*Virus Research*, 18 (1990) 99–108


*ADONIS* 0168170291000512

VIRUS 00622

# Localization of major neutralizing epitopes on the S1 polypeptide of the murine coronavirus peplomer glycoprotein

Sayaka Takase-Yoden [1], Tateki Kikuchi [1], Stuart G. Siddell [2] and Fumihiro Taguchi [1]

[1] *National Institute of Neuroscience, NCNP, 4–1-1 Ogawahigashi, Kodaira, Tokyo, Japan and* [2] *Institute of Virology, University of Wurzburg, Wurzburg, F.R.G.*

(Accepted 11 September 1990)

**Summary**

A recombinant baculovirus system has been used to express the amino terminal half of the murine coronavirus (JHMV) peplomer glycoprotein in insect cells. The expressed polypeptide is glycosylated and is recognized by a set of monoclonal antibodies (mAbs) specific for JHMV S protein. Three of these mAbs have a very high neutralizing activity for JHMV but not for other MHV strains. These results indicate that JHMV-specific, major neutralizing epitopes reside in the amino terminal S1 subunit of the peplomer glycoprotein.

Murine coronavirus; Peplomer; Monoclonal antibody

The peplomer or S protein of murine coronaviruses forms the projecting spikes on the surface of the virus particle. This protein is associated with attachment of the virus to the cell surface, the fusion of viral and cell membranes, and the elicitation of neutralizing antibodies (Collins et al., 1982; Wege et al., 1984; Vennema et al., 1990; Siddell et al., 1990). The S protein is also thought to be important for determining the pathogenic potential of the virus (Dalziel et al., 1986; Fleming et al., 1986; Wege et al., 1988).

*Correspondence to:* F. Taguchi, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan.

The murine hepatitis virus (MHV) S protein is a heterodimer consisting of two non-covalently bound subunits, the amino terminal S1 and the carboxy terminal S2, which are derived by proteolytic processing of an approximately 180,000 kDa precursor (Sturman et al., 1985). Cleavage is a host cell dependent event and is believed to be one of the processing steps necessary to activate the fusogenic capacity of the S protein (Frana et al., 1985).

In our attempts to understand the pathogenesis of MHV-JHMV (JHMV) infections, we consider it important to locate domains on the S protein which are immunodominant and responsible for inducing neutralizing antibodies. One approach, which is appropriate in the JHMV system, is to determine the binding sites of murine mAbs which are able to neutralize virus infectivity. In the experiments reported here we have prepared a series of mAbs specific for the JHMV S protein, selected those which are able to neutralize virus infectivity and determined their binding activities to the surface protein expressed by recombinant baculoviruses in insect cells.

A complete S gene cDNA was derived from mRNA isolated from DBT cells infected with the MHV/Wb1 isolate of JHMV. Within the S protein coding region this cDNA differs from the S gene sequence published by Schmidt et al. (1987) at positions 793, G → C; 794, C → G; 1090, T → C; and 1825, A → G (Siddell, unpublished data). The S gene was cut at a position located 18 bases downstream of the putative S protein cleavage site (Schmidt et al., 1987, Luytjes et al., 1987) and a universal translation terminator (ds [5′d (GCTTAATTAATTAAGC) 3′], Pharmacia) was ligated to the 3′ end of the 1.9 kb 5′ proximal S gene fragment, designated as $S^A$. *Bgl*II-linkers were added to the $S^A$ DNA and the fragment was inserted into the *Bam*HI site of the baculovirus transfer vector, pAcYMI (Matsuura et al., 1987), as shown in Fig. 1A.

The sequence of this construct, $pAcS^A$, was determined in the areas flanking the coronavirus cDNA and is shown in Fig. 1B. This sequence predicts that the initiation of the JHMV $S^A$ polypeptide should occur at the authentic S protein initiation codon and termination at a position within the universal terminator. This would add 5 amino acids (Gly-Ile-Trp-Leu-Asn) to the carboxyterminus of the $S^A$ polypeptide, which is 12 amino acids longer than the putative S1 subunit of the peplomer glycoprotein. The predicted length of the $S^A$ polypeptide was therefore 639 amino acids (71,800 kDa) and contained 10 putative N glycosylation sites. Using this construct recombinant baculoviruses were obtained using methods which have been described previously (Yoden et al., 1989).

In order to verify the expression of the $S^A$ polypeptide by the recombinant baculovirus in infected Spodoptera frugiperda (Sf) cells, we performed pulse-labeling/immunoprecipitation experiments. Briefly, Sf cells were infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 5 and pulse-labeled with $^3H$ leucine for 1 h at 24 h post-infection. Cytoplasmic lysates were immunoprecipitated with anti-JHMV sera and the labeled immune complex polypeptides were analysed by SDS polyacrylamide gel electrophoresis (Yoden et al., 1989, Taguchi et al., 1985). This analysis, shown in Fig. 2, demonstrates that an S protein specific polypeptide with an apparent molecular weight of approximately

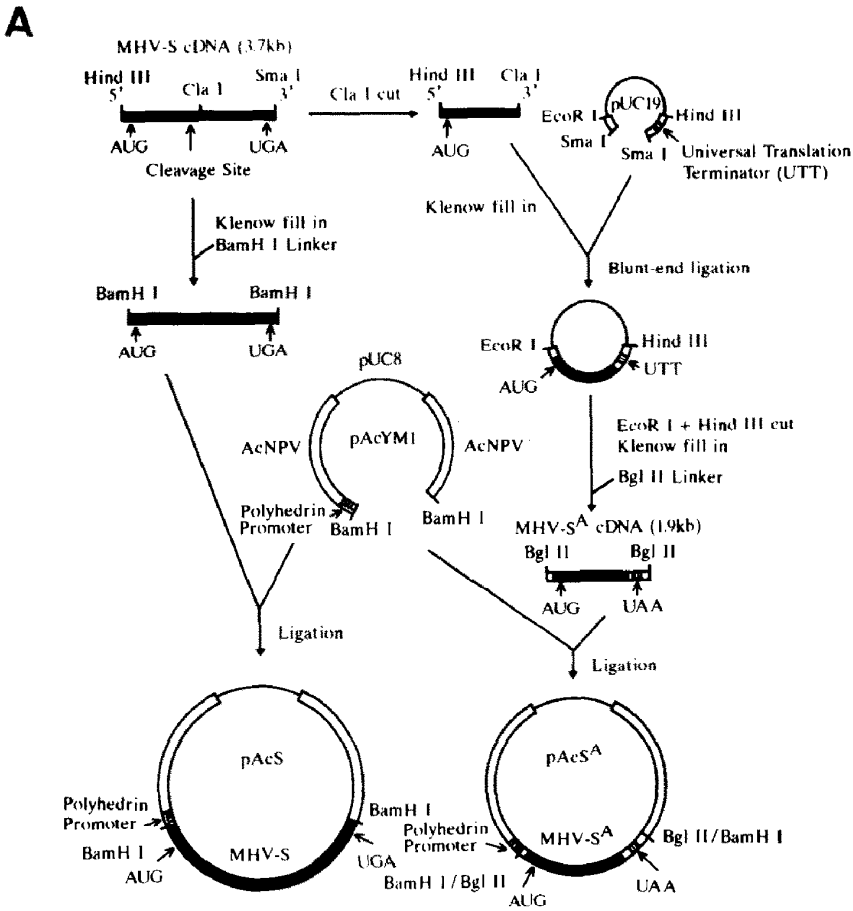


Fig. 1. Strategy to produce the recombinant baculovirus transfer vectors containing S or $S^A$ gene (pAcS, $pAcS^A$) (A) and nucleotide sequence of the regions flanking the coronavirus S gene cDNA in the construct $pAcS^A$ (B). The coronavirus S gene cDNA sequence is shown within brackets. The predicted amino acid sequence of the $S^A$ polypeptide carboxyterminus is also shown. Amino acids which are not encoded by coronavirus cDNA are underlined, as is the putative MHV S protein cleavage site.

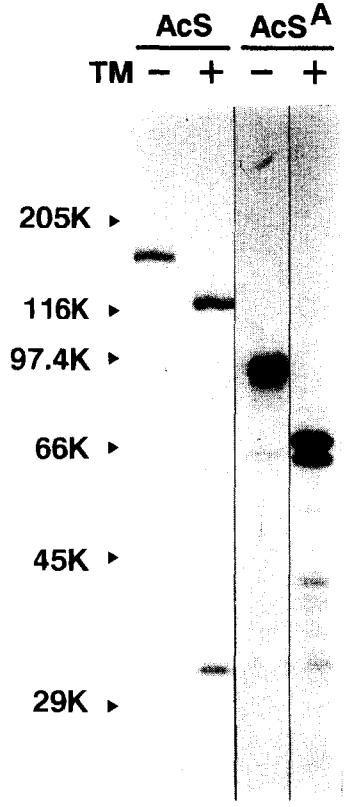


Fig. 2. Expression of the S and $S^A$ polypeptides by recombinant baculovirus in insect cells. The polypeptides synthesized in Sf cells infected with the recombinant baculoviruses, AcS and $AcS^A$, were labelled for 1 h with $^3$H-leucine (20 $\mu$Ci/ml, 140.5 Ci/mmol) at 24 h post-infection. Cell lysates were precipitated with anti-JHMV antibodies and analysed by 10% SDS-polyacrylamide gel electrophoresis. Sf cells infected with recombinant baculoviruses were untreated ($TM^-$) or treated with 10 $\mu$g/ml of tunicamycin ($TM^+$).

94,000 was synthesized in the recombinant baculovirus infected cells (lane $AcS^A$, $TM^-$). The complete JHMV S protein was also expressed using a recombinant baculovirus as has been described previously (Yoden et al., 1989). As is evident in Fig. 2 (lane, AcS, $TM^-$), the S protein is not cleaved in insect cells. Additionally, we have confirmed the expression of an immunoreactive S protein and $S^A$ polypeptide in insect cells by immunofluorescence (Fig. 3) and shown that the polypeptides are located on the cell surface (data not shown; Yoden et al., 1989). The molecular weight of the $S^A$ polypeptide synthesized in infected Sf cells suggests that carbohydrate side chains had been attached to the majority of the potential N-glycosylation sites. To confirm this conclusion the pulse-labeling experiments were repeated in the presence of tunicamycin at a concentration of 10 $\mu$g/ml. As shown in Fig. 2 (lane $AcS^A$, $TM^+$) the apparent mol. wt. of the $S^A$ polypeptide expressed in the presence of tunicamycin was reduced to approximately 70,000. This result is in

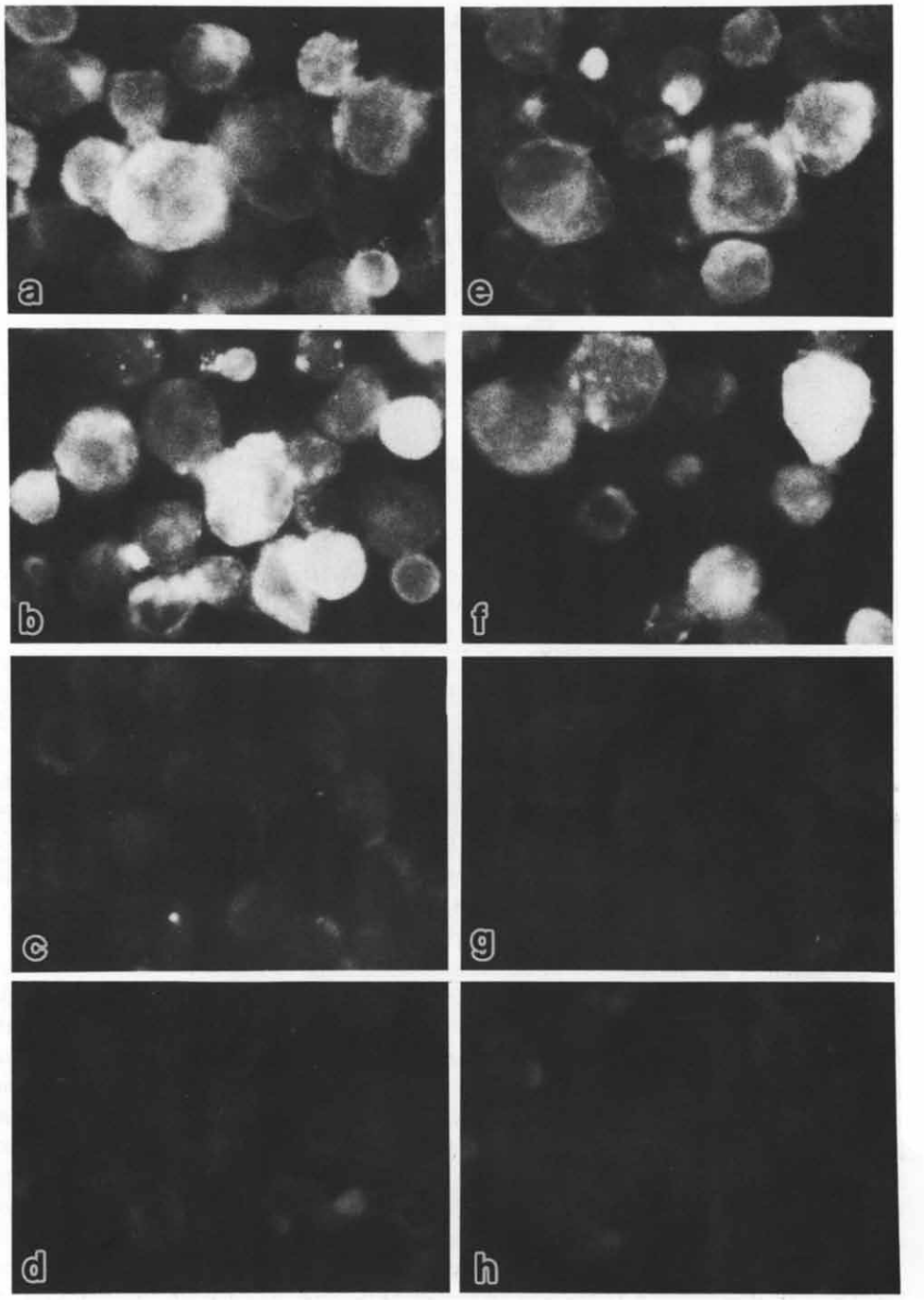


Fig. 3. Immunofluorescence staining of insect cells expressing S or $S^A$ protein by polyclonal anti-JHMV mouse serum and mAb (110-3). Sf cells were infected with recombinant baculoviruses carrying S gene (a and e), $S^A$ gene (b and f), or they were mock infected (d and h), or infected with wild type AcNPV (c and g). These cells were treated with polyclonal anti-JHMV mouse serum (a–d) or mAb (110-3) (e–h) and then stained with FITC-conjugated anti-mouse immunoglobulin for fluorescence microscopy.

TABLE 1

Reactivities of monoclonal antibodies to S proteins expressed by recombinant baculoviruses

| Monoclonal antibodies | Neutralization titer [a] | Reactivity to S proteins expressed by baculoviruses by immunofluorescence | | | | Reactivity to other MHVs [c] |
|---|---|---|---|---|---|---|
| | | S | $S^A$ | NPV [b] | Mock | |
| 32-4 | 100 | + | + | − | − | + |
| 110-3 | > 200,000 | + | + | − | − | − |
| 75-4 | > 200,000 | + | + | − | − | − |
| 8-1 | > 200,000 | + | + | − | − | − |
| 141-3 | < 50 | + | + | − | − | + |
| 104-2 | < 50 | + | + | − | − | − |
| 29-6 | < 50 | − | − | − | − | − |

[a] Neutralization was tested by 50% plaque reduction.
[b] Sf cells were infected with wild-type baculovirus.
[c] Other MHVs are MHV-A59, MHV-1, MHV-2 and MHV-3.

agreement with the size predicted for the non-glycosylated $S^A$ polypeptide from the cDNA sequence and also indicates that the polyclonal anti-JHMV serum contains components which recognized the non-glycosylated S polypeptide.

The monoclonal antibodies selected for this study are part of a set which have been prepared by immunization of BALB/c mice with the JHMV-cl-2 isolate (Taguchi et al., 1985). All of the these mAbs react in immunofluorescence and immuprecipitation with the S protein of JHMV-cl-2 and JHMV-sp4 (Taguchi and Fleming, 1989) and will be described in detail elsewhere (Taguchi et al., in preparation). As is shown in Table 1, all of mAbs except 32-4 and 141-3 have been shown to be JHMV specific, since they did not react with the other MHV strains so far examined. Three of four mAbs tested, 110-3, 75-4 and 8-1, had very high neutralizing activity (1 : > 200,000) and one mAb, 32-4, had low neutralizing activity (1 : 100) as judged by the 50% plaque reduction method (Taguchi et al., 1980). Three of the mAbs, 141-3, 104-2, and 29-6 were not able to neutralize virus infectivity. With the exception of mAb 29-6, all of the mAbs tested were shown by immunofluorescence to bind to the $S^A$ polypeptide expressed in recombinant baculovirus infected Sf cells (Table 1 and Fig. 3). These results clearly show that there are epitopes in the S1 polypeptide of the JHMV surface protein which are able to elicit the high neutralizing antibodies (major neutralizing epitopes) as well as epitopes for low- and non-neutralizing antibodies. At the present time we are not able to say why the mAb 29-6 failed to bind to the S protein synthesized in the baculovirus system. This might result, for example, from differences in the glycosylation of the S polypeptide in insect and mammalian cells.

The conclusions presented in this paper are in good agreement with a recent report by Buchmeier and colleagues who were able to locate neutralizing epitopes in the amino terminal half of the MHV-4 S protein (Parker et al., 1989). We believe that these combined results suggest that these epitopes are immunodominant. To

date, the binding site of all the neutralizing mAbs which we have mapped are located in the S1 polypeptide.

Recently, it has been reported that both the S1 and S2 subunits of the MHV-A59 S protein contain epitopes which bind neutralizing mAbs (Weismiller et al., 1990). This conclusion was based upon the binding specificities for a large set of S protein specific mAbs derived from immunizations with JHMV DL strain (Fleming et al., 1983) and MHV-A59 (Collins et al., 1982). However, it was conspicuous in this study that the strongly neutralizing, JHMV (DL) specific mAbs (J.1.2, J.2.2, J.7.2) did not react with MHV-A59, which could be due to the big antigenic difference between JHMV and A59 (Taguchi et al., 1982) and that the strongly neutralizing, MHV-A59 specific mAb (4B11.6) could not be assigned unequivocally to either the S1 or S2 subunit. Thus the binding specificities of the strongly neutralizing mAbs have not yet been determined. There is no doubt that neutralizing epitopes exist in both S1 and S2 subunits (Luytjes et al., 1989, Weismiller et al., 1990); however, we believe that our data are the first to show that MHV S protein epitopes which elicit strongly neutralizing antibodies are located in the S1 subunit. This conclusion is in agreement with similar studies on the avian infectious bronchitis virus (Cavanagh et al., 1986) and the porcine transmissible gastroenteritis virus (Delmas et al., 1990).

Finally, Makino et al (1987) have previously used the mAbs J.2.2 and J.7.2 to isolate recombinant viruses which escape neutralization. Originally, it was proposed, on the basis of T1 fingerprint analysis, that the crossover site of recombination in these isolates was located in the S2 subunit locus. However, recent sequence data (Banner et al., 1990) indicate that, in fact, recombination occurred in the S1 subunit locus. It therefore remains unclear whether the epitopes which bind J.2.2 and J.7.2 are located in the S1 or S2 subunits. It has recently been shown (Taguchi and Fleming, 1989) that these two mAbs do not react with the JHMV variants, sp-4 and JHM-X, which have been shown to contain genomic deletions in the S1 subunit region (Schmidt et al., 1987, Banner et al., 1990). It seems very likely, but not yet proven, that the epitopes for these mAbs reside in the region of the S1 subunit encompassed by these deletions.

## Acknowledgements

We thank Mrs H. Hirano for her technical assistance through the experiments.

## References

Banner, L.R., Keck, J.G. and Lai, M.M.C. (1990) A clustering of RNA recombination sites adjacent to a hypervariable region of the peplomer gene of murine coronavirus. Virology 175, 548–555.

Cavanagh, D., Davis, P.J., Darbyshire, J.H. and Peter, R.W. (1986) Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or hemagglutination-inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. 67, 1435–1442.

Collins, A.R., Knobler, R.L., Powell, H. and Buchmeier, M.J. (1982) Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral glycoprotein responsible for attachment and cell-cell fusion. Virology 119, 358–371.

Dalziel, R.G., Lampert, P.W., Talbot, P.J. and Buchmeier, M.J. (1986) Site specific alteration of murine hepatitis virus type-4 (MHV-4) peplomer glycoprotein E2 results in reduced neurovirulence. J. Virol. 59, 463–471.

Delmas, B., Rasschaert, D., Godet, M., Gelfi, J. and Laude, H. (1990) Four major antigenic sites of the coronavirus transmissible gastroenteritis virus are located on the amino-terminal half of spike glycoprotein S. J. Gen. Virol. 71, 1313–1323.

Frana, M.F., Benhke, J.N., Sturman, L.S. and Holmes, K.V. (1985) Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: host-dependent differences in proteolytic cleavage and cell fusion. J.Virol. 56–912–920.

Fleming, J.O., Trousdale, M.D., El-Zaatari, F.A., Stohlman, S.A. and Weiner, L.P. (1986) Pathogenicity of antigenic variants of murine coronavirus JHM selected with monoclonal antibodies. J. Virol. 58, 869–875.

Fleming, J.O., Stohlman, S.A., Harmon, R.C., Lai, M.M.C., Frelinger, J.A. and Weiner, L.P. (1983) Antigenic relationships of murine coronaviruses: analysis using monoclonal antibodies to JHM(MHV-4) virus. Virology 131, 296–307.

Luytjes, W., Geerts, D., Posthumus, W., Meloen, R. and Spaan, W. (1989) Amino acid sequence of a conserved neutralizing epitope of murine coronaviruses J. Virol. 63, 1408–1412.

Luytjes, W., Sturman, L.S., Bredenbeek, P.J., Charite, J., Van der Zeijst, B.A., Horzinek, M.C. and Spaan, W.J. (1987) Primary structure of the glycoprotein E2 of coronavirus MHV-A59 and identification of the trypsin cleavage site. Virology 161, 156–164.

Makino, S., Fleming, J.O., Keck, J.G., Stohlman, S.T. and Lai, M.M.C. (1987) RNA recombination of coronaviruses: localization of neutralizing epitopes and neuropathogenic determinants on the carboxyl terminus of peplomers. Proc. Natl. Acad. Sci. 84, 6567–6571.

Matsuura, Y., Possee, R.D. and Bishop, D.H.L. (1987) Expression of S-coded genes of lymphocytic choriomeningitis arenavirus using baculovirus vector. J. Gen. Virol. 68, 1233–1250.

Parker, S.E., Gallagher, T.M. and Buchmeier, M.J. (1989) Sequence analysis reveals extensive polymorphism and evidence of deletions within E2 glycoprotein gene of several strains of murine hepatitis virus. Virology 173, 664–673.

Schmidt, I., Skinner, M. and Siddell, S.G. (1987) Nucleotide sequence of the gene encoding the surface projection glycoprotein of coronavirus MHV-JHM. J. Gen. Virol. 68, 47–56.

Siddell, S.G., Stauber, R., Routledge, E. and Pfleiderer, M. (1990) Functional analysis of coronavirus MHV-JHM surface glycoproteins. In: M.A. Brinton, F.X. Heinz (Eds), New Aspects of Positive Strand RNA Viruses ASM Press, Washington, pp. 287–293.

Sturman, L.S., Ricard, C.S. and Holmes, K.V. (1985) Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusion activity of virions by trypsin and separation of two different 90 K cleavage fragments. J. Virol. 56, 904–911.

Taguchi, F. and Fleming, J.O. (1989) Comparison of six different coronavirus JHM variants by monoclonal antibodies against the E2 glycoprotein. Virology 169, 233–235.

Taguchi, F., Makino, S. and Fujiwara, K. (1982) Antigenic differentiation of mouse hepatitis viruses by neutralization test. Microbiol. Immunol. 26, 741–745.

Taguchi, F., Siddell, S.G., Wege, H. and ter Meulen, V. (1985) Characterization of a variant virus selected in rat brains after infection by coronavirus mouse hepatitis virus JHM. J. Virol. 54, 429–435.

Taguchi, F., Yamada, A. and Fujiwara, K. (1980) Resistance to highly virulent mouse hepatitis virus acquired by mice after low-virulence infection: enhanced antiviral activity of macrophages. Infect. Immun. 29, 42–49.

Vennema, H., Heijenen, L., Zijderveld, A., Horzinek, M.C. and Spaan, W.J.M. (1990) Intracellular transport of recombinant coronavirus spike proteins: implication for virus assembly. J. Virol. 64, 339–346.

Wege, H., Dorries, R. and Wege, H. (1984) Hybridoma antibodies to the murine coronavirus JHM: Characterization of epitopes on the peplomer protein (E2). J. Gen. Virol. 65, 1931–1942.

Wege, H., Winter, J. and Meyermann, R. (1988) The peplomer glycoprotein E2 of coronavirus JHM as a determinant of neurovirulence: definition of critical epitopes by variant analysis. J. Gen. Virol. 69, 87–98.

Weismiller, D.G., Sturman, L.S., Buchmeier, M.J., Fleming, J.O. and Holmes, K.V. (1990) Monoclonal antibodies to the peplomer glycoprotein of coronavirus mouse hepatitis virus identify two subunits and detect a conformational change in the subunit released under mild alkaline conditions. J. Virol. 64, 3051–3055.

Yoden, S., Kikuchi, T., Siddell, S.G. and Taguchi, F. (1989) Expression of the peplomer glycoprotein of murine coronavirus JHM using a baculovirus vector. Virology 173, 615–623.