Interference of Coronavirus Infection by Expression of Immunoglobulin G (IgG) or IgA Virus-Neutralizing Antibodies

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Immunoglobulin gene fragments encoding the variable modules of the heavy and light chains of a transmissible gastroenteritis coronavirus (TGEV)-neutralizing monoclonal antibody (MAb) have been cloned and sequenced. The selected MAb recognizes a highly conserved viral epitope and does not lead to the selection of neutralization escape mutants. The sequences of MAb 6A.C3 kappa and gamma 1 modules were identified as subgroup V and subgroup IIIC, respectively. The chimeric immunoglobulin genes encoding the variable modules from the murine MAb and constant modules of human gamma 1 and kappa chains were constructed by reverse transcriptase PCR. Chimeric immunoglobulins were stably or transiently expressed in murine myelomas or COS cells, respectively. The secreted recombinant antibodies had radioimmunoassay titers (i.e., the highest dilution giving a threefold increase over the background) higher than 10^3 and reduced the infectious virus more than 10^4 -fold. Recombinant dimeric immunoglobulin A (IgA) showed a 50-fold enhanced neutralization of TGEV relative to a recombinant monomeric IgG1 which contained the identical antigen binding site. Stably transformed epithelial cell lines which expressed either recombinant IgG or IgA TGEV-neutralizing antibodies reduced virus production by $>10^5$ -fold after infection with homologous virus, although a residual level of virus production ($<10^2$ PFU/ml) remained in less than 0.1% of the cells. This low-level persistent infection was shown not to be due to the selection of neutralization escape mutants. The implications of these findings for somatic gene therapy with recombinant antibodies are discussed.

The mucosal immune system and its predominant effector, secretory immunoglobulin A (IgA), provide the initial immunologic barriers against most pathogens that invade the body at a mucosal surface (22, 26, 30, 31, 40). This is especially true for viruses, since resistance to infection has been strongly correlated with the presence of specific IgA antibodies in mucosal secretions (2, 23).

Traditionally, the neutralization of viruses by immunoglobulins is thought to result from the binding of antibodies to virion attachment proteins, thereby preventing adherence to epithelial cells. In addition, mucosal antibodies interact intracellularly with viruses, preventing their replication, possibly by interfering with virus assembly (13, 19, 26–28).

Conventional approaches such as lactogenic immunity and artificial feeding may target the antibodies to epithelial surfaces, providing protection against enteric virus infections (10, 35, 41, 49). New strategies, including the introduction of antibody genes into cells, have been recently explored in model tissue culture systems. Sindbis virus transient-expression vector has been used to deliver single-chain variable antibody fragment (Fv) genes into vertebrate cells, leading to protection against virus infection (19). This strategy may potentially be applied to in vivo protection of mucosal surfaces by gene therapy with antibody-encoding genes since monoclonal antibodies (MAbs) against a vast range of viruses are now available. Virus-neutralizing MAbs may protect mucosal tissues against viral infections; however it is not known whether antibody-secreting cells will provide protection to the neighboring tissues.

Transmissible gastroenteritis coronavirus (TGEV) infects both enteric and respiratory tissues and causes a mortality of close to 100% when newborn animals are infected (14, 35). The immune response to this virus has been characterized (1, 5, 46), and full protection against TGEV can be provided by lactogenic immunity from immune sows (10, 35, 41, 49). It has also been shown that the oral administration of serum elicited by recombinant adenoviruses expressing the spike protein completely protects pigs against virulent virus challenge (44, 45).

The major antigenic sites of TGEV involved in the induction of virus-neutralizing antibodies are located in the globular portion of the spike (S) protein (8, 11, 17). Investigations by our laboratory into the mechanisms of TGEV neutralization (42) and of antigenic and genetic variability (36, 37) have led to the identification of a mouse MAb which neutralized all TGEV isolates tested and which also neutralized TGEV-related coronaviruses that infect at least three animal species: pigs, dogs, and cats. This MAb probably binds to an epitope essential for virus replication since no neutralization escape mutants (*mar* mutants) appeared when this MAb was employed (17).

We have studied the protection of epithelial cell monolayers against TGEV infection by using expression plasmids encoding virus-neutralizing MAbs. In this paper we describe the engineering of a recombinant TGEV-neutralizing MAb with an IgG1 isotype and the comparison of its protective activity with that of a recombinant dimeric antibody having identical variable modules and an IgA isotype. We found that cell lines stably transformed with these MAb-producing vectors were substantially protected against TGEV challenge; however, a small fraction of these cells continued to produce low levels of viral progeny. The implications of these findings with respect to

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the possible efficacy of recombinant MAb (rMAb)-mediated gene therapy are discussed.

MATERIALS AND METHODS

Cells and viruses. Swine testis (ST) (29) and simian virus 40 (SV-40)-transformed monkey kidney COS-1 cells (ATCC CRL-1650), nonsecreting murine myeloma Sp2/0 (ATCC CRL-1581) cells, and MAb 6A.C3- and MAb 1G.A7-secreting murine hybridomas (9, 20) were grown in Dulbecco's modified Eagle's medium supplemented with fetal calf serum. TGEV PUR46-MAD strain (17) was grown, purified, and titrated in ST cells as described previously (20). Vesicular stomatitis virus (VSV) was grown and titrated as described previously (6).

Immunofluorescence microscopy. Sp2/0 and ST cells expressing recombinant mouse-human (rMH) antibodies, recombinant mouse-swine (rMS) antibodies, and the MAb 6A.C3-secreting hybridoma were grown in microslide culture chambers (Miles Scientific) at a density of 1.5×10^5 cells per cm^2 . Cells were washed with phosphate-buffered saline (PBS) and fixed with methanol-acetone (1:1) at -20°C for 15 min. Cells were washed three times with PBS and once with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. All antibody dilutions used in the double immunofluorescence were performed in PBS containing 0.3% BSA (PBS-BSA) (4). ST cells expressing rMH antibodies were incubated with rhodamine-labeled sheep anti-human IgG diluted 1:100. ST cells expressing rMS antibodies were incubated first with goat anti-swine IgA (Cappel Laboratories) diluted 1:1,000 and then with fluorescein-labeled rabbit anti-goat IgG (Nordic) diluted 1:1,000.

ST cells expressing rMH and rMS antibodies and untransformed ST cells were infected with TGEV at a multiplicity of infection (MOI) of 0.5. At 48 h postinfection (p.i.), cell monolayers were washed and fixed as described above. To detect recombinant antibodies and viral proteins in the same cells, double immunofluorescence was performed. Infected cells expressing rMH antibodies were incubated with TGEV-specific polyclonal rabbit antiserum, then with fluorescein-labeled donkey anti-rabbit IgG (Amersham) diluted 1:1,000, and lastly with rhodamine-labeled sheep anti-human IgG diluted 1:100. Infected cells expressing rMS were first stained with TGEV-specific rabbit antibodies, then with rhodamine-labeled donkey anti-rabbit IgG (Amersham) diluted 1:1,000 and with goat anti-swine IgA diluted 1:1,000, and finally with fluorescein-labeled rabbit anti-goat antibody diluted 1:1,000.

RIA, virus neutralization, and Western blot analysis. The procedures for radioimmunoassay (RIA), virus neutralization, and Western blotting have been described previously (9). All antisera were diluted 1:1,000 in PBS-BSA and 0.1% Tween 20. The antisera used to develop the RIA assays to detect rMH immunoglobulins were rabbit anti-human kappa chain (Cappel) and rabbit anti-human IgG (Cappel). To detect rMS immunoglobulins, rabbit anti-swine IgA (Bethyl, Montgomery, Tex.) was used. To detect MAb 6A.C3, rabbit anti-mouse immunoglobulin was used.

To compare the neutralizing activity of rMH IgG with that of rMS IgA, the same antiserum (goat anti-mouse Fab₂) was used. The neutralizing activities of recombinant MAb dilutions with the same titer by RIA were compared.

RNA extraction. Total cytoplasmic RNA from hybridoma 6A.C3 was prepared by lysing 4×10^6 cells in 200 μl of TSM buffer (0.01 M Tris-hydrochloride [pH 7.6], 0.15 M NaCl, 5 mM MgCl₂) with 0.2% Nonidet P-40 and pelleting the nuclei by centrifugation at $13,000 \times g$ for 30 s. RNA was isolated by the addition of 200 μl of urea-sodium dodecyl sulfate (SDS) lysis buffer (8 M urea, 1.5% SDS, 15 mM EDTA, 0.24 M NaCl, 0.04 M Tris-hydrochloride [pH 7.6]), followed by vigorous vortexing and phenol-chloroform extraction. PolyA⁺ mRNA was isolated with the PolyAtract mRNA isolation system (Promega).

Sequencing and characterization of 6A.C3 V-module RNA. MAb 6A.C3 polyA⁺ mRNA was sequenced by oligodeoxynucleotide primer extension and dideoxynucleotide chain termination procedures (38) by using the protocol described previously (15). To sequence MAb 6A.C3 light (L)- and heavy (H)-chain genes the following primers were used: C_L 5'-CCAGAAGAGCGTGTCC-3'; V_L 5'-CCGTCCAGATCCACTGCC-3'; C_H 5'-CCAGGTCACTGTCACTGGC-3'; V_H 5'-GGAAGAAGATGACCCAGC-3'; V_H 5'-GGCCTTGCCCTGGAAC TTCCGGG-3'. Signal peptide and variable (V)-module sequences of L and H chains from MAb 6A.C3 were compared with other immunoglobulin sequences by using Kabat's database and the computer programs of the Genetics Computer Group (University of Wisconsin).

Synthesis of MAb 6A.C3 cDNA encoding the V module. To clone the MAb 6A.C3 V module, two cDNAs encoding the V modules of the L (VL; nucleotides 61 to 382; Fig. 1A) and H (VH; nucleotides 58 to 415; Fig. 1B) chains were synthesized from polyA⁺ mRNA isolated from hybridoma cells by reverse transcriptase (RT)-PCR with specific primers. *SalI* and *ClaI* restriction sites were introduced into VL-chain cDNA at the 5' and 3' ends, respectively. Similarly, *BamHI* and *ApaI* restriction sites were introduced into VH-chain cDNA at the 5' and 3' ends, respectively. All fragments were cloned into Bluescript SK⁻ (Stratagene) and sequenced (Sequenase 2.0). The primers used in MAb 6A.C3 amplification were as follows: flanking 5' V_H, 5'-CTTGGATCCCAAGCTTCTT ACAATG-3'; flanking 3' V_H, 5'-CAGATGGGCCCGCTGTTTGGC-3'; flanking 5' V_L, 5'-GGGTGACACACAAGCTTGGGC-3'; and flanking 3' V_L, 5'-GGGAAGATCGATACAGTTGGTGC-3'. Reverse transcriptase (AMV; Seikagaku America, Inc.) was used at a concentration of 0.4 U/ μl , and *Taq*

FIG. 1. Cloning of immunoglobulin L- and H-chain cDNAs into expression vectors. (A) Cloning of the rMH L-chain cDNA. PolyA⁺ RNA from hybridoma cells secreting MAb 6A.C3 was used as the template for an RT-PCR to obtain the cDNA encoding the VL module. The 5' end oligonucleotide corresponding to the RNA leader (L) and the 3' end oligonucleotide complementary to the first mouse constant-module nucleotides contained *SalI* and *ClaI* restriction endonuclease sites, respectively, to facilitate the cloning of the RT-PCR-derived cDNA into Bluescript SK⁻. The *ClaI* restriction endonuclease site starts at nucleotide 405 of the L-chain gene. The human L-chain (CL) module cloned into the expression vector pING2016E-gpt was modified by PCR to introduce a *ClaI* restriction site into the 5' end. The resulting VL and CL fragments were joined at the *ClaI* restriction site and cloned into the expression vector pINLC6A. (B) Cloning of the rMH H-chain cDNA. PolyA⁺ RNA from the hybridoma secreting MAb 6A.C3 was used as the template for an RT-PCR to obtain the cDNA encoding the H-chain VH module. The 5' end oligonucleotide corresponding to the RNA leader (L) and the 3' end oligonucleotide complementary to the first mouse H-chain constant-module nucleotides contained a *BamHI* and an *ApaI* site, respectively, to facilitate the cloning of the RT-PCR cDNA into pBluescript SK⁻. The *ApaI* restriction endonuclease site starts at nucleotide 426 of the H-chain gene. The human H-chain constant modules (CH1, CH2, and CH3) included in vector pGMH6 were digested with *ApaI*-*BamHI*, and the resulting fragment was ligated with the *BamHI*-*ApaI* fragment (containing the V module) into the *BamHI* restriction site of plasmid pING2003E-neo, yielding pINH6A. En, SV-40 enhancer; Pr, SV-40 promoter; A_n, polyA sequence; pA, SV-40 polyadenylation signal.

polymerase (Perkin-Elmer) was used at a concentration of 0.03 U/ μl . Amplifications were performed in a GeneAmp PCR System 9600.

Construction of immunoglobulin expression plasmids. The chimeric mouse-human L-chain expression vector was engineered (Fig. 1A) by modifying the human constant module from pING2016E-gpt (24, 50) by PCR mutagenesis with primers 5' CL (5'-CTGCATGATCAGTATCGATCTTCCCGCC-3') and 3' CL (5'-CCGAGCTCAGGCCCTGATGGG-3') containing *ClaI* and *SacI* restriction sites at the 5' and 3' ends, respectively, creating plasmid pING2016E-gpt*. The

SalI-*Clal* VL fragment was amplified by RT-PCR from mouse MAb 6A.C3 mRNA and cloned into Bluescript SK⁻. This fragment was subcloned into the *SalI*-*Clal* site of expression plasmid pING2016E-gpt⁺. The resulting plasmid was designated pINLC6A.

The chimeric mouse-human H-chain expression vector was engineered (Fig. 1B) by ligating the *Bam*HI-*Apa*I VH fragment amplified by RT-PCR from mouse MAb 6A.C3 mRNA to the *Apa*I-*Bam*HI fragment from the human IgG1 constant heavy-chain module isolated from plasmid pGMH6 (50) and cloning the resulting cDNA into expression plasmid pING2003E-neo (24, 50) previously digested with *Bam*HI. The resulting plasmid was designated pINH6A.

Transformation of Sp2/0 myeloma and ST cells with immunoglobulin gene expression plasmids. For the expression of rMH IgG1 antibodies, L-chain pINLC6A (5 μ g) and H-chain pINH6A (5 μ g) plasmids were cotransfected. To express rMS IgA antibodies, L-chain pINSLC6A (5 μ g) and H-chain pINSHC6A (5 μ g) plasmids, previously described (39), were used. Plasmids were linearized at unique restriction sites after the 3' ends of immunoglobulin genes (*Bgl*II for pINLC6A and pINSLC6A and *Aat*II for pINH6A and pINSHC6A) and were cotransfected into 10⁷ Sp2/0 or ST cells by electroporation (33). Cells were seeded in a M-24 well microplate at 4 $\times$ 10⁵ per well. Transformants were selected in the presence of G418 (0.8 mg/ml) (4). The supernatants from all wells were positive for TGEV-specific antibodies by RIA. Cells showing the highest expression level were cloned twice by limiting dilution.

Transient expression of immunoglobulin genes in COS-1 cells. COS-1 cells (8 $\times$ 10⁵) were transfected by the Lipofectin method (GIBCO BRL) with 5 μ g of circular DNA of the same expression vectors used in the stable transformation. Antibody levels were evaluated by RIA and neutralization in supernatants harvested at the indicated times posttransfection.

Interference of TGEV infection in ST cell lines expressing recombinant TGEV-neutralizing antibodies. Cloned and uncloned ST cell lines expressing recombinant TGEV-neutralizing antibodies with the IgG1 or IgA isotype were grown in M-24 wells. After extensive washing, cells were infected with TGEV (MOI, 0.5) at 4°C. After 1 h of virus adsorption, the inoculum was replaced with culture medium at 37°C. Supernatants from infected and noninfected cells were analyzed at 1 to 6 days (p.i.). Infections with VSV were performed in parallel as a control.

Nucleotide sequence accession number. The sequences obtained in this study have been assigned EMBL accession no. VH6AC3, AF001495, and VL6AC3, AF001496.

RESULTS

Sequences of genes encoding V modules of a TGEV-neutralizing MAb. In order to protect cell monolayers from virus infection by using MAbs, it is convenient to use an antibody that has a high titer for virus neutralization, that recognizes an epitope present in all virus isolates, and that does not lead to the selection of neutralization escape mutants. These conditions were fulfilled by MAb 6A.C3 (17, 20).

The sequences of MAb 6A.C3 V modules were determined by sequencing polyA⁺ RNA obtained from hybridoma cell line 6A.C3 and cloned cDNAs generated by RT-PCR. Both procedures gave the same result. The typical L- (Fig. 2A) and H-chain (Fig. 2B) organization was identified. L and H-chains of MAb 6A.C3 V modules contain three complementary determining regions, CDR1, CDR2, and CDR3, and four framework regions, FR1, FR2, FR3 and FR4. Kappa and gamma signal peptides were encoded by the first 60 and 57 nucleotides, respectively (Fig. 2). These sequences were compared with homologous immunoglobulin sequences of Kabat's database (21). The sequences of MAb 6A.C3 L- and H-chain V-module genes showed a high degree of homology with kappa (99.4%) and gamma 1 (92.7%) sequences of subgroup V and subgroup IIIC immunoglobulin genes, respectively. The J regions of the L and H-chains belong to the J₂ type.

Generation of rMAb 6A.C3. The construction of the recombinant antibodies required the fusion of VL or VH modules to constant modules, which necessitated the introduction of *Clal*I and *Apa*I restriction endonuclease sites, respectively, into the immunoglobulin genes (Fig. 1). The mutagenesis required to introduce the restriction endonuclease sites did not introduce amino acid changes.

Human kappa and gamma constant modules were flanked by the SV-40 early promoter and polyadenylation signals and were subcloned into plasmids pINLC6A and pINH6A, res-

spectively, which carry the mouse immunoglobulin enhancer at the 5' ends of the expression cassettes (Fig. 1). Sequencing confirmed that V and constant immunoglobulin modules were correctly joined.

The engineering of recombinant IgA (rIgA) with the same V modules as those of rIgG1 has been described previously (39).

Functional analysis of recombinant MAbs with gamma 1 and alpha isotypes. To verify the functionality of 6A.C3 rMAbs with IgG1 or IgA isotypes, COS-1 cells were transiently cotransfected with plasmids encoding chimeric H and L-chains. To study antibody oligomerization Western blot analysis was performed under nonreducing conditions and demonstrated that MAb 6A.C3 with the IgG1 isotype was monomeric (molecular mass, 150 kDa), while rIgA presented a dimeric structure of about 300 kDa (results not shown). Antibody expression levels ranged between 20 and 40 μ g/ml in the supernatant of transformed cells after 48 h; these levels were comparable to the antibody levels produced in other cell systems (47, 48). The secreted chimeric immunoglobulins bound TGEV, as determined by RIA, with titers (i.e., the highest dilution giving a threefold increase over background) up to 10³ and neutralized virus infectivity around 10⁴-fold (neutralization indices [NIs], around 4) (Table 1).

Murine Sp2/0 myeloma cells, which do not secrete endogenous immunoglobulins, were stably transformed by electroporation with constructs encoding the chimeric H and L-chains. Cell transformation frequencies with genes encoding both the H and L immunoglobulin chains ranged between 10⁻³ to 10⁻⁴ in the different experiments. Binding of the rMAbs to TGEV was determined by RIA of the supernatants from clones secreting the highest levels of antibodies. Titers obtained by RIA were similar to those obtained by transient transfection (Table 1).

The antibody H-chain frequently has a higher contribution to antigen binding than the L-chain. In fact, virus neutralization by purified antibody H-chains has been described previously (18). In the RIA, culture supernatants containing the rMAb 6A.C3 H-chain alone did not bind the virus or neutralize its infectivity, indicating that either the H-chain was not secreted efficiently or the contribution of the MAb 6A.C3 L-chain to TGEV binding must be essential.

The final aim of this work is to express the rMAbs in the swine enteric tract to examine their protective effect on mucosal surfaces against viral challenge. In these experiments IgA isotype antibodies are known to be more stable than those with the IgG isotype (22). To compare the neutralizing activities of rIgG1 and rIgA, supernatants containing recombinant antibodies at the same titer, as determined by RIA, were used in neutralization assays. Recombinant IgA neutralized TGEV 50-fold more effectively than rIgG1, as expected for a dimeric immunoglobulin (Fig. 3).

Generation of ST cells expressing rIgG1 and rIgA and evaluation of their resistance to TGEV infection. Porcine epithelial ST cells susceptible to infection with TGEV were transfected with DNA constructs encoding the chimeric H and L-chains to produce either the rIgG1 or the rIgA TGEV-specific antibodies. Twenty-four stably transformed cell lines expressing the recombinant MAb with each isotype were selected. Titers of the recombinant MAbs in supernatants from the cell lines obtained by RIA ranged from 10² to 10³. Immunofluorescence microscopy studies using immunoglobulin-specific antibodies revealed that, before the stably transformed cells were cloned, 10 to 15% of the cells of each line expressed the recombinant MAbs (results not shown).

The 48 ST cell lines expressing rIgG1 or rIgA TGEV-specific antibodies were infected with TGEV (MOI, 0.5). Transformed cell lines were apparently resistant to TGEV infection (Fig.

FIG. 2. Sequences of rMH L- and H-chain cDNAs of TGEV-neutralizing MAb. (A) Nucleotide sequence of the chimeric mouse-human kappa L-chain. Both the L and H-chains of MAb 6A.C3 V modules contain three complementary determining regions called CDR1, CDR2, and CDR3 and four framework regions called FR1, FR2, FR3, and FR4. The first 60 nucleotides encode the signal peptide. The J region included the last four nucleotides of CDR3 and the entire FR4. The first 24 nucleotides of the recombinant constant L-chain correspond to the MAb 6A.C3 sequence (mouse CL) and are joined in frame to the sequence encoding the constant module of the human kappa L-chain. The first coding ATG and the TAG termination signal are shown in boldface. (B) Sequence of rMH gamma H-chain cDNA. The first 57 nucleotides of the sequence encode the signal peptide. The J region included the last four nucleotides of CDR3 and the entire FR4. The first 14 nucleotides of the chimeric constant H-chain correspond to the mouse MAb 6A.C3 sequence (mouse CH1) and are joined in frame to the constant-module sequence of the human gamma 1 H-chain. Start and stop codons are shown in boldface.

4A) since no cytopathic effect was observed 48 h p.i., while untransformed ST cell monolayers were completely lysed. TGEV-infected ST cells produced 10^7 PFU/ml, while IgG1- and IgA-transformed ST cell supernatants had titers which dropped to 10^3 PFU/ml (Fig. 4B). This inhibition in viral synthesis was specific, since VSV grew to the same titer in transformed and untransformed cells (Fig. 4B). While there was a significant (10^4 -fold) reduction in virus synthesis in the anti-

body-producing cells, they were not fully resistant to TGEV infection since a residual level of virus synthesis (10^3 PFU/ml) persisted in the absence of any discernible cytopathic effect.

Interference of TGEV infection in cloned ST cell lines expressing recombinant antibodies. To determine whether the residual infection of transformed ST cell lines by TGEV was due to the presence of a large proportion of cells that did not produce the antibody, two cell lines producing the highest

TABLE 1. Functional characterization of recombinant antibodies

Antibody ^a	Titer determined by:	
	RIA ^b	NI ^c
MAb-6A.C3	10 ⁴	>4
rIgG-Sp2/0 cells	10 ² –10 ³	2–3
rIgA-Sp2/0 cells	10 ² –10 ³	4
rIgG-COS cells	10 ² –10 ³	4
rIgA-COS cells	10 ³ –10 ⁴	3
r γ	5	<0.3

^a Chimeric immunoglobulins rIgG1 and rIgA expressed in COS monkey kidney cells and in Sp2/0 myeloma cells were analyzed by RIA and virus neutralization assay. MAb-6A.C3, antibody secreted by the original hybridoma; rIgG-Sp2/0 and rIgA-Sp2/0, rMH and rMS antibodies, respectively, secreted by transformed Sp2/0 myeloma cells; rIgG-COS and rIgA-COS, rMH and rMS antibodies, respectively, secreted by transformed COS cells; r γ , rMH gamma chain.

^b RIA titer is the highest dilution giving a threefold increase over the background. Similar results have been obtained in more than five independent evaluations of the antibodies secreted by the selected cell lines.

^c NI titer is the log₁₀ of the ratio of the PFU obtained after incubating the virus in the presence of medium to that obtained after incubating the virus in the presence of the indicated MAb. Similar results have been obtained in the evaluation of more than five independent cultures of transiently transformed COS cells.

levels of either rIgG1 or rIgA antibodies were cloned by limiting dilution and infected (MOI, 0.5). All cloned cells expressed the expected rMAbs, either rIgG1 or rIgA, as determined by immunofluorescence microscopy (Fig. 5a and c), and >99.9% were free of viral antigens (Fig. 5b and d). No cytopathic effect was observed at 72 h p.i. in the transformed cells, in contrast to the lysis observed in the untransformed ST cell monolayer, which was similar to what was observed (Fig. 4) in the uncloned cells (results not shown).

The kinetics of antibody production, viral synthesis, and cytopathic effects were determined in rIgG1- and rIgA-transformed and untransformed cloned cell lines (Fig. 6). Virus production in the supernatants of transformed cultures was reduced from approximately 10^{7.5} to <10² PFU/ml. Cytopathic effects were not detected in rIgG-producing cells (Fig. 6C), and only minor effects were observed at 72 h p.i. in rIgA-producing cells. Nevertheless, when IgA-producing cells were passed the new cell monolayers reached confluence. A large reduction in the virus titer was detected (>10⁵-fold), but a residual infectivity (<10² PFU/ml) persisted in the supernatants. Double immunofluorescence labeling showed the presence of viral antigens in a minor fraction (<0.1%) of the antibody-producing cells (data not shown).

FIG. 3. TGEV neutralization by recombinant antibodies. Neutralization assay results for chimeric rIgG1 and rIgA antibodies at the same titer, as determined by RIA, are compared. C⁻, supernatant from myeloma Sp2/0. ●, rIgA; ■, rIgG1; ◆, negative control MAb. The means $\pm$ the standard deviations of three experiments are shown.

FIG. 4. Resistance of rMAb-producing ST cells to virus infection. (A) Phase-contrast microscopy of rIgG1- (a and b) and rIgA (c and d)-producing ST cells, and of untransformed cells (e and f). Cells were infected with TGEV (MOI, 0.5) (b, d, and f) or uninfected (a, c, and e). (B) Specificity of the virus growth inhibition in antibody-producing cell lines. Untransformed ST cells or ST cells producing TGEV-neutralizing rIgA or IgG1 antibodies derived from MAb 6A.C3 were infected (MOI, 0.5) with TGEV or VSV. Supernatants were harvested 48 h p.i. and titrated on ST cells. Thin vertical bars, standard error. ST, untransformed cells; ST-rIgG and ST-rIgA, cells producing TGEV-neutralizing rIgG and rIgA, respectively. The means $\pm$ the standard deviations of three experiments are shown.

Low levels of infectious virus particles were produced by the transformed ST cells in the presence of virus-neutralizing antibodies (Fig. 6A and B). One possible explanation for these findings is that a neutralization-resistant virus could have been selected for. To test this possibility, the rMAb-producing ST cells were infected with TGEV (MOI, 0.5), the virus produced

FIG. 5. Immunofluorescence of TGEV-infected ST cells expressing neutralizing MAb. Control ST cells or cells expressing MAb were infected with TGEV. At 48 h p.i. cells were fixed with methanol and stained by two-color immunofluorescence to detect antibodies and viral proteins. Recombinant antibodies were detected in transformed ST cells (a and c) but not in untransformed cells (e). Conversely, viral antigens were detected in untransformed cells (f) but not ($<0.1\%$ of cells) in rIgG- (b) or rIgA (d)-transformed cells. ST, untransformed cells; ST-rIgG and ST-rIgA, cells producing TGEV-neutralizing rIgG and rIgA, respectively; anti-MAb and anti-TGEV, cells stained by immunofluorescence with antibodies specific for immunoglobulins or TGEV.

at 1, 2, 3, and 6 days p.i. was harvested, and neutralization by MAb 6A.C3 and by MAb 1G.A7, which recognizes a different antigenic subsite of the spike protein, was studied. Both MABs were equally effective at neutralizing TGEV collected at different days p.i. (results not shown). Furthermore, MAb 6A.C3 completely neutralized the virus collected at the different time points. These results demonstrate that a neutralization escape mutant was not selected.

DISCUSSION

The genetic engineering and the anti-viral efficacies of two TGEV-neutralizing MABs with the same binding site and IgG1 and IgA isotypes are described. The dimeric rMAB with the IgA isotype had a 50-fold higher efficiency in virus neutralization than the monomeric IgG1. The transformation of ST cells with plasmids encoding TGEV-neutralizing rMABs with the IgG1 or IgA isotype reduced virus infectivity $>10^5$ -fold and prevented the appearance of cytopathic effects. A low level of virus production was detected in a few antibody-producing cells.

TGEV-neutralizing recombinant antibodies. The rMH antibodies secreted by the immunoglobulin gene-transformed ST cells had titers, as determined by RIA, that were higher than 10^3 and that are comparable to those of the original hybridoma and NIs >3 , indicating that a high level of functional chimeric antibodies was produced. Similar antibody production levels were achieved in myeloma (Sp2/0) and in epithelial (COS)

cells, indicating that, in principle, the DNA constructs could be used to transform epithelial cells at mucosal surfaces for somatic gene therapy.

The specific activity of rIgA in TGEV neutralization was about 50-fold higher than that of monomeric rIgG1 with the same virus binding site. This was probably due to a combination of the higher avidity of the dimeric rIgA and to a higher steric hindrance of recombinant dimeric IgA in comparison with the monomeric rIgG1. In the influenza virus system, polymeric IgA neutralizes influenza virus to a larger extent than IgG (12, 32). Evidence has been presented (43) indicating that immunoglobulin polymerization affects the mechanism of neutralization. The larger polymeric structure of IgA-viral complexes may inhibit the endocytosis of attached neutralized virus by preventing the formation of a critical number of cell receptor-virus attachment site interactions needed for internalization.

Protection of antibody-producing ST cell monolayers against virus infection. A highly significant ($>10^5$ -fold) reduction in virus recovery in rIgG- or rIgA-producing cell lines has been demonstrated. This reduction in virus production may suffice in vivo to provide protection against virus infection, in collaboration with other antiviral mechanisms, including nonspecific immune system effector molecules such as interferon and cellular immune responses that could cope with the residual virus or the virus-producing cells.

ST cell lines expressing a TGEV-neutralizing rMAB, in which only 10 to 15% of the cells were transformed, were

FIG. 6. Kinetics of antibody production, virus titer, and cytopathic effects in recombinant antibody-producing cells. Untransformed ST cells or cell lines producing recombinant IgG1 or IgA were infected with TGEV (MOI, 0.5). (A) Antibody titers for TGEV were determined by RIA. (B) Virus titers were determined with ST cells. (C) The cytopathic effect was calculated by counting the cells that remained bound to culture plates and was expressed as the percentage of the cells that were detached. ST, untransformed cells; ST-rIgG and ST-rIgA, cells producing TGEV-neutralizing rIgG and rIgA, respectively. One representative experiment selected from three assays with similar results is shown.

largely protected against TGEV infection. Nevertheless, protection was not complete, because low, but significant, levels of infectious TGEV were produced.

Supernatants of transformed ST cell clones had virus titers that were $<10^2$ PFU/ml, very low in comparison with the 10^7 PFU/ml observed in untransformed cells under the same conditions. The decrease in virus titer probably was not due to a nonspecific reduction in the capacity of the transformed cells to produce virus, since the transformed ST cells produced VSV as efficiently as the untransformed cells. In addition, the TGEV titers were more similar in the supernatants of cell lines in which only 10 to 15% of the cells were transformed than in the cloned transformed cell lines in which 100% of the cells were antibody producers. The most likely explanation for this residual virus persistence is that TGEV neutralization is reversible and at the antibody concentrations present in the supernatants of the rMAb-transformed cells a small virus fraction (10^2 to 10^3 PFU/ml) remains as free infectious virus while most of the virus (10^4 to 10^5 PFU/ml) is in the form of noninfectious virus-antibody complexes (42).

Three mechanisms may be responsible for the reduction in virus production: (i) extracellular neutralization, (ii) intracellular neutralization, and (iii) modulation of virus production by antibodies (16). Extracellular neutralization is likely to be responsible for the results of this study, because the antibodies

are continuously released into the medium, even during virus infection, and in the immunoglobulin gene-transformed uncloned cells about 85 to 90% of the cells did not produce the antibodies yet were still protected from TGEV infection.

Intracellular neutralization of virus in cultured cells has been demonstrated in the influenza and Sendai virus systems during transcytosis of dimeric IgA from the basal to the apical side of polarized epithelial cells (26). This mechanism may also operate in antibody-producing ST cells infected with TGEV, since during virus and antibody synthesis these proteins could colocalize, leading to an intracellular interference of protein transport as described previously for other systems (3, 7, 13, 25, 27, 34). It is not known whether intracellular neutralization is a mechanism that also operates *in vivo*.

Modulation of virus production by antibody binding to viral antigens on the surface of virus-infected cells is a phenomenon that operates in several virus systems (16). This mechanism may modulate TGEV synthesis in antibody-producing ST cells. This modulation of virus synthesis probably leads to the establishment of a persistent infection, since residual virus production was often observed for at least 3 weeks p.i. (results not shown). Antibody-induced modulation is a mechanism by which other viruses can persist and escape immunologic surveillance (16). Experimental evidence has suggested such a mechanism for measles virus, herpes simplex virus, and retrovirus.

The majority of the virus produced by the transformed cells was probably identical to the original one used for infection. A neutralization escape mutant was not selected for because the virus produced at days 1 and 6 p.i. was neutralized with the same efficiency by MAb 6A.C3, produced by the transformed ST cells, and by MAb 1G.A7, which binds to a different antigenic subsite of the TGEV spike protein (9, 17). This is in agreement with our previous results indicating that the epitope recognized by MAb 6A.C3 is an interspecies conserved epitope, present in porcine, canine, and feline coronaviruses, which seems to be essential for virus replication, and for which neutralization escape mutants have never been observed (37).

The transient expression of virus-neutralizing antibodies in mucosal surfaces could be used to provide immediate protection in the mucosal areas. This type of somatic gene therapy could be of potential benefit to protect nonvaccinated individuals such as newborn animals. In addition, this type of therapy could be used to prevent the allergic immune responses produced by the predominance of an IgE isotype response by using recombinant virus vectors encoding antibodies with IgG isotypes.

The transformation of virus-susceptible cells with genes encoding virus-neutralizing antibodies has led to a $>10^5$ -fold reduction in virus recovery. Although some residual virus synthesis persists in less than 0.1% of the cells in culture, it is possible that *in vivo* defense mechanisms apart from virus neutralization, such as cytolytic T cells, could completely control virus infection. This new strategy may be particularly useful in infections where fast immune intervention is required in a defined tissue, since viral vectors could express antibody genes within 2 to 3 h and since this expression may be targeted to specific tissues.

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