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.

Journal of Virological Methods, 6 (1983) 255–265
Elsevier

A SENSITIVE RADIOIMMUNOASSAY FOR THE DETERMINATION OF ANTIBODIES TO MOUSE HEPATITIS VIRUS

J.L. LEIBOWITZ[1], L.S. FUNG[2] and G.A. LEVY[2]

[1]*Department of Pathology, University of California, San Diego, La Jolla, CA 92093, U.S.A.*, [2]Liver Disease *Unit, Sunnybrook Medical Centre, University of Toronto, Toronto, Ontario, Canada M4N 3M5*

(Accepted 8 February 1983)

A solid-phase radioimmunoassay is described for the detection of antibodies to mouse hepatitis virus. Viruses were purified by velocity and isopycnic gradient centrifugation and 96-well plastic plates were coated with viral antigens. To allow the detection of most serotypes of low titered antisera, a pool of antigens from several viral serotypes were employed. The second antibody, an affinity-purified goat antimouse immunoglobulin, detects IgG, IgM and IgA antibodies. This assay is more sensitive than either the plaque reduction assay or the commercially available enzyme-linked immunosorbant assay and proved to be useful for screening mouse colonies for the presence of mouse hepatitis virus, following seroconversion in experimental animals and in the production of monoclonal antibodies to both structural and nonstructural proteins.

mouse hepatitis virus radioimmunoassay coronavirus

INTRODUCTION

Coronaviruses are a group of enveloped RNA viruses that cause a broad spectrum of diseases in their natural hosts (Tyrell, 1978; Robb and Bond, 1979). These diseases include encephalitis, hepatitis, interstitial pneumonitis, nephritis and enteritis (Piazza, 1969). The spectrum and severity of disease produced by coronaviruses vary with age and genetic background of the host, route of infection and size of the virus inoculum. Resistance to severe acute infection appears to depend, at least in part, on the potential for replication of mouse hepatic virus (MHV) in cells of the reticuloendothelial

Abbreviations: MHV, mouse hepatitis virus; PBS, phosphate buffered saline; RIA, radioimmunoassay; SPRIA, solid-phase radioimmunoassay; ELISA, enzyme-linked immunosorbant assay; Ig, immunoglobulin; MOPS, 3-(*N*-morpholine)-3-propanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; TES, *N*-Tris (hydroxymethyl)-3-methyl-2-aminoethane sulfonic acid; DMEM, Dulbecco's modified Eagle's medium; PFU, plaque forming units; EDTA, disodium ethylene diamine tetraacetate; BSA, bovine serum albumin; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PEG, polyethylene glycol; GAMG, goat antimouse immunoglobulin.

system, a genetically restricted event (Virelizier and Allison, 1976; Weiser and Bang, 1976). Recently, interest in MHV as a model for human hepatitis has been revived as the result of observations implicating variables at the level of the host as important in the pathogenesis of the disease (Krzystyniak and Dupuy, 1981; Levy et al., 1982).

A major problem in MHV research has been the difficulty in obtaining experimental animals which have not been previously exposed to the virus as indicated by the presence of antibodies. Previously, three methods utilized for determining antibody to MHV have been a plaque neutralization assay, immunofluorescence and complement fixation (Robb and Bond, 1979). Neutralization and immunofluorescence, although highly sensitive, are time consuming and complement fixation is too insensitive to be useful. Recently an enzyme-linked immunosorbant assay (ELISA; Kraaijeveld et al., 1980) has been described and has become commercially available, but is too expensive for screening large numbers of mice to be used in a research laboratory working on MHV. We have therefore developed a highly sensitive and reproducible radioimmunoassay which can be used for both screening large numbers of mice and studying the molecular biology and immunopathology of MHV.

MATERIALS AND METHODS

Cells

The origin and growth of the murine cell lines 17 CL1, DBT and L2 cells have been described previously (Levy et al., 1981a). The cells were routinely propagated in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories Inc., Rockville, MD) supplemented with 10% newborn calf serum (Biocell Laboratories, Carson City, CA) and 25 μg/ml chlortetracycline hydrochloride grade II (Sigma Chemical Co., St. Louis, MO) and buffered with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 3-(*N*-morpholine-3-propanesulfonic acid (MOPS), *N*-Tris (hydroxymethyl) -3-methyl-2-aminoethane sulfonic acid (TES) and 4 mM glutamine (Sigma Chemical Co.).

Virus

The origin and growth of the A59 and JHM strains of MHV have previously been described (Robb and Bond, 1979). MHV-1, MHV-S and MHV-3 were obtained from the American Type Culture Collection, Rockville, MD. Viruses were plaque purified twice on monolayers of DBT cells and seed stocks prepared.

Working stocks were grown in 17 CL1 cells infected at a multiplicity of infection (MOI) of 10^{-4} plaque forming units (PFU)/cell. Viruses were assayed on monolayers of L2 cells in a standard plaque assay as described previously (Levy et al., 1981a).

Viral purification

A59, MHV-1, MHV-3 and MHV-S were grown to high titer on monolayers of 17 CL1 cells. Viruses were harvested by 1 cycle of freeze thawing and clarified by centrifugation at 4500 × *g* for 1 at 4°C. For some preparations an aliquot of [^{32}P]orthophosphate labelled virus was added to follow the virus through the purification. The supernatant was recovered and the virus was precipitated by adding to each 10 ml, 1.1 ml 5M NaCl and 5.55 ml 30% (w/v) polyethylene glycol (PEG) 6000 in MOPS-saline disodium ethylene diamine tetracetate (EDTA), pH 6.8 (10 mM MOPS, 1 mM EDTA, 150 mM NaCl, Baker Scientific Co., Philipsburg, NJ; Wege et al., 1978). The solution was stirred on ice for 30 min and the virus pelleted by centrifugation at 9250 × *g* at 4°C for 30 min. The pellet was resuspended in a small volume of MOPS-saline-EDTA and overlayed onto 11 ml of a 5–25% (w/w) K-tartrate gradients and centrifuged at 35,000 rpm in an SW 41 rotor for 45 min at 4°C. The gradient was fractionated and aliquots counted in a liquid scintillation counter. The fractions containing virus were pooled, diluted to a volume of 4 ml with MOPS-saline-EDTA, overlayed onto 8 ml of a 10–40% (w/w) K-tartrate gradient and centrifuged for 4 h at 4°C at 35,000 rpm in an SW41 rotor. The gradient was fractionated and the virus peak located as before. The pooled virus was diluted to 5 ml with MOPS-saline-EDTA and pelleted at 45,000 rpm for 30 min at 4°C in an SW50.1 rotor. The viral pellet was resuspended in 0.5 ml carbonate buffer by 3 bursts of sonication at 4°C. Viral protein concentration was determined in a modified Lowry assay as described previously (Levy et al., 1981b).

Plaque neutralization assay

Virus was assayed on monolayers of L2 cells in a standard plaque assay as described previously (Levy et al., 1981a). For neutralization, sera which was heat inactivated at 56°C for 30 min was incubated with 100 PFU of MHV for 30 min at 4°C. Two-hundred µl of the mixture were then layered onto confluent monolayers of L2 cells in 6-well plastic cluster dishes (Linbro Plastics, McLean, VA) for 30 min at 22°C and then overlayed with 1% agarose in DMEM supplemented with 2% fetal calf serum (FCS). The plates were incubated for 48 h at 37°C in a 5% CO_2 incubator and then stained with 0.1 gm% crystal violet in 20% ethanol for enumeration of plaques.

Solid phase radioimmunoassay (SPRIA)

Flexible 96-well microtiter plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with either MHV strain A59 or a mixture of MHV-A59, MHV-1 and MHV-S virus which had been purified by gradient centrifugation. Individual wells were coated with antigen diluted in 100 µl of carbonate buffer pH 9.6 and were incubated overnight at 4°C in a humid chamber. The antigen was cross-linked using 10

μl of a 10 mg/ml solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Calbiochem-Behring Corp., La Jolla, CA). The following morning, the plates were washed repeatedly with 200 μl aliquots of 1% bovine serum albumin (BSA), 0.05% Tween 20 and 0.02% sodium azide in phosphate buffered saline (PBS, pH 7.4) (SPRIA buffer) and the carbodiimide was inactivated with 0.1 M NH_4Cl for 1 h and the plate was repeatedly washed with SPRIA buffer. ^{125}I-labelled affinity purified goat anti-mouse immunoglobulin (GAMG) (specific activity 3.2×10^{10} cpm/mg) was then added and the plates incubated for 1 h at 37°C. The plates were then washed and counted in a gamma counter. Appropriate positive and negative sera were included in all assays as controls and all samples were assayed in triplicate.

RESULTS

Purifications

Viruses were grown on 17 CL1 cells as described. MHV-A59 grew to a tenfold higher titer than MHV-JHM, MHV-1, MHV-S and MHV-3. The virus was followed through purification by the presence of small amounts of ^{32}P-labelled virus. However at each step a distinct opalescent band could be seen which corresponded to the ^{32}P-labelled virus. The combination of velocity and isopycnic sedimentation K-tartrate density gradients purified virus to apparent homogeneity (Fig. 1). The purity

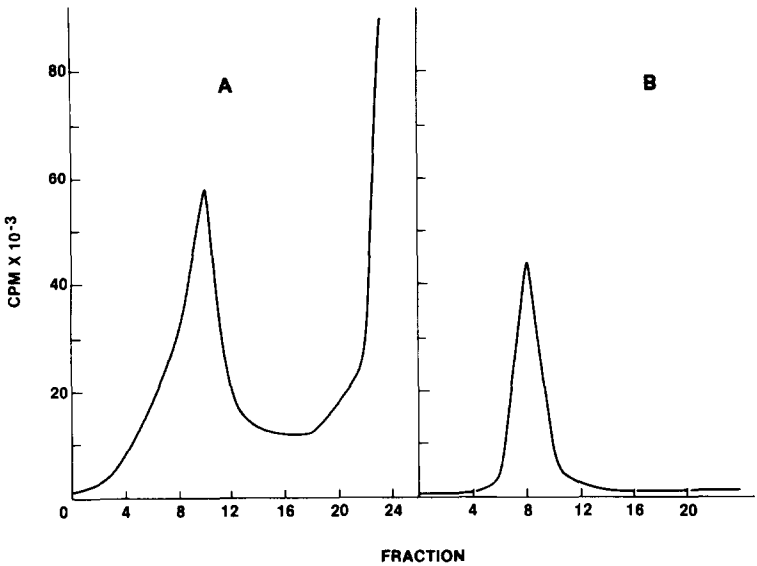

Fig. 1. Purification of MHV on K-tartrate gradients. (A) MHV was layered on a 5–25% K-tartrate gradient and centrifuged at 35,000 rpm for 45 min. CPM represents the mean of triplicate samples of gradient fractions. (B) Fractions 8–10 from gradient in A. were layered over 10–40% K-tartrate gradient and centrifuged at 35,000 rpm for 4 h. CPM represents the mean of triplicate samples of gradient fractions. Virus was recovered in fractions 6–10.

of the virus preparations was determined by polyacrylamide gel electrophoresis according to the method of Maizel (1971). On a sodium dodecyl sulfate (SDS) resolving polyacrylamide gel (10%) with a 3% stacking gel, the viral protein nucleocapsid (N), and the E2 glycoprotein were evident in high amounts. Only trace amounts of E1 glycoprotein could be visualized. No contaminating proteins were detected on the gel

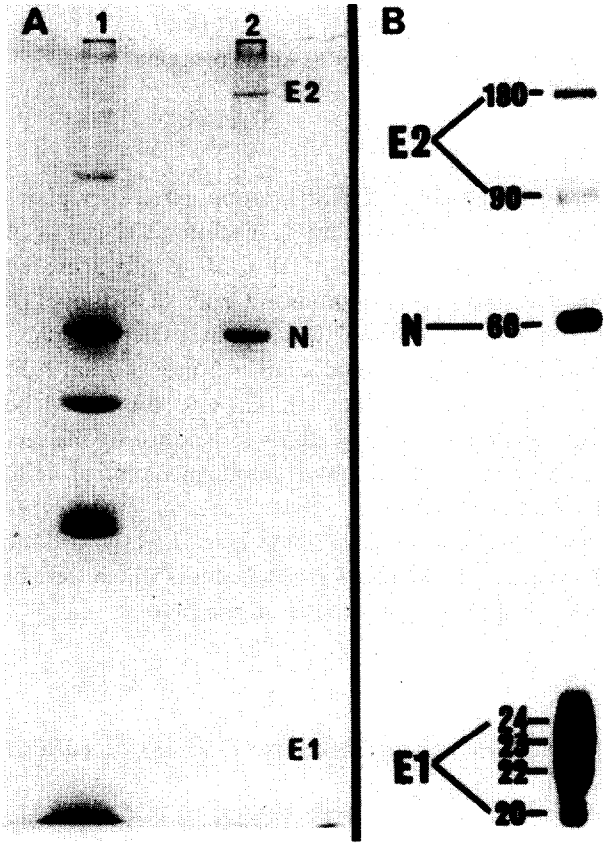

Fig. 2. Polyacrylamide gel analysis of viral preparations. (A) Twelve μg of purified MHV-A59 in SDS (non-reduced) were applied to a 3% stacking gel and electrophoresed through a 10% resolving gel at 60 V for 18 h. The gel was fixed in 10% glacial acetic acid and 30% methanol and stained with 0.05% Coomassie blue in 10% glacial acetic acid and 10% methanol. Lane 1 represents molecular weight standards. Top to bottom: Myosin (200,000 daltons), β-galactosidase (130,000 daltons), BSA (68,000 daltons), actin (42,000 daltons) and DNAse 1 (31,000 daltons). Lane 2 shows E2 glycoprotein (180,000 daltons) and nucleocapsid protein (N) (50,000 daltons) with an amount of E1 glycoprotein (24,000 daltons). (B) 17 CL1 cells were infected with MHV strain A59 at an MOI of 0.2 and labelled from 6 to 18 h post-infection with [^{35}S]methionine, 100 μCi/ml in methionine-free medium. Virus was then purified from the culture fluids as described in the Materials and methods. The purified virus (reduced) was electrophoresed on a 10% polyacrylamide gel and fluorographs prepared as described previously (Maizel, 1971). In reduced form, E2 glycoprotein (180,000 and 90,000 daltons), nucleocapsid protein (N) (60,000 daltons) and E1 glycoprotein (20,000 to 24,000 daltons) are easily seen.

(Fig. 2). To further rule out contamination in the preparation, [^{35}S]methionine labelled virus was electrophoresed through a 10% polyacrylamide gel and analysed by autoradiography. All three of the structural viral proteins were visualized and no contamination was noted (Fig. 2). We were able to recover 10–15% of the infectious virus but this probably represents a minimum estimate of the total recovery of viral antigen as it is based on biologic activity, and certainly much of this could be lost by chemical and physical treatment during purification. By the modified Lowry technique, 4×10^9 PFU was found to correspond to 220 ± 30 μg of protein.

Antibody specificity

To determine the specificity of the ^{125}I-labelled GAMG probe, microtiter plates were coated with 0.732 ng of mouse IgG, 1 ng of IgM lambda myeloma 104-E, 1 ng of IgM kappa myeloma HP-76 and 1.5 ng of IgA myeloma MOP-C 315 kindly provided by Dr. Brian Underdown (Table 1). Two-hundred thousand cpm of ^{125}I-labelled GAMG was added and the wells counted. The antibody was capable of detecting IgG most effectively and also detected IgM as determined against 2 monoclonal IgM molecules. The detection of kappa was greater than lambda and this could be consistent with the antibody having greater anti-kappa characteristics or by the fact that kappa myeloma protein binds better to the plates than lambda myeloma protein. The probe also detected IgA antibody, although at a lower efficiency than other immunoglobulin classes. The GAMG probe was shown not to react directly with the viral protein coated plates as demonstrated by low background counts which were of the same magnitude as those when antigen was omitted from the plate.

Radioimmunoassay (RIA)

The microtiter plates were coated with varying amounts (1–50 ng) of purified

TABLE 1

Characterization of ^{125}I-labelled goat antimouse immunoglobulin probe

Antibody tested	CPM $\times 10^{-2}$
PBS	6.5 ± 2.8
IgG	$1{,}428 \pm 33$
IgM Lambda	378 ± 21
IgM Kappa	$1{,}043 \pm 71$
IgA	282 ± 8

Individual wells of multiwell flexible plates (Dynatech Laboratories Inc., Alexandria, VA) were coated with 0.732 ng of normal mouse IgG, 1 ng of IgM lambda myeloma 104-E, 1 ng of IgM kappa myeloma HP-76 and 1.5 ng IgA myeloma MOP-C 315 in triplicate kindly provided by Dr. Brian Underdown. 200,000 CPM of ^{125}I-goat antimouse Ig was added to each plate. CPM represents the mean of samples in triplicate.

MHV-A59 and tested with a positive antibody against MHV-A59 to determine the optimal antigen concentration. Antibody could be detected with as little as 1 ng of antigen (data not shown) and could be easily detected with 10 ng (Fig. 3) but optimal sensitivity was observed at 25 ng antigen (Fig. 3), with no significant increase noted at 50 ng of antigen per well (Fig. 3). Therefore, in all subsequent determinations an antigen concentration of 25 ng/100 μl per well was used. Known positive and negative sera to MHV-A59 were titered against antigen-coated plates. The assay was optimal at a serum dilution of 1/200 to 1/400 and a dilution of 1/250 was chosen to screen all sera. A positive sera was defined as greater than 2 SD above the mean of 5 negative control sera.

As shown in Fig. 4, under standard conditions of our assay, a known positive sera could be detected at a dilution of 1/51,200. When the RIA was compared to a plaque reduction assay (Robb and Bond, 1979) and the commercially available ELISA (Microbiological Associate, Walkersville, MD), it proved significantly more sensitive to either of these assays (Table 2), being 150-fold more sensitive than the plaque reduction assay, and greater than 50-fold more sensitive than the commercially available ELISA. Furthermore, in following seroconversion of MHV-3 infected A/J mice, we were able to detect antibody by the RIA at 3 days when both the plaque neutralization assay and ELISA were negative. At 7 days following infection the RIA was 120 times more sensitive than the ELISA and 400 times more sensitive than the plaque neutralization assay. In addition, using this assay, we were able to detect antibody to a variety of MHV serotypes. Antibody to MHV-3 could be detected with equal efficiency against MHV-3 or MHV-A59 as the target antigen. MHV-A59 was

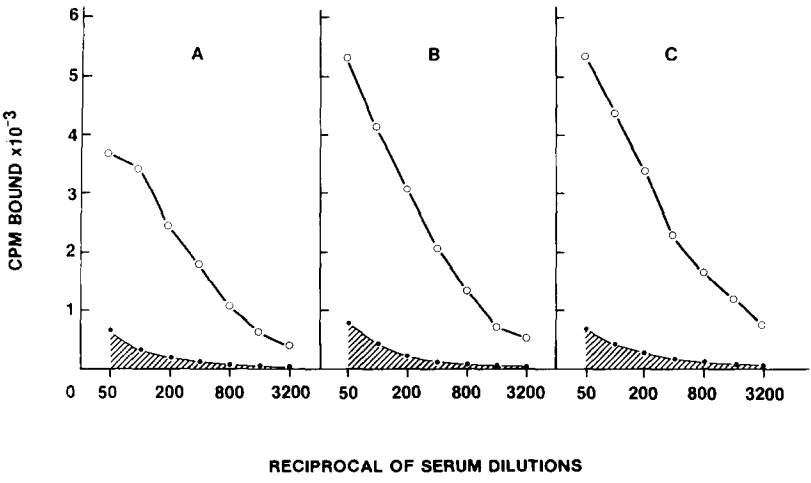

Fig. 3. Optimal antigen requirements of MHV radioimmunoassay. Individual wells in triplicate were coated with (A) 10 ng (B) 25 ng or (C) 50 ng of purified MHV-A59 virus protein. ○———○ Each point is the mean of triplicate samples of a known positive serum to MHV-A59. ●———● Each point represents the mean plus 2 SD for 5 negative sera.

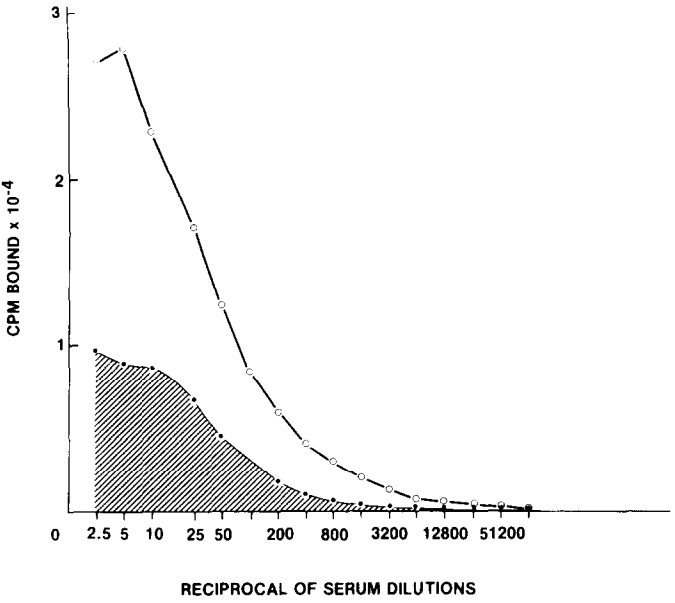

Fig. 4. Antibody titration of anti MHV-A59 serum. ○———○ Each point is the mean of a known positive serum and its dilutions were run in triplicate. ●———● Each point is the mean plus 2 SD of 5 negative control sera and their dilutions.

also an adequate target for anti-JHM sera. However, with sera having low levels of antibody to MHV-S (neutralization titers $< 1/10$) the results of the RIA were erratic using either MHV-A59 or MHV-3 as the target. Using a pool of purified antigens including MHV-A59, MHV-S and MHV-1 we were able to detect sera with neutralization titers as low as 1/5 with ease. Hierholzer et al. (1979) demonstrated previously, by a complement-fixation assay, that A59 antigen reacted with antisera directed against a

TABLE 2

Relative sensitivities of assays for antibody* to MHV

Assay	Titer
Plaque neutralization	1/320**
ELISA	1/1000***
RIA	1/51,200****

* A known positive antiserum to A59 was used in this experiment.

** The antiserum (heat-inactivated at 56°C) was incubated with A59 virus for 30 min at 4°C and then added to a monolayer of L2 cells and overlayed with agarose. Enumeration of plaques was determined following a 2-day incubation at 37°C. Positivity was defined as greater than 50% plaque reduction.

*** The ELISA was performed by Microbiological Associates, Walkersville, MD.

**** The RIA was performed as outlined previously.

wide variety of strains of MHV. However, there were relative differences in sensitivity to antisera to MHV with antisera to MHV-1 and MHV-S being the least reactive of those examined. The RIA, however, appears to be significantly sensitive so that this is not a problem in most cases. Sera with very low titers ($<$ 1/10) by a sensitive homologous plaque reduction assay to MHV-S (kindly provided by Dr. Abigail Smith, Yale University) were occassionally negative by RIA when using only A59 as the target antigen. However, we were able to detect antibody to MHV in the majority of cases. By using a pool of antigens including MHV-S in addition to A59, we are able to improve the sensitivity of the assay for this strain.

DISCUSSION

The RIA which we describe in this paper, has proven to be a fast, highly sensitive, economical and a reproducible assay. It is suitable for screening large numbers of samples and therefore is useful in screening for hybridomas. We have used it in screening hybridomas against specific viral proteins. In addition, we have also employed this assay for the detection of outbreaks of MHV in breeding colonies and have found it to be most satisfactory. In one case, the assay was positive for MHV which was later confirmed by the commercially available ELISA, although the plaque reduction assay was negative. In a second outbreak, the ELISA was negative while the plaque reduction assay and the RIA were both positive.

In two other cases, the assay was negative for MHV and in fact, further investigations revealed that the outbreaks were the result of ectromelia and Sendai viruses. We chose A59 to be the target antigen because of its growth to high titer in tissue cultures and its adequate antigenic spectrum cross reacting with most MHV serotypes. However, we recommend using a pool of purified antigens (A59, MHV-S and MHV-1) in order to maximize the sensitivity of the assay to other strains of MHV because we were unable to detect low titers of antibody to MHV-S using only MHV-A59 as the target antigen.

As well as being useful in screening for antibody to MHV, the assay can be used to quantitate this antibody. The method chosen to determine the antibody titer is analogous to other standard serologic end-point dilution assays such as complement fixation, hemagglutination inhibition and plaque neutralization. Because of the shape of the curve (Fig. 3), it is necessary to do an end-point dilution assay, since the linear portion of the curve is only between 1/5 and 1/400.

The increased sensitivity of the RIA compared to the ELISA is probably due to the use of purified viral antigens and an affinity-purified goat antimouse probe with high specific activity. This probe could detect IgG with high sensitivity as can be seen in Table 1. In addition, anti-μ activity was detected as seen by reactivity to two monoclonal IgM molecules. It appears that the antibody has higher anti-kappa than anti-lambda activity, but it is possible to explain this result on increased binding of kappa myeloma to the plate. IgA was also detected but less efficiently than IgG or

IgM. The fact that this probe has all specificities might explain the ability to detect early seroconversion.

MHV contains three structural proteins, nucleocapsid protein, peplomer glycoprotein (E2) and a glycoprotein analogous to matrix protein of negative stranded viruses (E1) (Sturman, 1977; Sturman and Holmes, 1977; Hierholzer et al., 1979; Sturman, 1981). NP-40 treated virion was no more effective than non-detergent treated virus as a target antigen (data not shown). This suggests that the Tween 20 in the buffers is making the viral envelope more permeable, thus allowing internal antigens to be accesssible to antibody. Although the major component of MHV is the nucleocapsid protein (Fig. 2) (Robb et al., 1979) at the dilution chosen for screening (1/250), the system is unsaturated and therefore probably detects antibodies to all three proteins in the proportions in which they are present. Further investigations are now under way to rigorously establish whether this is in fact true.

ACKNOWLEDGEMENTS

The authors thank Dr. H. Soule, Scripps Clinic and Research Foundation, La Jolla, California, and Dr. B. Underdown, Division of Immunology, University of Toronto, Toronto, Canada, for their advice and critical comments and C. Underwood for her excellent technical assistance. The authors also express their gratitude to E. Kroeker for her assistance in the preparation of the manuscript.

This work was supported by grants CA-28166 from the National Cancer Institute, NS 15211 from the NINCDS, a grant from the National Multiple Sclerosis Society, a grant from the Canadian Liver Foundation and Medical Research Council of Canada Grant MA-6787. J.L.L. is the recipient of Teacher Investigator Award number NS 0048 from the NINCDS and G.A.L. is the recipient of a Medical Research Council of Canada Scholarship.

REFERENCES

Hierholzer, J.C., Broderson, J.R. and Murphy, F.A., 1979, Infect. Immunol. 24, 508–514.

Kraaijeveld, C.A., Madge, M.H. and MacNaughton, M.R., 1980, J. Gen. Virol. 49, 83–91.

Krzynstyniak, K. and Dupuy, J.M., 1981, J. Gen. Virol. 57, 53–61.

Leibowitz, J.L., Wilhelmsen, K.C. and Bond, C.W., 1981, Virology 114, 39–51.

Levy, G.A., Leibowitz, J.L. and Edgington, T.S., 1981a, J. Exp. Med 154, 1150–1163.

Levy, G.A., Schwartz, B.S., Curtiss, L.I.T. and Edgington, T.S., 1981b, J. Clin. Invest. 67, 1614–1622.

Levy, G.A. Leibowitz, J.L. and Edgington, T.S., 1982, in: Progress in Liver Diseases, Vol. VII, eds. H. Popper and F. Schaffner, pp. 393–409.

Maizel, J.V., Jr., 1971, in: Methods in Virology, Vol. 5, eds. K. Maramorosh and H. Koprowski (Academic Press Inc., New York) pp. 179–246.

Piazza, M., 1969, Experimental Viral Hepatitis (Charles C. Thomas, Springfield, IL) p. 1348.

Robb, J.A. and Bond, C.W., 1979, Virology 94, 352–369.

Robb, J.A., Bond, C.W. and Leibowitz, J.L., 1979, Virology 94, 385–392.

Sturman, L.S., 1977, Virology 77, 637–643.

Sturman, L.S., 1981, in: Biochemistry and Biology of Coronaviruses. Advances in Experimental Medicine and Biology, Vol. 142, eds. V. ter Meulen, S. Siddell and H. Wege (Plenum Press, New York) pp. 1–17.

Sturman, L.S. and Holmes, K.V., 1977, Virology 77, 650–655.

Tyrell, D.A.J., 1978, Intervirology 10, 321–336.

Virelizier, T.L. and Allison, A.C., 1976, Arch. Virol. 50, 279–285.

Wege, H., Muller, A. and ter Meulen, V. 1978, Genomic RNA of the murine coronavirus. J. Gen. Virol. 41, 217–227.

Weiser, W. and Bang, F.B., 1976, J. Exp. Med. 143, 690–695.