to different sites involved in neutralization (results not shown), as could be expected for MAbs which did not interfere in their binding to the virus.

Coronaviruses, such as mouse hepatitis virus and infectious bronchitis virus, attach to host cells through the S glycoprotein (Sturman and Holmes, 1983; Cavanagh and Davis, 1986; Holmes *et al.*, 1989). Similarly, this must also be the case for TGEV, as MAbs specific for the S glycoprotein, but not MAbs specific for the N or M protein, inhibited the binding to ST cells. MAbs specific for antigenic sites A and D were the best inhibitors of binding (Table 3), suggesting that the domain recognized by the cellular receptor on ST cells must be located spatially close to these sites.

To study the relationship between neutralization and binding inhibition by TGEV-specific MAbs (Table 3), it was essential to show first that the binding of virus to ST cells was specific. Three types of results indicated that this was the case: (i) the binding was saturable (Figs. 3A and 5A); (ii) binding of ^{3}H-labeled or of ^{35}S-labeled virus was inhibited up to 90% by a 40-fold higher concentration of the unlabeled one, but not by the serologically unrelated mouse hepatitis coronavi-

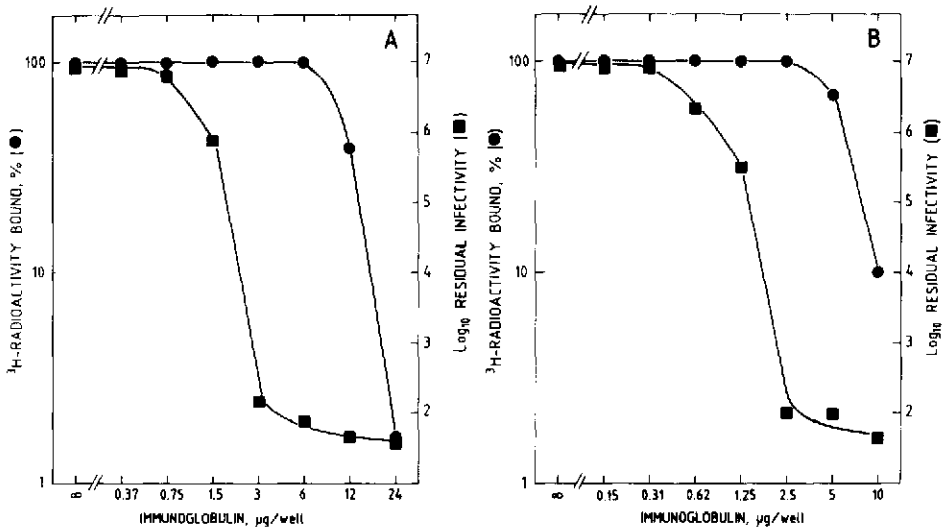

FIG. 4. Relationship between neutralization of TGEV and inhibition of its binding to cells. Binding inhibition and neutralization of ^{3}H-labeled purified TGEV (see Materials and Methods) by MAbs 1C.C12 (A) and 6A.C3 (B) were determined using the same virus preparation. The radioactivity bound (●) was determined by mixing the indicated dilutions of hybridoma supernatants with [^{3}H]TGEV and incubating at 4° for 15 min, adding the mixture to ST cell monolayers, and then incubating for 2 hr at 4°. Residual infectivity (■) was determined after similar treatment, by infecting ST cell monolayers with dilutions of the virus–MAb mixtures immediately after they were prepared. The results shown represent the average values of three experiments, which, individually considered, gave the same pattern. One hundred percent binding represented the virus bound in the absence of antibody, and normally was around 30–40% of the total radioactivity added.

rus or the Moloney leukemia virus (Figs. 3B, 5B); (iii) binding and internalization were inhibited only by MAbs of certain specificities (Table 3, Fig. 5B).

The internalized virus was defined as the amount of radiolabeled virions that remained cell associated after incubation in the presence of a pH 3 buffer. Use of a low-pH buffer to differentiate between exposed and internalized virions has previously been described (Huang and Wagner, 1964; Highlander *et al.*, 1987). In the TGEV system this assay was valid on the following bases (Fig. 5B): (i) cell viability was not affected by incubation with the pH 3 buffer; (ii) by incubating at 4°, the virions were bound but not internalized, because at this temperature, cell-bound virions are not detached from cells with PBS but are removed with pH 3 buffer; (iii) after incubation at 37° the virions were internalized, because after this incubation, the virions were not detached by washing either with PBS or with the pH 3 buffer; (iv) the amount of virus remaining cell associated after treatment with the pH 3 buffer was dependent on the temperature of incubation, being maximum at 37°C, as expected for internalized virions; (v) the internalization defined by this procedure was a specific process, since it was inhibited by MAbs specific for site A but not for site C, and by unlabeled TGEV, but not by the serologically unrelated MHV.

A correlation has been established between radiolabeled and infectious virions on the following basis. A decrease of 10^5-fold in virion infectivity was detected (Fig. 4), indicating that 99.999% of the virions were neutralized, while the binding of radiolabeled virions to the cells was inhibited 0%. The simplest explanation is that neutralization under those conditions was not due to blocking of the binding. The possibility that only the binding of the infectious virus was inhibited, which would represent a minor proportion, cannot be excluded. Nevertheless, it seems unlikely since the noninfectious virus was rendered noninfectious by different defects. A large proportion (up to 40%) of the virions were specifically bound to the cells. Obviously, the infectious virions were included among them. These results strongly suggest that both infectious and noninfectious virions have a functional spike protein mediating the binding of the virus to the cells. The limitations derived from the inhability to follow isolated infectious virions have been accepted in all the studies analyzing the interaction between viruses and cells, by electron microscopy or using radiolabeled virions (Fuller and Spear, 1985; Fuller *et al.*, 1989; Highlander *et al.*, 1987; Miller and Hutt-Fletcher, 1988). In all cases, the observed virions, or most of the radioactivity, corresponds to noninfectious particles.

The number of antibody molecules interacting with each virus particle during neutralization can be estimated assuming that 1% of the coronavirus particles are infectious. This value was estimated to be between 4×10^3 and 1×10^4 immunoglobulin molecules of the MAbs 6A.C3 and 1C.C12, per virion, for high antibody-

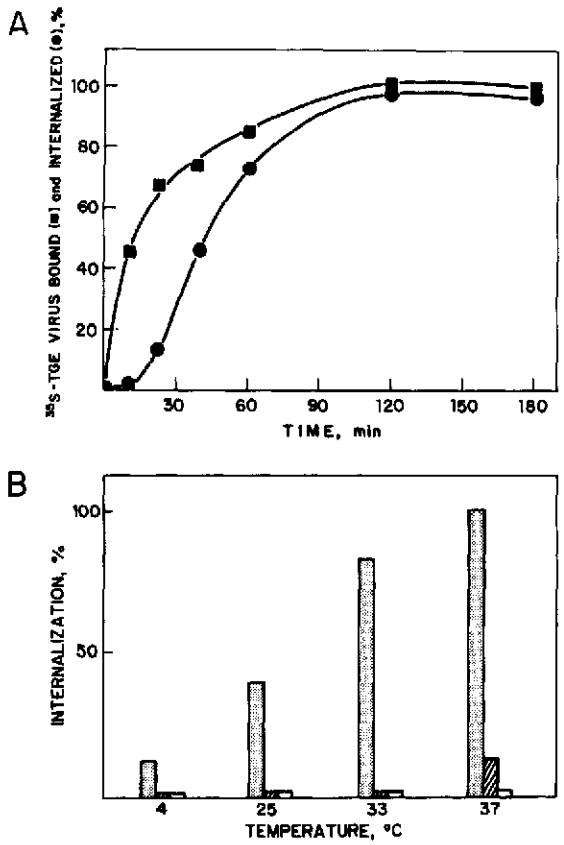

FIG. 5. Kinetics of TGEV binding and internalization, optimum temperature for these processes, and specific inhibition. (A) Binding and internalization of ^{35}S-labeled purified TGEV were studied on ST cell monolayers grown on 24-well plates, by adding the virus (see Materials and Methods), incubating at 37°, immediately cooling the plates on ice at the indicated times, and removing the unbound virus by washing with binding medium to determine the cell-associated virus (■) or with pH 3 buffer to determine the internalized virus (●). (B) Internalization of ^{35}S-labeled purified TGEV on ST cell monolayers grown on 24-well plates was determined at the indicated temperatures, in the presence of undiluted hybridoma supernatants containing the TGEV S protein site C-specific MAb 6A.A6 (shaded columns), the site A-specific MAb 1C.C12 (hatched columns), or 40-fold excess of unlabeled TGEV (blank columns). The extent of internalization was not affected by the presence of a control coronavirus (MHV) or by the absence of a MAb in the medium (results not shown). Maximum radioactivity bound or internalized at 37° in the absence of antibody and competing virus was set as 100% binding or internalization, respectively, and usually was 50% or 37%, respectively, of the total radioactivity added.

to-virus ratios (undiluted supernatants). Assuming that TGEV has 10^3 spike glycoproteins per virion [the same number as influenza virus (Dimmock, 1984)], at high antibody:virus ratios there will be 4 or 10 molecules of the MAb 6A.C3 or 1C.C12 per glycoprotein, which could interfere with virus-to-cell binding.

To analyze the neutralization of TGEV by MAbs, three situations have to be considered with respect to the antibody-to-virus ratio. At high ratios there was a good correlation between the neutralizing activity of a MAb and its ability to inhibit the binding of TGEV to ST cells, as most MAbs fit this correlation (Table 3), indicating that at high antibody concentrations the binding of virus to the cellular receptor was prevented and a pseudoneutralization resulting from steric hindrance probably occurred (Mandel, 1979). This observation was confirmed by studying the effect of different MAb concentrations on binding, both at 4° (Fig. 4) and at 37° (Fig. 6). As expected, neutralization was slightly more efficient at 37°. This is a phenomenon observed with TGEV on a regular basis.

At intermediate antibody-to-virus ratios, the predominant cause of virus neutralization was, probably, the inhibition of internalization, as the virus bound to and accumulated in the cell surface (Fig. 6 and results not shown).

At low antibody-to-virus ratios (Fig. 4), TGEV was neutralized 10^5-fold, and 100% of the virus was still cell associated, indicating that the step inhibited at this dilution ought to be different from the binding. At these concentrations, the binding of virus to ST cells was clearly increased, probably as the result of some aggregation of the virus by the antibody. If the neutralization

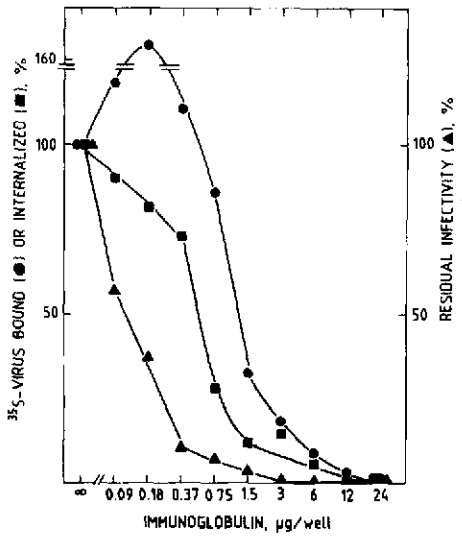

FIG. 6. Relationship between neutralization of TGEV by MAbs and inhibition of its binding and internalization on ST cells. ^{35}S-labeled purified TGEV was incubated at 4° for 15 min with the indicated dilutions of MAb 1C.C12, and the virus–MAb mixtures were added to ST cell monolayers grown on 24-well plates, which were incubated at 37° for 1.5 hr. Cell-associated (●) or internalized virus (■) was determined after washing the monolayers either with binding medium or with a pH 3 buffer, respectively. Residual infectivity (▲) was determined on the same virus preparation by performing dilutions after the incubation at 4°, which were immediately used to infect cell monolayers grown on 24-well plates. The results shown represent the average values from three experiments, which, individually considered, gave the same pattern.

TABLE 4

TGEV NEUTRALIZATION BY MAbs BEFORE AND AFTER ITS BINDING TO ST CELLS

MAb	Specificity	Antigenic subsite[a]	Neutralization[b]		
			By first adsorbing the virus to the cells and then adding the MAb		By first mixing the MAb with the virus and then adding the mixture to the cells
			%	NI	NI
Medium	—	—	0	0	0
6A.C3	S	Ac	97	1.5	8.0
1B.B1	S	Aa	90	1.0	5.5
1E.H8	S	Aa	90	1.0	4.0
1D.E8	S	Ab	75	0.6	3.6
6A.A6	S	—	<50	<0.3	<0.3
3B.D3	M	—	<50	<0.3	<0.3
3D.H10	N	—	<50	<0.3	<0.3

[a] Antigenic sites and subsites were defined by competitive RIA and by characterization of TGEV-derived *mar* mutants with MAbs (Correa *et al.*, 1988), respectively.

[b] Neutralizing activity of the MAbs was determined by two procedures: (i) by first adsorbing different numbers of PFU to the cells, incubating 60 min at 4°, adding the MAb, incubating 60 min at 4°, removing the excess MAb, washing with medium, adding the culture medium, and incubating at 37° for plaque development; (ii) by mixing the MAb with virus, incubating at 4° for 15 min, adding the mixture to the cells, incubating different numbers of PFU with the MAb for 60 min at 4° on the cell monolayer, washing with medium, and proceeding as above.

of TGEV at low antibody concentrations was not due to the blocking of the binding, once the virus was bound, it could still be neutralized. In fact, 75 to 95% of TGEV already bound to ST cells, was neutralized by MAbs (Table 4). Under these conditions (4°C), about 10% of the virus has been protected from cell dissociation at pH 3 (Fig. 5B) (Highlander *et al.*, 1987), and was not accessible to MAb, which prevented the extensive neutralization observed when the virus interacted first with the antibody (Table 4).

The results at low antibody-to-virus ratios (Fig. 6) suggest that all neutralization is not due to inhibition of virus internalization. The neutralization detected at these ratios could in part be the result of the aggregation of a portion (10–20%) of the virus caused by MAbs 1C.C12 and 6A.C3, as determined by electron microscopy (results not shown). Also, the alterations observed in virus structure could be responsible for the inhibition of internalization and other steps taking place after this. In other viral systems such as adenovirus, neutralization was due to the blocking of a replication cycle step that occurs after internalization. In fact, adenovirions already attached to HeLa cells could be neutralized with anti-hexon or anti-penton base antisera, which did not prevent its internalization (Wohlfart, 1988; Wohlfart *et al.*, 1985). Similarly, influenza virus neutralized by saturating amounts of monoclonal IgG directed against the hemagglutinin enters the cells and undergoes primary but not secondary uncoating *in vivo* (Rigg *et al.*, 1989). In the herpes simplex virus system, MAbs have been described with potent neutralizing activity not associated with the inhibition of virus adsorption but with the inhibition of internalization (Fuller and Spear, 1985; Fuller *et al.*, 1989).

Virus neutralization and binding assays were conducted with the same virus and MAb stocks and under very similar conditions. One difference between assays was that for neutralization, the virus–antibody mixtures were diluted and, under those conditions, some neutralized virus might have been dissociated from the antibody, recovering its infectivity (Table 2). These results imply that the neutralization values presented are minimum, and that the correlation between neutralization and binding inhibition is even lower than the one shown in Figs. 4, 6, and 7, strengthening the conclusion that direct inhibition of binding is not the mechanism of viral neutralization at low antibody-to-virus ratios.

In summary, neutralization of TGEV, and probably of other coronaviruses, may be the consequence of the inhibition of several steps of its replication cycle. These viruses may be neutralized by a few antibody molecules bound per virion, because of the small aggregation detected, the slight inhibition of internalization, and the blocking of a step that would take place after internalization. At intermediate antibody:virus ratios, inhibition of internalization is the predominant mechanism of neutralization. When the studies are performed at high antibody concentrations, the binding of virus is prevented, no further step takes place, and binding inhibition appears to be the mechanism of neutralization.

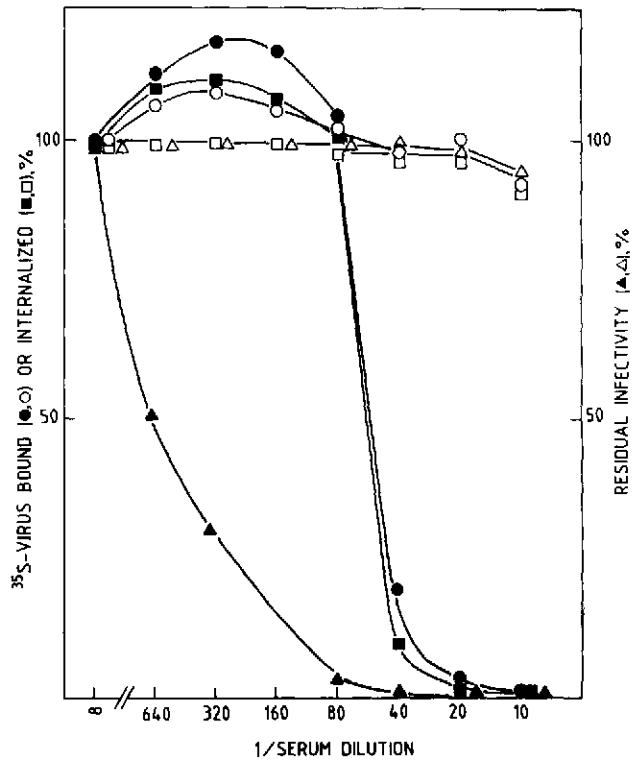

FIG. 7. Relationship between neutralization of TGEV by porcine antiserum and inhibition of its binding and internalization on ST cells. The study was performed as indicated in the legend to Fig. 6. Circles, squares, and triangles indicate cell-associated virus, virus internalized, and residual infectivity, respectively, after treatment with TGEV-specific antiserum (full symbols) or with the serum from nonimmune animals (blank symbols).

The study of the inhibition of virus-to-cell binding and of the internalization, using MAbs, permitted the selective inhibition of each of these steps of the virus cycle (Fig. 6), while, with polyvalent antibodies (Fig. 7), this differentiation was not possible. Murine (results not shown) or porcine polyvalent antisera which neutralized TGEV also inhibited the binding of virus to ST cells at high concentrations. In contrast, at lower antibody-to-virus ratios the virus could be neutralized by polyclonal antiserum without inducing inhibition of binding or internalization. These results appear at variance with those of Nguyen *et al.* (1986), who reported that neutralization of TGEV by polyvalent antibodies is due to inhibition of internalization.

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