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MHV S Peplomer Protein Expressed by a Recombinant Vaccinia Virus Vector Exhibits IgG Fc-Receptor Activity

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We have previously shown that cells infected with mouse hepatitis virus (MHV) bind rabbit, mouse, and rat IgG by the Fc portion of the IgG molecule. This Fc-binding activity appeared to be mediated by the MHV S protein. S protein could also be precipitated from MHV-infected cells by a monoclonal antibody directed against the murine Fc γ receptor (FcγR). To prove definitively that the S protein mediates Fc-binding activity, we have expressed the MHV S protein utilizing recombinant vaccinia viruses. The anti-FcγR monoclonal antibody, 2.4G2, precipitated recombinant S protein in cells of murine, human, and rabbit origin. Since the anti-Fc receptor monoclonal antibody does not react with human and rabbit Fc receptors these results demonstrate that the epitope recognized by this antibody is carried on the MHV S protein and is not murine in origin. Examination of various MHV isolates and escape mutants failed to identify the precise sequences in S responsible for the molecular mimicry of the murine FcγR. These data are consistent with the hypothesis that a previously identified region of similarity between the S protein and the FcγR mediates this activity. The Fc binding activity of S was expressed on the cell surface, since MHV-JHM-infected cells, but not uninfected cells, formed rosettes with anti-sheep red blood cell (SRBC) antibody-coated SRBC. The anti-FcγR monoclonal antibody neutralized MHV-JHM and inhibited syncytium formation induced by the MHV S protein. © 1992 Academic Press, Inc.

INTRODUCTION

Murine hepatitis virus (MHV), JHM strain, is a neurotropic member of the coronaviridae (Spaan et al., 1988). Mice or rats infected intracerebrally or intranasally with MHV-JHM may develop persistent infection leading to the development of chronic demyelinating inflammatory lesions of the brain and spinal cord (Weiner, 1973; LePrevost et al., 1979; Sorensen et al., 1982, 1984; Perlman et al., 1990). MHV-induced neurological disease in rodents provides an experimental model for demyelinating diseases of the human nervous system, such as multiple sclerosis. The S protein (formerly called E2) forms spikes or peplomers on the surface of the virus (Cavanagh, 1983). Each peplomer consists of two or three S molecules, each with a molecular weight of 180 kDa. Analysis of the primary nucleotide sequence of the S gene revealed that it encodes a type 1 membrane protein (Luytjes et al., 1987; Schmidt et al., 1987).

The S protein is responsible for virus-induced cell fusion (Storz et al., 1981; Sturman et al., 1985), attachment of the virus to specific receptors on plasma membranes, (Williams et al., 1990), induction of neutralizing

antibodies (Collins et al., 1982), and cell-mediated immunity (Holmes et al., 1986; Stohlman et al., 1986; Yamaguchi et al., 1988; Wysocka et al., 1989). The S protein is 1363 amino acids long, and just before maturation of the virus, S is cleaved, apparently by a host cell protease, to two nonidentical subunits, designated S1 (amino-terminal subunit) and S2 (carboxy-terminal subunit) (Holmes et al., 1981; Frana et al., 1985; Sturman et al., 1985). S1 and S2 are held together on the virion in a noncovalent manner (Cavanagh, 1983). The cleavage occurs between amino acids 768 and 769 and is required for the fusion activity of S protein (Luytjes, 1987; Schmidt, 1987; Sturman et al., 1985). The biosynthesis of the S protein occurs on ribosomes bound to the rough endoplasmic reticulum (RER). The S protein is N-glycosylated. (Holmes et al., 1981; Niemann et al., 1982).

Several distinct antigenic and structural changes of S have been described during persistent MHV infections of rodents (Fleming et al., 1983; Talbot and Buchmeier, 1985; Taguchi et al., 1985, 1989). Variants which differ from the original population by possessing either larger or smaller S glycoprotein have been isolated from persistent CNS infections of rats or from rat astrocytes in vitro (Taguchi et al., 1985; Morris et al., 1989). Recently, it has been demonstrated that neutralizing antibodies induced by immunization with affinity purified MHV-A59 S protein were protective in vitro and

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in vivo (Daniel and Talbot, 1990). Therefore, the peplomer S protein plays a central role in the outcome of MHV infections.

We have recently reported molecular mimicry between the MHV S protein and the Fc γ receptor (FcγR) (Oleszak and Leibowitz, 1990). Rabbit, mouse, and rat IgGs were shown to bind to the MHV S protein, and this binding was dependent upon the Fc portion of the immunoglobulin molecules. Furthermore, the 2.4G2 antimouse FcγR monoclonal antibody (Unkeless, 1979; Mellman and Unkeless, 1980) precipitated S protein from MHV-infected cells and goat anti-S antibodies precipitated a 75 to 77 kDa molecule from uninfected WEHI-3 cells, a FcγR-bearing cell line. These results suggest that the MHV S protein cross-reacts immunologically with the mouse FcγR and is able to function as an Fc receptor.

In this report we unequivocally demonstrate that the Fc binding activity and immunologic cross-reactivity with FcγR resides on the S glycoprotein. MHV S protein expressed by a recombinant vaccinia virus reacts with anti-FcγR monoclonal antibody. Furthermore, MHV-JHM-infected cells form rosettes with IgG-coated sheep red blood cells, demonstrating that the FcR site is present on the cell surface. Finally, we demonstrate that the anti-FcγR 2.4G2 monoclonal antibody neutralizes MHV-JHM and inhibits cell fusion in a contact fusion assay.

MATERIALS AND METHODS

Cells and viruses

RK-13 (Bearle, 1963) and HeLa S3 cell lines were originally purchased from the American Tissue Culture Collection (ATCC, Rockville, MD) and propagated in Dulbecco modified Eagle medium (DMEM, GIBCO Laboratories, Grand Island, NY) with 10% fetal bovine serum (FBS) and 100 U of penicillin G and streptomycin per milliliter. The origin and growth of the L-2 (Rothels, 1959), DBT (Hirano *et al.*, 1974), and 17CL-1 (Sturman and Takemoto, 1972) cell lines have been previously described. MHV-JHM, MHV-A59, and MHV-3 were routinely propagated on 17CL-1 cells as described previously (Robb *et al.*, 1979). Vaccinia virus strain IHD was obtained from the ATCC. Isolation and characterization of anti-S neutralizing antibody-resistant MHV variants V5A13, V5A13.1, and V4B.11 have been previously described (Parker *et al.*, 1989; Gallagher *et al.*, 1990).

Construction of recombinant vaccinia virus expressing MHV-JHM S glycoprotein

Clones pJMS1010, pJS112, and pJS92, which together encompass the entire S protein coding region

were generously provided by Dr. S. Siddell, Institut fur Virologie and Immunobiologie, der Universitat Wurzburg. All of these clones were flanked by G-C tails and *Pst*I sites. To assemble a complete S gene, pJMS1010 was digested with *Pst*I (nucleotide 2555 from the S protein AUG) and *Xba*I (nucleotide 3779) and subcloned into pSP18 (Bethesda Research Laboratories). pJS112 was restricted with *Pst*I and subcloned into the *Pst*I site of pSP18-pJMS1010 (clone 1).

The entire *Pst*I insert of pJS92 was subcloned into pSP19, which is identical to pSP18 except that polylinker region is in reverse order. pJS92 is flanked by G-C tails which were found in initial construction to decrease S protein expression in eukaryotic cells. The G-C tail at the 5' end of pSP19pJS92 was removed with *Bal*31, and *Sma*I linkers were placed on each end. A clone in which the initiator AUG of the S protein was 23 nucleotides from the *Sma*I linker was selected and propagated (clone 2). Clones 1 and 2 were combined using a procedure described by Schubert *et al.* (1985). Briefly, both clones 1 and 2 were restricted with *Hind*III (a unique *Hind*III site is present only in the polylinker) and then treated with exonuclease III (20 μg linearized plasmid with 200 U of exonuclease III in 125 μl of 10 mM Tris, pH 7.5, 10 mM MgCl$_2$, 5 mM dithiothreitol for 15 min at 24°). Each clone was restricted with *Bgl*I (unique site present in pSP18 and pSP19), and the two fragments of each plasmid were separated by agarose gel electrophoresis. The appropriate fragments were mixed, ligated with T4 DNA ligase, and used to transform competent HB101 cells.

Ampicillin-resistant colonies were selected, plasmid DNA was isolated, and the junction between pJS92 and pJSM1010-pJS112 resultant clones was sequenced and shown to be identical to published sequences (Schmidt *et al.*, 1987). The resultant S gene was subcloned into pTM3, a vaccinia virus transfer plasmid which includes the T7 promoter, the untranslated region of encephalomyocarditis virus, and the gene for xanthine-guanine phosphoribosyl-transferase under the control of the vaccinia virus P7.5 promoter (Elroy-Stein *et al.*, 1989). These sequences were flanked by vaccinia thymidine kinase sequences for the purpose of homologous recombination. The *Nco*I site was removed with T4 DNA polymerase so that the AUG of the S protein was used to initiate translation. HeLa cells infected with vaccinia virus, strain WR, were transfected with the S protein construct and recombinant virus was selected and propagated as described previously (Falkner and Moss, 1988).

Metabolic labeling of cells and immunoprecipitation

Monolayers of DBT or L-2 cells in 6-well Costar plates were infected with appropriate viruses using

one to five plaque forming units (PFU)/cell. Cells were labeled with 400 μCi/ml [^{35}S]methionine (ICN Radiochemicals, Irvine, CA) either 8 hr after infection (wt MHV) or 5 hr after infection (recombinant vaccinia viruses) in methionine-free medium. Cytosolic extracts of infected and control cells were prepared as described previously (Oleszak and Leibowitz, 1990). Aliquots of 50 μl (corresponding to 4 × 10^5 cells) were immunoprecipitated with appropriate antibodies using *Staphylococcus aureus* Cowan strain (SAC) cells (Calbiochem, San Diego, CA). The immune complexes were disrupted in sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) sample buffer at 70° for 5 min and analyzed by electrophoresis on 8% SDS–PAGE as described by Laemmli and Favre (1973) followed by autoradiography.

Antibodies

The 1.38.1 and 1.43.2 monoclonal antibody specific for the MHV S glycoprotein were developed in this laboratory (Leibowitz *et al.*, 1987). A hyperimmune goat serum which recognizes the S protein was a generous gift from Dr. K. Holmes (Uniformed Services University for Health Sciences, Bethesda, MD). The rat antimouse FcγR 2.4G2 monoclonal antibody was purchased from ATCC. Rat anti-surfactant 8H monoclonal antibody (IgG$_{2b}$) was provided by Dr. David Strayer (University of Texas Medical School, Houston, TX). All monoclonal antibodies were used as ammonium sulfate concentrates of tissue culture supernatant. The concentration of the 1.38.1 anti-S monoclonal antibody was determined by mouse immunoglobulin specific ELISA, by Dr. Rosenblum of the M.D. Anderson Cancer Center, Houston, TX. The concentrations of the rat anti-mouse FcγR 2.4G2 monoclonal antibody (IgG$_{2b}$) and of rat anti-surfactant 8H monoclonal antibody (IgG$_{2b}$) was determined by a radial immunodiffusion assay specific for rat (IgG$_{2b}$) (The Binding Site, Inc., San Diego, CA). Purified whole rabbit IgG specific for *Micrococcus lysodeikticus* and it's F(ab')$_2$ fragment was a generous gift of Dr. S. Rodkey (University of Texas Medical School, Houston, TX). Affinity purified goat anti-rat IgG and goat anti-mouse IgG were heavy and light chain specific and were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Rabbit anti-sheep red blood cell IgG were purchased from Cappel (West Chester, PA).

Virus neutralization assay

Twofold dilutions of hybridoma antibodies in DMEM supplemented with 2% heat-inactivated FBS were incubated with an equal volume of an appropriate virus containing approximately from 10^3 to 10^4 PFU/ml. The mixtures were incubated at 37° for 1 hr. Residual infec-

tivity was evaluated by a plaque assay. Neutralizing titer was expressed as the concentration of monoclonal antibody (micrograms/milliliter) that gave 50% reduction in the number of plaques as compared to the control virus preparation.

Binding of IgG-coated sheep red blood cells (SRBC) to MHV-infected cells

SRBC, provided by Dr. T. Blasdel (University of Texas Medical School, Houston, TX) were resuspended at 10^9 cells/ml, washed three times with phosphate buffer saline (PBS), and incubated for 30 min at 37° with the maximum subagglutinating concentration of rabbit anti-SRBC IgG (dilution 1:10,000). The sensitized SRBC were washed three times with PBS and resuspended in DMEM containing 2% heat-inactivated FBS to a final concentration of 1%. The sensitized SRBC (0.5 ml per well) were added to cultures of MHV-JHM-infected cells, when approximately 60% of the cells were involved in syncytia, or to mock-infected L-2 or DBT cells. Unsensitized SRBC were used as negative controls. An hour later when the SRBC had formed a uniform layer above the cells, the monolayers were washed several times with PBS until all free erythrocytes were removed. The L-2 cells were fixed in methyl alcohol stained for 3–5 min with eosin (1% solution), differentiated in methyl alcohol, and photographed. DBT cells were directly photographed.

Contact fusion assay

L-2 or DBT cells were plated on 6-well Costar plates (5 × 10^4/well) and were either mock infected or infected with MHV-JHM at a multiplicity of infection (m.o.i.) = 1–3. At 5 to 6 hr after infection the cultures were overlaid with a 10-fold excess of uninfected L-2 or DBT cells (Mizzen *et al.*, 1983). Antibodies were added either at 1 or 2 hr after adsorption (final concentration 1:10) and were present throughout the experiment. Fusion was assessed 5 hr after the addition of the indicator cells. Cultures were fixed with cold methanol and stained with Giemsa. Syncytia were counted using a colony counter (National Instrument Laboratory, Rockville, MD).

RESULTS

The anti-FcγR 2.4G2 recognizes an epitope of S expressed by vaccinia virus recombinants

We chose to express the MHV S gene utilizing a vaccinia virus vector in which the S gene is under the control of the bacteriophage T7 promoter. Thus cells infected with this vector, VV-E2, do not express S unless they are coinfected with a second recombinant
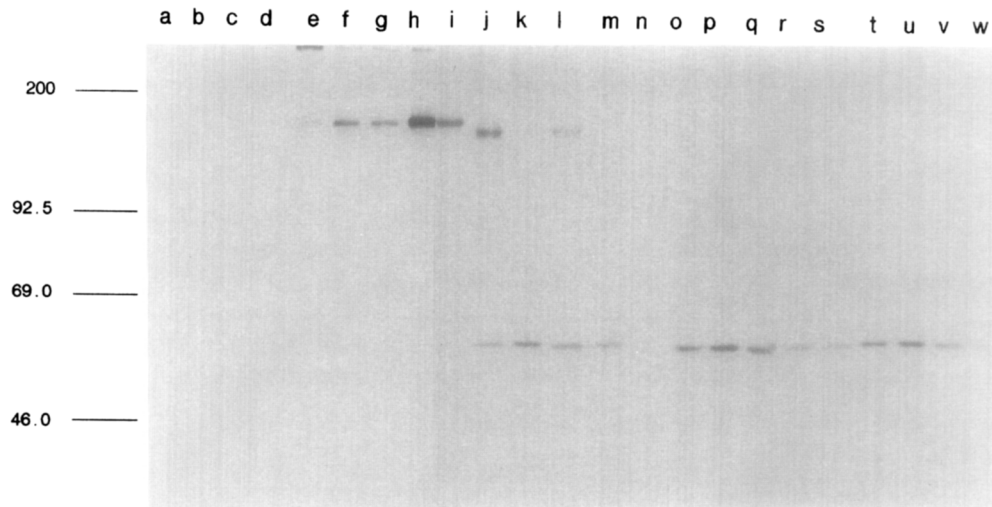
FIG. 1. Immunoprecipitation of MHV S peplomer protein expressed by recombinant vaccinia virus by rat anti-FcγR 2.4G2 monoclonal antibody in DBT cells. DBT cells were: (1) mock-infected (lanes a, b, c, and d); (2) infected with wt MHV-JHM (lanes e, f, g, h, and i); (3) infected simultaneously with two recombinant viruses vTF3 and VV-E2 (lanes j, k, l, m, and n); (4) infected with recombinant VV-E2 only (lanes o, p, and q); (5) infected with wt vaccinia virus (lanes r, s, and t); or (6) infected with recombinant vTF3 only (lanes u, v, and w). Infection with vaccinia viruses was normalized for the overall m.o.i. Thus m.o.i. of 5 was used for infection with single virus (vTF3 or VV-E2) and m.o.i. of 2.5 was used for cells infected with these two recombinant viruses (vTF3 and VV-E2). Construction of the recombinant vaccinia viruses is described under Materials and Methods. Cells were labeled with [35S]methionine and cytoplasmic extracts were prepared. Aliquots (10 µl) of cell extract were immunoprecipitated with goat anti-S antibody (lanes a, h, i, j, o, r, and u), mouse anti-S monoclonal antibody 1.38.1 (lanes c, f, and m); mouse anti-S monoclonal antibody 1.43.2 (lanes b and g); the anti-FcγR 2.4G2 monoclonal antibody (lanes d, e, l, q, t, and w); goat anti-rat IgG (lanes k, p, s, and v); or goat anti-mouse IgG (lane n). Immunoprecipitates were analyzed by SDS-PAGE using an 8% gel. The molecular weight standards (kDa) are shown on the left margin.

vaccinia virus, vTF3, which provides the T7 RNA polymerase needed to transcribe the S gene. Radioactively labeled cell lysates were prepared from uninfected DBT cells; MHV-JHM-infected DBT cells; DBT cells singly infected with wild-type vaccinia virus, VV-E2, or vTF3; or DBT cells coinfected with VV-E2 and vTF3. In agreement with our previous results (Oleszak and Leibowitz, 1990) the anti-FcγR 2.4G2, two monoclonal antibodies specific for S (1.38.1 and 1.43.2), and a goat anti-S antibody immunoprecipitated a polypeptide of 180 kDa (Fig. 1) from MHV-JHM-infected DBT cells. This polypeptide was not immunoprecipitated from control, uninfected DBT cells (Fig. 1).

Cells simultaneously infected with vTF3 and VV-E2 synthesized a 165-kDa polypeptide corresponding to recombinant S protein (Fig. 1) which was immunoprecipitated by goat anti-S antibody and by the anti-FcγR 2.4G2 monoclonal antibody. The recombinant protein precipitated by the anti-FcγR 2.4G2 monoclonal antibody had a mol wt identical to that precipitated by goat anti-S antibody. This recombinant S protein was 15 kDa smaller than wt MHV S protein (Fig. 1, compare lanes j and l with lanes h and i). This difference in size has been noted previously and reflects a deletion in the S gene of the virus from which these clones were derived (Schmidt et al., 1987; Parker et al., 1989). The 1.38.1 and 1.43.2 anti-S neutralizing monoclonal anti-

bodies, which immunoprecipitated the 180 kDa wt MHV-JHM S protein (Fig. 1), did not recognize the recombinant vaccinia virus synthesized S protein (Fig. 1 and data not shown). The inability of these anti-S monoclonal antibodies to recognize recombinant S may be due to deletions of certain epitopes in the recombinant S or due to conformational differences between wt and recombinant S which are a result of this deletion. Precipitation of recombinant S protein expressed by vaccinia virus was not via the secondary goat anti-rat IgG antibody. This antibody as well as goat anti-mouse IgG did not precipitate any protein of this size from DBT cells infected simultaneously with vTF3 and VV-E2 (Fig. 1). In addition these results demonstrate that irrelevant goat IgG does not immunoprecipitate recombinant S, in agreement with our previously published findings with wt MHV S. As expected, the 165-kDa polypeptide could not be detected by immunoprecipitation from lysates of cells infected either with recombinant vTF3 alone, with VV-E2 alone, or with wt vaccinia virus (Fig. 1). Recombinant 165 kDa S protein was also immunoprecipitated by a purified rabbit IgG specific for M. lysodeikticus (but not F(ab)₂ fragments) and by an irrelevant rat monoclonal antibody specific for lung surfactant (data not shown).

To demonstrate beyond any doubt that the FcR activity we observed in MHV-infected cells was mediated by
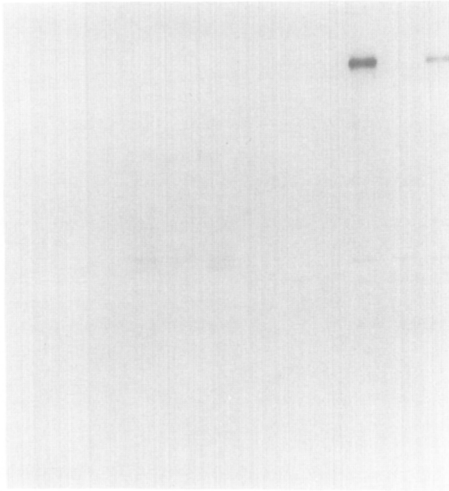
a b c d e f g h i j k l

FIG. 2. Immunoprecipitation by the anti-FcγR 2.4G2 monoclonal antibody of MHV-S peplomer protein expressed in HeLa cells using recombinant vaccinia virus. HeLa cells were infected with recombinant vTF3 only (lanes a, b, and c), wt vaccinia virus (lanes d, e, and f), single recombinant VV-E2 only (lanes g, h, and i), or simultaneously with both recombinant vaccinia viruses vTF3 and VV-E2 (lanes j, k, and l). Cells were labeled with [^{35}S]methionine and cytoplasmic extracts were prepared. Aliquots (10 μl) of cell extracts were immunoprecipitated with goat anti-S antibody (lanes a, d, g and j), goat anti-rat IgG (lanes b, e, h, and k), or with the anti-FcγR 2.4G2 monoclonal antibody (lanes c, f, i and l). Immunoprecipitates were analyzed by SDS–PAGE using an 8% gel.

S protein rather than mouse FcR associated with S, we infected cells of human (HeLa) and rabbit (RK-13) origin with recombinant viruses vTF3 and VV-E2 and immunoprecipitated cell lysates with goat anti-S antibody and the anti-FcγR 2.4G2 monoclonal antibody (Fig. 2). Goat anti-S antibody and anti-FcγR 2.4G2 monoclonal antibody immunoprecipitated a 165-kDa protein from both HeLa (Fig. 2) and RK-13 cells (data not shown) infected with recombinant vTF3 and VV-E2 viruses. Goat anti-rat IgG, which was employed as secondary antibody, did not immunoprecipitate any polypeptide chains from these cells. Goat anti-S and anti-FcγR 2.4G2 monoclonal antibody did not immunoprecipitate any polypeptides from cells infected separately either with vTF3 or VV-E2 or wt vaccinia virus (Fig. 2). Since the anti-FcγR 2.4G2 monoclonal antibody did not react with rabbit or human FcR, these results demonstrate that the site recognized by this antibody resided on the S peplomer protein.

Fc binding ability of S proteins of MHV-JHM deletion mutants

We have previously identified regions of sequence similarity between the N-terminal (S1) portion of S and

FcγR (Oleszak and Leibowitz, 1990). Although linear sequences may be responsible for the molecular mimicry that we observed between FcγR and S protein, it is equally possible that conformational determinants were responsible for this effect. To investigate the latter possibility, we studied the Fc binding of the S protein of three variants (V5A13, V5A13.1, and V4B.11) of MHV-JHM kindly provided by Dr. Buchmeier. These three variants, originally selected for resistance to neutralization, have deletions of 447, 477, and 426 bases, respectively, within the S1 region of the parental MHV-JHM sequence (Parker et al., 1989; Gallagher et al., 1990). The region of sequence similarity between MHV S and FcγR (domain 1), (Oleszak and Leibowitz, 1990) is preserved in all three MHV variants and is located directly adjacent to the region of deletion. The alignment of amino acid residues of S of these three variants with the parental MHV-JHM (Parker et al., 1989; MHV-A59 (Luytjes et al., 1987) and MHV-JHM (originally received from Dr. S. G. Siddell; Schmidt et al., 1987) are depicted in Fig. 3.

To determine whether these deletions had an effect on FcR activity, cells were infected with variant virus and the S proteins immunoprecipitated with the anti-FcγR 2.4G2 monoclonal antibody or anti-S antibodies (Fig. 4). Goat anti-S antibody immunoprecipitated a 165-kDa polypeptide approximately 15 kDa smaller than that of wt MHV S (Fig. 4). These variant S proteins were also immunoprecipitated by anti-FcγR 2.4G2 monoclonal antibody (Fig. 4) but not by secondary goat anti-rat IgG (data not shown). The finding that these variants express an S protein smaller than that of the wt is in agreement with reports of others (Parker et al., 1989; Gallagher et al., 1990). Furthermore, our anti-S monoclonal antibodies 1.38.1 and 1.43.2 failed to
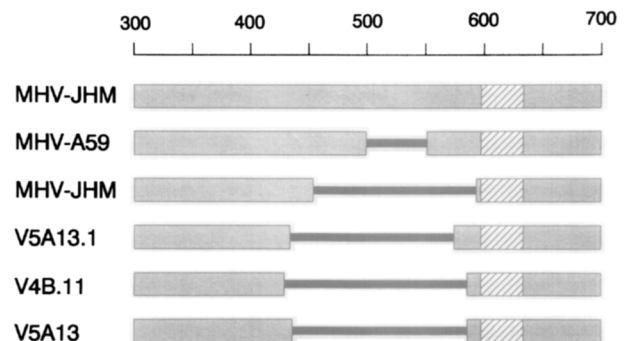

FIG. 3. Alignment of amino acid residues 300 through 700 from MHV-JHM S (Parker et al., 1989) with the comparable amino acid sequences from MHV-A59 (Luytjes et al., 1987), MHV-JHM (Schmidt et al., 1987) and MHV-JHM deletion variants V5A13.1; V4B.11 and V5A13 (Parker et al., 1989). Black, solid bars represent deletions. The region of sequence similarity between the MHV S protein and FcR (Oleszak and Leibowitz, 1990) is indicated (hatched bar).
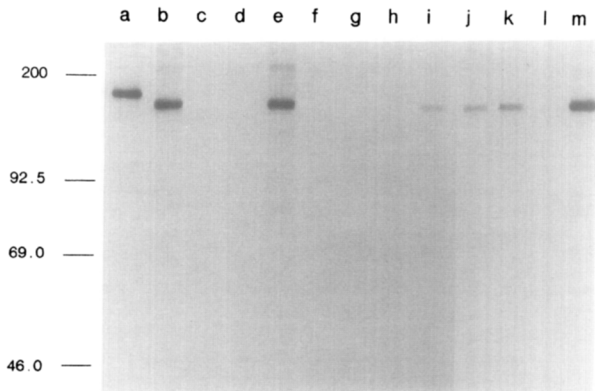
FIG. 4. Immunoprecipitation of MHV-JHM variants S peplomer protein by the anti-FcγR 2.4G2 monoclonal antibody. DBT cells were infected with wt MHV-JHM, V5A13, V5A13.1, or V4B11, or were mock infected, labeled with [³⁵S]methionine, and cytoplasmic extracts were prepared as described under Materials and Methods. wt MHV-JHM-infected cell extracts were immunoprecipitated with goat anti-S antibody (lane a); V5A13.1-infected cell extracts were immunoprecipitated with goat anti-S antibody (lane b), anti-S monoclonal antibody 1.43.2 (lane c), anti-S monoclonal antibody 1.38.1 monoclonal antibody (lane d), or anti-FcγR 2.4G2 monoclonal antibody (lane k). V4B.11-infected cell extracts were immunoprecipitated with goat anti-S antibody (lane e), anti-S monoclonal antibody 1.43.2 (lane f), anti-S monoclonal antibody 1.38.1 (lane g), or the anti-FcγR 2.4G2 monoclonal antibody (lane i). V5A13-infected cell extracts were immunoprecipitated with goat anti-S antibody (lane m), anti-S monoclonal antibody 1.38.1 (lane n), anti-S monoclonal antibody 1.43.2 (lane l), or the anti-FcγR 2.4G2 monoclonal antibody (lane j). Immunoprecipitates were analyzed by SDS–PAGE using an 8% gel. The molecular weight standards (kDa) are shown on the left margin.

react with S proteins from these deletion variants (Fig. 4). These results show that the Fc-binding activity does not require the sequences present between residues 429 to 586.

Binding of IgG-coated SRBC to MHV-infected cells

In order to determine whether the FcR binding site on S is expressed on the cell surface of infected cells we performed an Fc rosetting assay using IgG-coated SRBC. L-2 or DBT cells, either mock or MHV-JHM infected, were incubated with antibody coated SRBC as described under Materials and Methods. MHV-JHM-infected cells formed rosettes with anti-SRBC antibody coated SRBC (Figs. 5a and 6a), whereas, uninfected cells did not (Fig. 5c and 6b). Furthermore, MHV-JHM-infected cells did not form rosettes with SRBC incubated with normal rabbit serum (Fig. 5b). Rosetting was carried out when approximately 60 to 70% of infected cells were involved in syncytia (about 8 hr post-infection). Preincubation of MHV-JHM-infected DBT cells for 1 hr with goat anti-S antibody inhibited rosette formation with rabbit anti-SRBC antibody-coated SRBC (Fig. 6c). These results demonstrate that MHV-JHM in-

fected cell express an Fc binding activity on the cell surface.

Neutralization of MHV strains by the anti-FcγR 2.4G2 monoclonal antibody

To determine whether the anti-FcγR 2.4G2 monoclonal antibody could reduce the titer of MHV, various concentrations of the monoclonal antibody were incubated with constant concentrations of virus for 1 hr at 37°, prior to titration by a plaque reduction assay. Wild-type MHV-JHM and the three MHV-JHM variants, V5A13, V5A13.1, and V4B.11, were all neutralized by the anti-FcγR 2.4G2 monoclonal antibody. The neutralization titer of the anti-FcγR 2.4G2 monoclonal antibody was lower than that of the neutralizing anti-S 1.38.1 monoclonal antibody (Table 1). The irrelevant rat anti-surfactant monoclonal antibody 8H (IgG$_{2b}$), that immunoprecipitated S protein (Oleszak and Leibowitz, 1990) from MHV-JHM-infected cells, also decreased the titer of MHV-JHM, although perhaps not as efficiently as the 2.4G2 monoclonal antibody. Fetal bovine serum had no effect on virus titer.

Next, we attempted to select anti-FcγR 2.4G2 monoclonal antibody-resistant variants. Multiple attempts to select variants by in vitro neutralization with 2.4G2 failed. We then used the protocol successfully em-
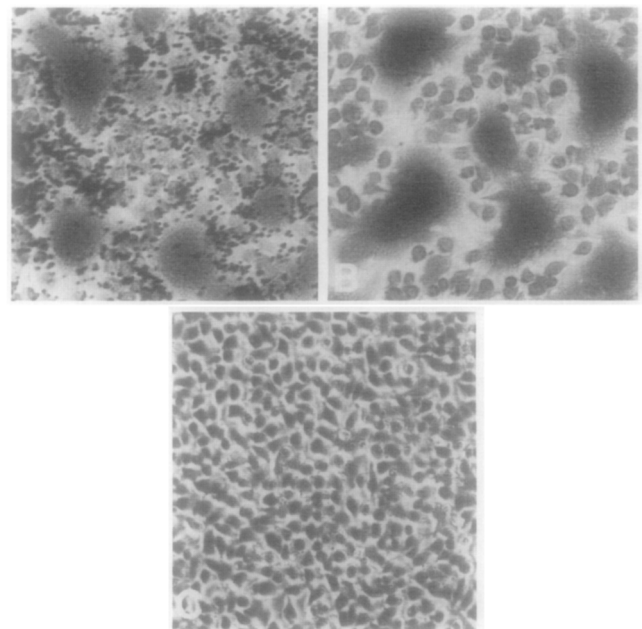

FIG. 5. Rosette formation of MHV-JHM-infected L-2 cells with anti-SRBC antibody-coated SRBC. (A) Rosettes of MHV-JHM-infected L-2 cells with rabbit anti-SRBC antibody-coated SRBC. (B) MHV-JHM-infected L-2 cells do not form rosettes with unsensitized SRBC. (C) Uninfected L-2 cells do not form rosettes with rabbit anti-SRBC antibody-coated SRBC.
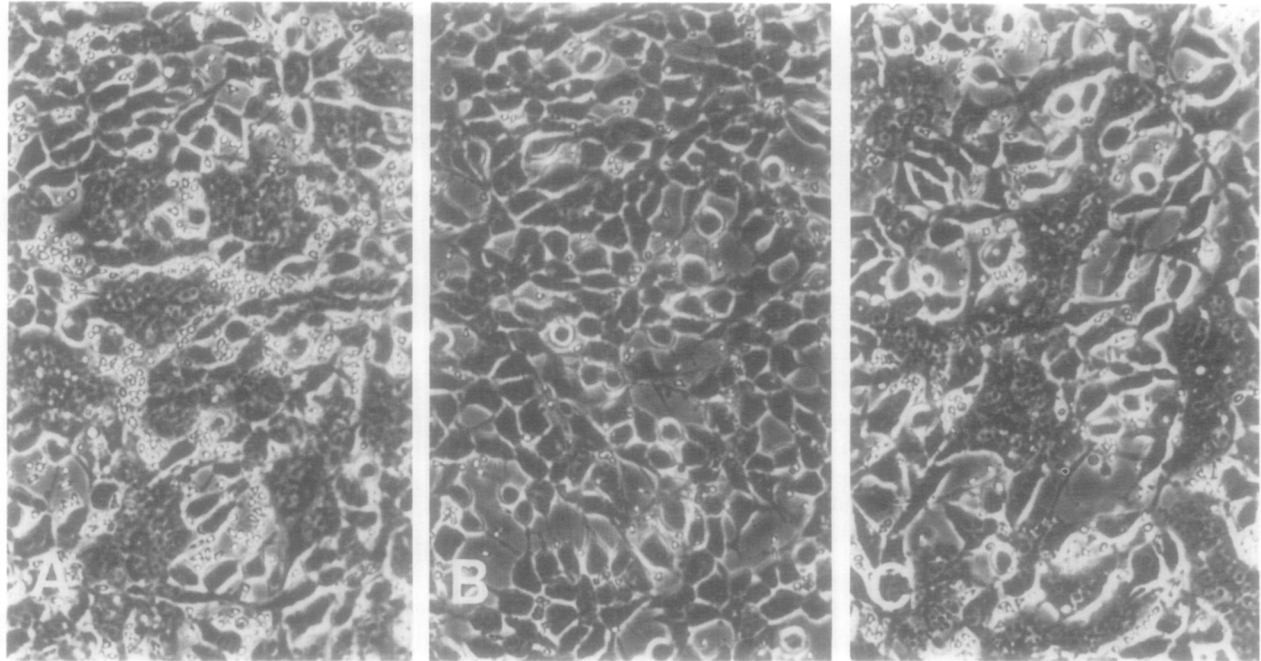
FIG. 6. Rosette formation of MHV-JHM-infected DBT cells with anti-SRBC antibody-coated SRBC. (A) Rosettes of MHV-JHM-infected DBT cells with rabbit anti-SRBC antibody-coated SRBC. (B) Uninfected DBT cells do not form rosettes with rabbit anti-SRBC antibody-coated SRBC. (C) Goat anti-S antibody inhibited rosette formation of MHV-JHM-infected DBT cells.

ployed by Wege *et al.* (1988) to isolate MHV-JHM variants resistant to anti-S neutralizing antibodies. They isolated their variants from supernatants of MHV-JHM-infected hybridoma cell cultures which were secreting neutralizing monoclonal antibodies. We infected 10^7 hybridoma cells producing the anti-FcγR 2.4G2 monoclonal antibody with wt MHV-JHM at an m.o.i. = 1.

TABLE 1

NEUTRALIZATION OF MHV BY THE ANTI-FcγR 2.4G2 MONOCLONAL ANTIBODY

Virus	Titer (PFU/ml)	Antibody	50% Plaque reduction titer (μg/ml)
MHV-JHM	1.00×10^4	Anti-S 1.38.1 monoclonal antibody	0.82
MHV-JHM	6.00×10^3	Fetal bovine serum	1:2 dilution
MHV-JHM	3.00×10^3	Anti-surfactant monoclonal antibody	6.42
MHV-JHM	7.50×10^3	Anti-FcγR 2.4G2 monoclonal antibody	4.03
V5A13	1.25×10^3	Anti-FcγR 2.4G2 monoclonal antibody	8.06
V5A13.1	2.00×10^3	Anti-FcγR 2.4G2 monoclonal antibody	4.03
V4B11.3	2.25×10^3	Anti-FcγR 2.4G2 monoclonal antibody	8.06

After adsorption the hybridoma cells were washed and cultured in suspension for 9 days. Samples of the hybridoma supernatants were collected every 24 hr, frozen at $-70°$ and the titer of each sample was determined in the presence or absence of the anti-FcγR 2.4G2 monoclonal antibody. All viral samples were neutralized by the anti-FcγR 2.4G2 monoclonal antibody, suggesting that neutralization resistant variants were not selected (data not shown).

Inhibition of virus-induced cell fusion by anti-FcγR 2.4G2 monoclonal antibody

In order to assess the ability of the anti-FcγR 2.4G2 monoclonal antibody to inhibit MHV-JHM-induced cell fusion, we employed the contact fusion assay of Mizzen *et al.* (1983). In this assay, MHV-JHM-infected cells which express S (L-2 or DBT cells) were placed in contact with a 10-fold excess of uninfected indicator cells (also L-2 or DBT cells). Anti-FcγR 2.4G2 monoclonal antibody or goat anti-S antibody or anti-S 1.38.1 monoclonal antibody was added either after 1 or 2 hr after adsorption and were present through out the experiment. Fusion was assessed 5 hr after the addition of the indicator cells (Table 2). Contact with MHV-JHM-infected cells resulted in fusion of the indicator cells. Anti-FcγR 2.4G2 monoclonal antibody, goat anti-S antibody and anti-S 1.38.1 monoclonal antibody signifi-

Antibodies	Percentage inhibition	
	Exp. 1 (L-2 cells)	Exp. 2 (DBT cells)
None	—	—
Anti-Fcγ R 2.4G2		
monoclonal antibody[a]	72	93
Goat anti-S[b]	N.D.	94
Anti-S 1.38.1		
monoclonal antibody[a]	79	97

[a] Used at a concentration of 26 μg/ml.
[b] Used at a final dilution of 1:10.

cantly inhibited this process. This inhibition of spread of syncytia was not due to the secondary infection of indicator cells with virus, because at this m.o.i. cells did not show fusion earlier than 7 to 8 hr postinfection while our experiments were terminated 5 hr postinfection. Thus, the anti-FcγR 2.4G2 monoclonal antibody possessed the biological properties of the anti-S antibodies: all were able to neutralize the virus and to inhibit spread of syncytia in the contact fusion assay.

DISCUSSION

We have used recombinant vaccinia viruses to express the MHV S protein in cells of mouse, human, and rabbit origin. S protein synthesized in these diverse cell types all bind the anti-Fc receptor monoclonal antibody 2.4G2. Since this monoclonal antibody does not react with either human or rabbit FcR these results rule out the possibility that Fc receptor activity is due to the association of a cellular murine Fc receptor with the S protein. Furthermore, fibroblastic L cells do not contain FcR gene transcripts (Ravetch et al., 1986). It is almost certain that the Fc binding activity and the epitope recognized by 2.4G2 both reside on the S protein.

The molecular weight of the recombinant MHV S protein was 15 kDa lower than that of our wt MHV-JHM S protein in all three cell lines tested. This difference in size is due to a deletion in the MHV-JHM cDNA clones that were used to construct our recombinant vaccinia viruses (Schmidt et al., 1987; Parker et al., 1989; Gallagher et al., 1990). This deletion is from residues 453 to 595 using the sequence from MHV (Parker et al., 1989) as a point of reference (see Fig. 3). Thus, it is clear that the sequences between residues 453 and 595 do not contain the epitope responsible for molecular mimicry between S and FcR.

These results were verified by analyses performed on cells infected with 3 mutants deleted within the N-terminal half of the S gene (Fig. 4). The three variants and the S recombinant vaccinia virus are all deleted adjacent to a region of sequence similarity between MHV-JHM and FcR (Fig. 3) which has been suggested as a candidate for the domain conferring molecular mimicry of the FcR (Oleszak and Leibowitz, 1990). These results are consistent with the hypothesis that the Fc binding site is located in this domain.

Anti-Fc receptor antibody 2.4G2 has weak neutralizing activity, which cannot be detected using unconcentrated tissue culture supernatants. Multiple attempts to select mutants resistant to neutralization with anti-Fc receptor 2.4G2 (ammonium sulfate concentrated tissue culture supernatants) were unsuccessful, suggesting that the sequences involved in the molecular mimicry of the Fc receptor were essential for infectivity. This is also supported by our previous observation that the Fc binding activity of S is conserved amongst several strains of MHV.

A number of human and animal herpesviruses induce the expression of IgG Fc receptor activity on the surface of infected cells and enveloped virions (Watkins, 1964; Yasuda and Milgrom, 1968; Eizuru and Minamishima, 1988). For HSV-1, the FcR activity resides on a complex of two glycoproteins, gE and gI (Longnecker et al., 1987; Johnson et al., 1988). Expression of gE alone was sufficient for FcR activity; however, Fc binding was greatly enhanced by coexpression of gI (Bell et al., 1990; Hanke et al., 1990). Therefore, it has been proposed that HSV-1 induces two Fc receptors with different affinities. The Fc receptor activity of MHV S protein more closely resembles that of HSV gE since it appears to be of relatively low affinity. Furthermore, the activity seems to reside on only one MHV protein (this work, Oleszak and Leibowitz, 1990).

The anti-FcγR 2.4G2 monoclonal antibody is able to neutralize MHV infectivity and inhibit spread of syncytia (fusion from within) in MHV-infected L-2 or DBT cells. The regions of the S protein important for fusion have recently been identified (Gallagher et al., 1990; Weismiller et al., 1990; Routledge et al., 1991; Yoo et al., 1991). Additional experiments are needed to determine whether the FcR-like epitope is important for both neutralization and cell fusion, or if 2.4G2 directly neutralizes the virus, but interferes indirectly with another domain involved in virus-mediated fusion. Alternatively, the neutralizing activity of 2.4G2 may be due to blocking the membrane fusing activity of S, rather than inhibiting binding of S to the cellular receptor for MHV. The precise mechanism of coronavirus penetration and uncoating is unclear at this time, making it difficult to determine the precise stage of entry of MHV-JHM

that is blocked by the anti-FcR 2.4G2 monoclonal antibody.

Anti-S monoclonal antibodies that neutralize virus infectivity and/or inhibit spread of syncytia have been used to map epitopes involved in these functions. The data are somewhat contradictory, suggesting that monoclonal antibodies that neutralized the virus bond to different epitopes than those that inhibited cell fusion in some cases (Collins et al., 1982; Talbot et al., 1984; Daniel and Talbot, 1990) while other sets of monoclonal antibodies were able to simultaneously neutralize MHV-JHM and inhibit cell fusion (Wege et al., 1988). They concluded that domains responsible for cell fusion are probably in close proximity to the binding sites of neutralizing monoclonal antibody. Furthermore, it has been suggested that both activities may be associated with conformation-dependent epitopes.

Receptors for the Fc portion of IgG are present on a variety of cell types including macrophages, B lymphocytes, some T cells, or K cells and polymorphonuclear leucocytes (Mellman et al., 1983; Adams et al., 1984; Leslie, 1985). They perform a central role in immune defenses by providing a bridge between the humoral and cellular aspects of the immune response.

The antigenic mimicry observed here between E2 viral antigen and FcR may have important biological implications. It has been postulated that binding of non-specific polyclonal IgG to viral antigens exposed on the cell surface could mask these antigens from specific antibody by sterically hindering the attachment of such antibody (Adler et al., 1978). Such binding could reduce complement and cell mediated lysis of infected cells. These effects might allow virus-infected cells to avoid the host immune response, especially ADCC-mediated destruction, and potentially play a role in MHV persistent infections. This may be especially important in experimentally produced persistent infection of rats, since the binding of rat IgG to E2 appears to be of a higher affinity than the binding of mouse IgG.

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