



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

# Increased viral titers and enhanced reactivity of antibodies to the spike glycoprotein of murine coronavirus produced by infection at pH 6

Claude Daniel, Alain Lamarre, Pierre J. Talbot \*

*Virology Research Center, Institut Armand-Frappier, Université du Québec, 531 boulevard des Prairies, Laval, Québec, Canada, H7N 4Z3*

Accepted 22 July 1994

---

## Abstract

Infection of cell monolayers by murine coronavirus A59 at pH 6 rather than 7 yielded a ten-fold increase in the infectious titer and a remarkable enhancement of the reactivities of monoclonal and polyclonal antibodies against the spike glycoprotein in immunoblotting, immunoprecipitation and enzyme-linked immunosorbent assays. These observations are very useful for detecting antibodies against the S glycoprotein of coronaviruses and enhancing infectious titers.

*Keywords:* Coronavirus; Infectivity; S glycoprotein; Antibody; Antigenicity

---

## 1. Introduction

Murine hepatitis viruses (MHV) are members of the *Coronaviridae*, a family of enveloped, positive-stranded RNA viruses. These pathogens are associated with various respiratory, gastrointestinal and neurological infections in mammals and fowls (Wege et al., 1982). Indeed, infection of mice by the neurotropic strains A59 and JHM provides an excellent animal model of virus-induced diseases of the central nervous system (ter Meulen et al., 1989).

---

\* Corresponding author. Fax: (514) 686-5531. E-mail: Pierre\_Talbot@iaf.quebec.ca

Three structural proteins were identified on MHV-A59 virions (Sturman and Holmes, 1983): the nucleoprotein (N, 50 kDa), the membrane glycoprotein (M, 23 kDa) and the spike glycoprotein (S, 180 kDa; S1/S2, 90–96 kDa). The S glycoprotein is essential for infectivity; as it mediates viral attachment to a cell receptor (Williams et al., 1991) and virus-induced cell fusion (Vennema et al., 1990). Recent studies have shown that fusion induced in cell monolayers infected with MHV-A59 was reduced in slightly acidic culture medium (Sturman et al., 1990). The conformation of S was also shown to be affected by pH variations. This was demonstrated by aggregation of the C-terminal membrane-anchored S2 and the release of the N-terminal S1 after treatment of virions at pH 8.0 and 37°C (Sturman et al., 1990). Moreover, this treatment modified the antigenicity of S1 (Weismiller et al., 1990). In this study, we show that infection by MHV-A59 in culture medium at pH 6, instead of the usual pH 7, not only increased infectious viral titers, but also greatly enhanced the reactivity of the spike glycoprotein to antibodies.

## 2. Results and discussion

### *Increase of viral titers by infection at pH 6.0*

The murine hepatitis virus, strain A59, was obtained from the American Type Culture Collection (Rockville, MD), plaque purified twice, and passaged on DBT cells, a murine cell line established from a delayed brain tumor in a CDF1 mouse inoculated intracerebrally with strain Schmidt-Ruppin of Rous sarcoma virus (Hirano et al., 1974), as described previously (Daniel and Talbot, 1987). Viral growth kinetics in media initially adjusted to pH 6 or 7 were compared. DBT cell monolayers of identical surface and cell passage were infected at a multiplicity of infection of 0.001 in medium at either pHs. Media from different culture flasks were harvested at 2 h intervals after infection and frozen at  $-80^{\circ}\text{C}$  until assayed. Cell monolayers were lysed by three cycles of freezing and thawing in an equivalent volume of medium at the appropriate pH. Viral titers were determined by a plaque assay (Daniel and Talbot, 1987). As shown in Fig. 1, growth kinetics at either pHs were similar, with optimal titers reached between 16 and 18 h post-infection. However, viral titers were reproducibly 5–10 times higher in pH 6 samples throughout the course of the infection. Examination of virions in cell supernatants and lysates under the electron microscope did not reveal significant morphological changes that would indicate a variation in peplomer density or conformation (data not shown). Interestingly, cytopathic effects observed after infection, which consisted of cell-to-cell fusion into multinucleated syncytia, were very similar (extent and time course of syncytia formation) when cells were infected in medium at pH 6 or 7. Indeed, the DBT cells were almost completely fused at the time of optimal infectious viral titers. This is in contrast to the previously reported decreased virus-induced fusion index of 17-Cl-1 cells infected at pH 6 (Sturman et al., 1990). Presumably, different cell types are differentially susceptible to the effect of pH on the development of virus-induced cytopathic effects. Indeed, host-dependent differences in MHV-A59-induced cell fusion have been reported (Frana et al., 1985).

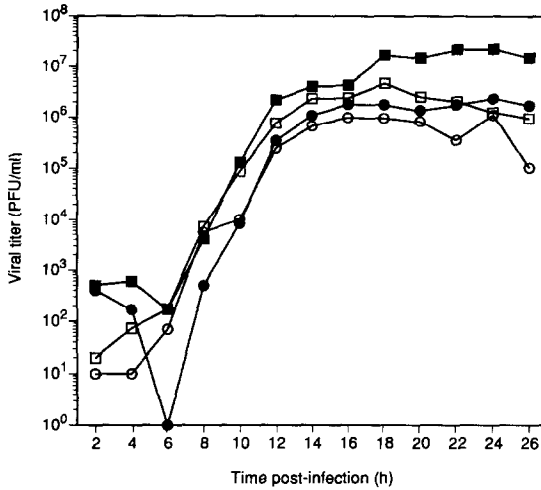


Fig. 1. MHV-A59 growth kinetics in medium at pH 6 or 7. Culture flasks containing DBT cell monolayers were infected with MHV-A59 in medium at pH 6 (■, □) or pH 7 (●, ○). At the indicated time, media from infected cell cultures were harvested and monolayers disrupted by freeze-thaw cycles. Culture supernatant (filled symbols) and cell-associated (open symbols) viral titers were evaluated by a plaque assay.

The antigenicity of the structural proteins of viruses produced by infection at pH 6 or 7 were evaluated by Western immunoblotting, immunoprecipitation, and ELISA. The monoclonal antibodies (MAbs) used in these experiments were raised against MHV-JHM or MHV-A59 as described previously (5B19: Collins et al., 1982; 7-10A, 4-11G and 1-9F: Daniel and Talbot, 1990; E17: Wege et al., 1984). The MAbs 5B19 and E17 were kindly provided by Drs. M.J. Buchmeier (Research Institute of Scripps Clinic, La Jolla, CA) and H. Wege (University of Würzburg, Germany), respectively. MAbs 5B19, 7-10A, 4-11G and 1-9F are neutralizing *in vitro* and, with the exception of 1-9F, inhibit fusion in MHV-infected cell cultures. The epitope recognized by MAb 1-9F has been mapped to S1, and those recognized by MAbs 5B19, E17, 7-10A and 4-11G to S2 (Daniel et al., 1993; Luytjes et al., 1989).

#### *Enhanced reactivity of antibodies in Western immunoblotting*

To prepare antigen for Western immunoblotting, DBT cell monolayers were infected as described above, and medium harvested at 18 h post-infection. Concentrated viral proteins (Daniel and Talbot, 1990) were solubilized in a sample buffer containing SDS and  $\beta$ -mercaptoethanol, separated by SDS-PAGE on a 7–15% linear polyacrylamide gel (Laemmli, 1970), and electro-transferred to nitrocellulose paper for 1 h at 35 V. This was sufficient to transfer all proteins, as shown by the absence of Coomassie blue staining of the gels after electrotransfer. Immunoblotting was performed with a mouse hyperimmune serum specific for MHV-A59 (diluted 1/1000), or with anti-S MAbs (5B19, culture supernatant diluted 1/10; E17, ascites fluid diluted 1/100), according to Talbot et al. (1984a), except that 0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS) was used as blocking and washing buffer, and bound MAbs were revealed by

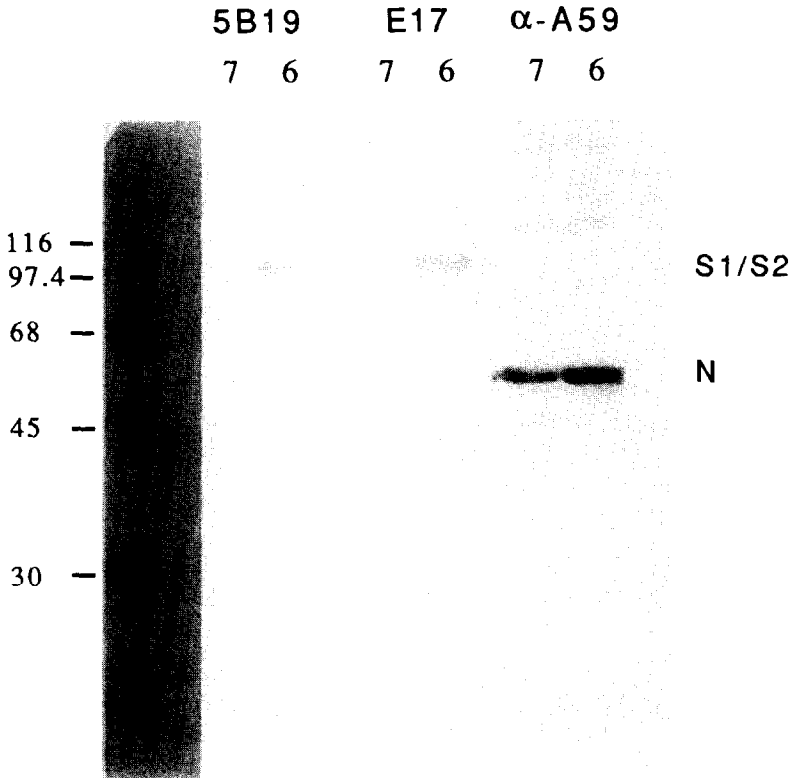


Fig. 2. Immunoblots of MHV-specific antibodies against viral proteins produced at pH 6 or 7. Bands at 96 kDa represent reactivity of antibodies ( $\alpha$ -A59: hyperimmune serum; 5B19 and E17: anti-S MAbs) against S1/S2 whereas bands at 50 kDa show reactivity against the nucleoprotein. Molecular mass standards (kDa) are shown on the left lane.

incubation with horseradish peroxidase-labeled, goat antibodies to mouse Ig (Kirkegaard & Perry Laboratory, Gaithersburg, MD; diluted 1/1000), followed by incubation with hydrogen peroxide and 4-chloro-1-naphthol (Hawkes et al., 1982). The reactivity of the anti-S MAbs (shown here: 5B19 and E17) against the S1/S2 (96 kDa) subunits of virus produced by infection at pH 6 was evident, whereas reaction against viral antigen produced by infection at pH 7 was barely detectable (Fig. 2). The MHV-A59 hyperimmune serum also reacted more intensely with antigen produced by infection at pH 6, although reactivity with the S glycoprotein was very faint. The prominent reactivity of this antiserum in Western blot against the nucleoprotein (50 kDa) suggests a greater immunogenicity of this protein or its inherent property to bear more linear epitopes.

#### *Enhanced reactivity of antibodies in radioimmunoprecipitation assay*

To determine whether this enhanced reactivity could be observed with antibodies that bind to discontinuous, conformation-sensitive epitopes, immuno-precipitation was carried out as described previously (Daniel and Talbot, 1990). Lysates of [ $^{35}$ S]methionine-labeled DBT cells infected with MHV-A59 at either pHs were immunoprecipitated with

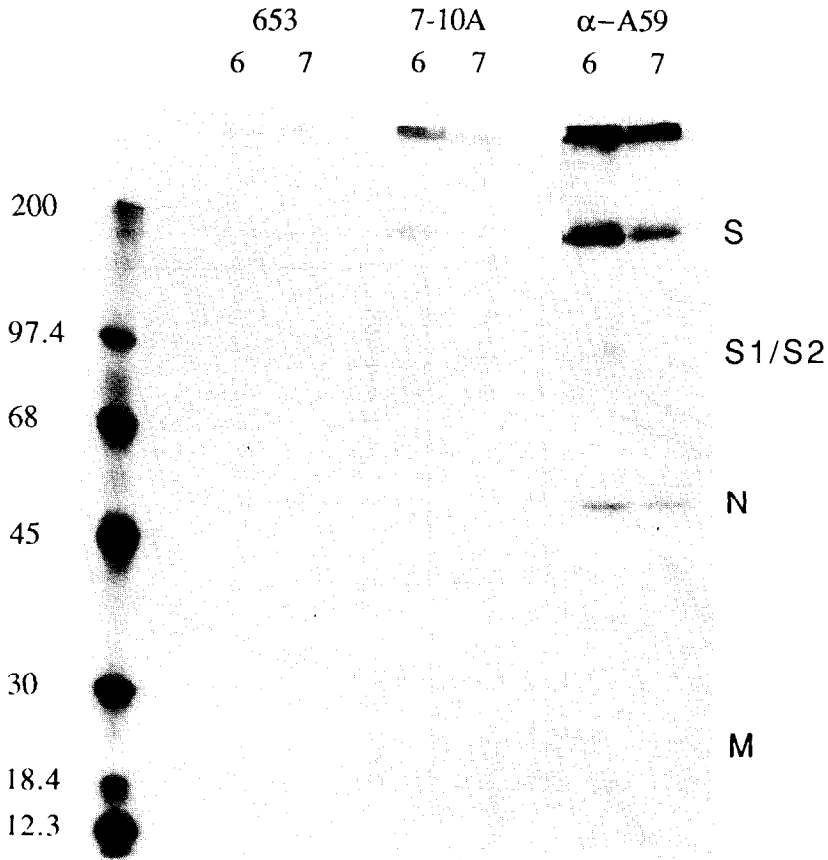


Fig. 3. Radio-immunoprecipitation of virus-infected cell lysates produced at pH 6 or 7 with MHV-specific antibodies. Metabolically labeled virus-infected cell lysates were immunoprecipitated with MHV-specific antibodies ( $\alpha$ -A59: hyperimmune serum; 7-10A: anti-S MAb; 653: control ascites fluid) and resolved by SDS-PAGE on a 7–15% gel.

a hyperimmune serum against MHV-A59 (10  $\mu$ l of a 1/10 dilution), anti-S or control hybridoma ascites fluids (10  $\mu$ l). From this experiment (Fig. 3), it appears that a large proportion of the anti-S antibodies in the MHV-A59 hyperimmune serum recognize conformational epitopes, since this antiserum reacted strongly with S in immunoprecipitation (Fig. 3) but not in Western immunoblotting (Fig. 2). Reactions against the nucleoprotein (N) were similar between the two antigens, whereas the membrane protein (M) was hardly detectable. It is also obvious that the enhanced reactivity was observed with discontinuous epitopes, since MAb 7-10A and MHV-A59 hyperimmune serum reacted more strongly with S and S1/S2 of virus produced at pH 6.

#### *Enhanced reactivity of antibodies in ELISA*

Finally, the reactivity of antisera or MAbs against antigens produced by infection at pH 6 or 7 were compared in ELISA. The antigen used in the ELISA was prepared by

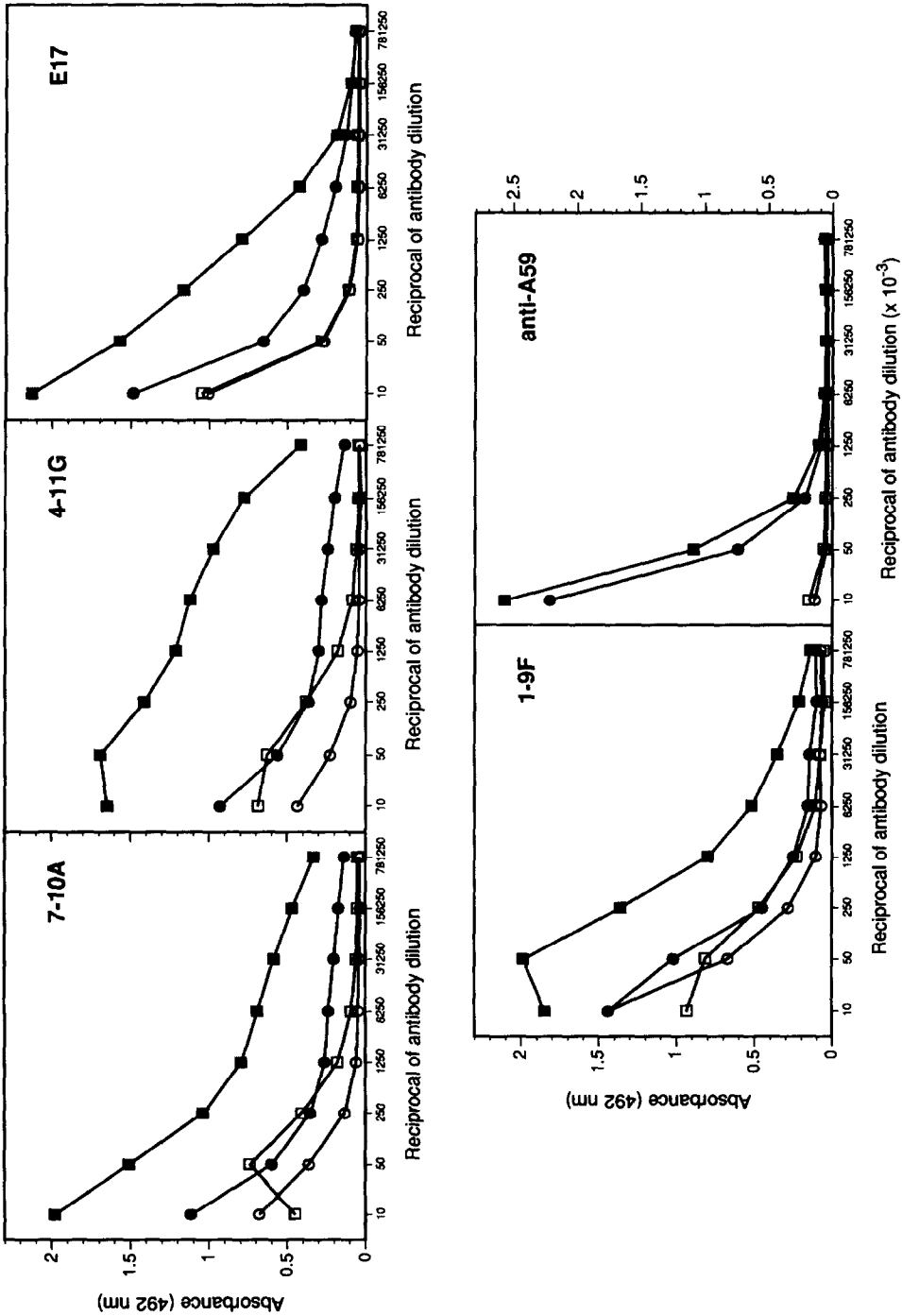


Fig. 4. Reactivity of MHV-specific antibodies against viral antigens produced at pH 6 or 7. Microplates were coated with virus or mock-infected (filled and open symbols, respectively) cell lysates produced at pH 6 (■) or pH 7 (●) and incubated with serial five-fold dilutions of ascites fluids of various MHV-A59 anti-S MAbs or hyperimmune serum (anti-A59), as indicated on the figure.

infecting and mock-infecting L929 cell monolayers with MHV-A59 in medium at pH 6 or 7. Confluent monolayers were lysed by freezing and thawing, sonicated  $2 \times 1$  min in a BranSonic 2000 sonicating water bath (Branson Cleaning Equipment Company, Shelton, CT), and clarified by centrifugation at  $10\,000 \times g$  for 30 min. Membranes (viral and cellular) were pelleted by ultracentrifugation at  $100\,000 \times g$  for 1 h, resuspended in PBS (1/50 of the original volume) and total protein concentrations evaluated. ELISA with hyperimmune serum against MHV-A59, or anti-S MAbs was performed according to previously published procedures (Talbot et al., 1984b). As shown in Fig. 4, the anti-S MAbs reacted much more strongly with antigen produced by infection at pH 6, in contrast to the MHV-A59 hyperimmune serum, which reacted equally well with both antigens. In the representative experiments shown in Fig. 4, the reactivity of MAb 7-10A was enhanced 625-fold, and those of MAbs 1-9F, E17, and 4-11G, 60-fold, 25-fold and 5-fold, respectively. In ELISA, MHV-JHM antigen produced by infection at pH 6 also showed enhanced antigenicity, in comparison with pH 7-produced antigen, using JHM-specific MAbs (data not shown).

### 3. Conclusion

From these results, it is concluded that the optimal virus stability at pH 6 observed previously (Daniel and Talbot, 1987) contributed to the higher titer observed for virus produced at this pH, since prolonged cell culture survival resulting from reduced cell fusion was not observed in DBT cells. Furthermore, antibody reactivity to the S glycoprotein produced after infection in acidic medium was much enhanced. Comparison of viral antigens produced at pH 6.0 or 7.0 by SDS-PAGE and Coomassie blue staining did not reveal significant differences in the total amount of S glycoprotein (data not shown). However, further studies using the S protein purified in native conditions will be needed to demonstrate that a structural modification of the protein produced at pH 6.0 is responsible for the enhanced reactivity of antibodies.

This study shows that the production of murine coronavirus antigen in slightly acidic medium would be very advantageous for the detection of anti-S reactivity in sera containing antibodies of low titers and/or low affinity. Indeed, this procedure was successfully used to reveal cross-reactivity between anti-peptide antibodies and the S glycoprotein (Daniel et al., 1994). Moreover, the increased infectious viral titers observed after infection at pH 6 will have an important practical value for studies with these viruses.

### Acknowledgments

We thank Francine Lambert for excellent technical assistance. We also thank M.J. Buchmeier and H. Wege for the generous gift of their respective MAbs. This study was supported by grant OGP0042619 from the Natural Sciences and Engineering Research Council of Canada (NSERC) and in part by grant MT-9203 from the Medical Research Council of Canada (MRC) to P.J.T., who also acknowledges a scholarship from NSERC.C.D. and A.L. are grateful to NSERC and 'Fond de la Recherche en Santé du Québec', and 'Fond pour la Formation des Chercheurs et l'Aide à la Recherche', respectively, for studentship support.



## References

- 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.
- Daniel, C. and Talbot, P.J. (1987) Physico-chemical properties of murine hepatitis virus, strain A59. *Arch. Virol.* 96, 241–248.
- Daniel, C. and Talbot, P.J. (1990) Protection from lethal coronavirus infection by affinity-purified spike glycoprotein of murine hepatitis virus, strain A59. *Virology* 174, 87–94.
- Daniel, C., Anderson, R., Buchmeier, M.J., Fleming, J.O., Spaan, W.J.M., Wege, H. and Talbot, P.J. (1993) Identification of an immunodominant linear neutralization domain on the S2 portion of the murine coronavirus spike glycoprotein and evidence that it forms part of a complex tridimensional structure. *J. Virol.* 67, 1185–1194.
- Daniel, C., Lacroix, M. and Talbot, P.J. (1994) Mapping of linear antigenic sites on the s glycoprotein of a neurotropic murine coronavirus with synthetic peptides: a combination of nine prediction algorithms fails to identify relevant epitopes and peptide immunogenicity is drastically influenced by the nature of the protein carrier. *Virology* (in press).
- Frana, M.F., Behnke, 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.
- Hawkes, R., Niday, E. and Gordon, J. (1982) A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119, 142–147.
- Hirano, N., Fujiwara, K., Hino, S. and Matumoto, M. (1974) Replication and plaque formation of mouse hepatitis virus (MHV-2) in mouse cell line DBT culture. *Arch. Ges. Virusforsch.* 44, 298–302.
- Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Luytjes, W., Geerts, D., Posthumus, W., Melen, R. and Spaan, W. (1989) Amino acid sequence of a conserved neutralizing epitope of murine coronaviruses. *J. Virol.* 63, 1408–1412.
- Sturman, L.S. and Holmes, K.V. (1983) The molecular biology of coronaviruses. *Adv. Virus Res.* 28, 35–112.
- Sturman, L.S., Ricard, C.S. and Holmes, K.V. (1990) Conformational change of the coronavirus peplomer glycoprotein at pH 8.0 and 37°C correlates with virus aggregation and virus-induced cell fusion. *J. Virol.* 64, 3042–3050.
- Talbot, P.J., Knobler, R.L. and Buchmeier, M.J. (1984a) Western and dot immunoblotting analysis of viral antigens and antibodies: application to murine hepatitis virus. *J. Immunol. Methods* 73, 177–188.
- Talbot, P.J., Salmi, A.A., Knobler, R.L. and Buchmeier, M.J. (1984b) Topographical mapping of epitopes on the glycoproteins of murine hepatitis virus-4 (strain JHM): correlation with biological activities. *Virology* 132, 250–260.
- ter Meulen, V., Massa, P.T. and Dorries, R. (1989) Coronaviruses. In: R.R. McKendall (Ed), *Viral Disease. (Handbook of Clinical Neurology, vol. 12)*, pp 439–451. Elsevier Science, Amsterdam.
- Vennema, H., Heijnen, L., Zijderfeld, A., Horzinek, M.C. and Spaan, W.J.M. (1990) Intracellular transport of recombinant coronavirus spike proteins: Implications 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., Siddell, S.H. and ter Meulen, V. (1982) The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* 99, 165–200.
- 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.
- Williams, R.K., Jiang, G.S. and Holmes, K.V. (1991) Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. *Proc. Natl. Acad. Sci. USA* 88, 5533–5536.