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Hybridoma Antibodies to the Murine Coronavirus JHM: Characterization of Epitopes on the Peplomer Protein (E2)

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SUMMARY

A panel of hybridoma antibodies that react with the surface peplomer glycoprotein (E2) of the murine coronavirus JHM were produced to characterize major antigenic domains associated with functions related to virulence. Three groups of hybridoma antibodies were differentiated by immunoprecipitation of lysates from JHM-infected cells. One group precipitated the virion structural proteins gp170 and gp98 together with the intracellular form of E2, gp150. A second group reacted with gp98 and gp150, and a third group precipitated gp150 only. Competition assays with biotinylated hybridoma antibodies allowed the definition of at least six different epitope groups. Only those antibodies which immunoprecipitated both gp170 and gp98 neutralized infectivity, inhibited cell fusion and protected infected rats against acute disease. Another class of antibodies binding to gp170 and gp98 also neutralized JHM virus, but did not inhibit fusion and did not protect against disease. Antibodies that immunoprecipitated gp150 and gp98 revealed only weak neutralization and did not inhibit cell fusion or protect animals. Four epitopes were defined by antibodies that immunoprecipitated gp150, but revealed no biological activity. These data indicate that the site responsible for cell fusion is associated with an epitope group carried by gp170 and gp98. Neutralizing antibodies bind to this and another epitope. Furthermore, protection of JHM-infected rats against acute disease requires both inhibition of cell fusion and neutralization of virus.

INTRODUCTION

Many diseases of clinical and economic importance are associated with coronavirus infections and intensive studies have been carried out to define this virus group (Wege *et al.*, 1982; Siddell *et al.*, 1983; Sturman & Holmes, 1983). Infections by murine coronaviruses are valuable tools in studying mechanisms leading to acute and chronic diseases. In this context, infections of rodents with the murine coronavirus JHM are used to study the process of demyelination in the central nervous system (Nagashima *et al.*, 1979; Stohlman & Weiner, 1981; Knobler *et al.*, 1982; Watanabe *et al.*, 1983; Wege *et al.*, 1984). During the course of persistent infections in cell cultures, virus variants were selected which differ in structural polypeptides or virulence (Makino *et al.*, 1983; Baybutt *et al.*, 1984). Therefore, a detailed knowledge of the major structural polypeptides with respect to their antigenic domains and the role of these domains in determining virulence, is required to analyse the complexity of virus–host interactions *in vivo* and *in vitro*.

Coronavirus particles are composed of four to six polypeptides, which fall into three different functional classes, namely the nucleocapsid protein (N) and two glycosylated proteins in the form of a relatively small transmembrane or matrix protein (E1) and a large complex protein (E2) which forms the surface peplomers. For JHM virus, E2 consists mainly of two related glycoproteins with mol. wt. of approximately 170000 and 98000 (termed gp170 and gp98). These glycoproteins are processed from an intracellular glycosylated form of E2 with a mol. wt. of 150000 (here denoted gp150) which is primarily translated as a polypeptide with a mol. wt. of

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120000 (Wege *et al.*, 1979; Siddell *et al.*, 1981; Siddell, 1982). Gel electrophoresis under reducing conditions of lysates from JHM-infected cells separates E2 as the glycoproteins designated as gp170, gp150 and gp98. An additional glycoprotein, gp65, was found which may belong to the E2 family although the precise relationships are unknown.

It had been shown that E2 of the related murine coronavirus A59 is cotranslationally glycosylated, transported to the Golgi apparatus and further processed by addition of sugar moieties and palmitic acid. Proteolytic cleavage may lead to two glycosylated polypeptides of approximately half the size (Sturman *et al.*, 1980; Holmes *et al.*, 1981; Niemann *et al.*, 1982). Sturman & Holmes (1983) suggested that this peplomer forms a trefoil-like structure held together by disulphide bonds whereby the acylated subunit anchors the peplomer to the viral envelope. The peplomers of coronaviruses are responsible for cell fusion as well as attachment to cells and elicit neutralizing antibodies (Garwes *et al.*, 1978/1979; Hasony & Macnaughton, 1981; Holmes *et al.*, 1981; Schmidt & Kenny, 1982; Collins *et al.*, 1982). However, where these biologically active sites are located on the E2 protein family is unknown and it is conceivable that the structure of the peplomers significantly influences the virulence and the type of disease.

In this study we describe hybridoma antibodies directed against the surface peplomer of JHM virus and define the major epitopes on this complex protein. It could be shown that the site responsible for cell fusion carried a specific epitope on gp170 and gp98. Antibodies to this and another epitope neutralize the virus. For protection of animals against JHM virus-induced acute disease, neutralizing and cell fusion-inhibiting antibodies are required.

METHODS

Viruses and cells. The murine coronavirus JHM was grown on Sac(−) cells, a murine Moloney sarcoma line, and density gradient-purified from the medium of infected suspension cultures, by precipitation with polyethylene glycol 6000 and sucrose density centrifugation as described previously (Wege *et al.*, 1979; Siddell *et al.*, 1980). Virus for primary immunization of mice was propagated by intracerebral infection of BALB/c mice (4 to 6 weeks old) and used as a 10% homogenate of brain tissue from diseased animals. For production of hybridoma cell lines, the mouse (BALB/c) myeloma line P3-X63-Ag8 (IgG1, kappa secretor) was cultured in RPMI 1640 medium (Gibco) containing 20% inactivated foetal calf serum, 1% glutamine, 1% Eagle's non-essential amino acids, 1% sodium pyruvate and 10^{-5} M-2-mercaptoethanol, antibiotics and 1% 8-azaguanine.

Immunization and production of hybridoma cell lines. BALB/c mice (8 to 10 weeks old; Versuchstierzucht, Hannover, F.R.G.) were immunized intraperitoneally with JHM virus-containing brain homogenate at doses ranging from 10 to 0·1 p.f.u./animal. Three weeks later, surviving mice were boosted by intravenous inoculation with purified JHM virus (120 μg/mouse). The mice were killed 4 days later by anaesthesia and the spleen cells were fused with P3-X63-Ag8 cells at a ratio of 4:1 with polyethylene glycol 1500 (Roth, Karlsruhe, F.R.G.) by the method of Galfré *et al.* (1977). After fusion the cells were distributed in 24-well microplates (Costar) in aliquots of 1×10^6 cells/well and normal spleen cells (1×10^6/well) were added. Growing colonies were expanded after HAT selection.

Anti-JHM virus antibody-secreting colonies were detected by radioimmunoassay and positive colonies were passaged once in mice that had been primed by intraperitoneal inoculation of Pristane (2,6,10,14-tetramethylpentadecane; Roth, Karlsruhe, F.R.G.) 8 to 12 days before. After development of ascites the hybridoma cells were cloned using the soft agar method (Cotton *et al.*, 1973). Tissue culture supernatants were concentrated with a serum concentrator (minicon B125; Amicon) for tests requiring higher antibody titres.

Radioimmunoassay. To screen for anti-JHM virus antibody-secreting hybridomas, the solid phase radioimmunoassay described by Dörries & ter Meulen (1980) was used. Virus antigen and control antigen were made from infected or uninfected Sac(−) cells by ultracentrifugation of disrupted cells as described previously (Wege *et al.*, 1984). For detection of bound mouse antibodies, goat anti-mouse $F(ab')_2$ antibodies (Cappel Laboratories, Cochranville, Pa., U.S.A.) were labelled with ^{125}I (Amersham Buchler) with chloramine-T.

Purification and biotinylation of hybridoma antibodies. Ascites fluid containing hybridoma antibodies was clarified by centrifugation for 5 min at 4000 *g*. The globulin fraction was concentrated by addition of ammonium sulphate to 40% saturation (4 °C overnight) and then dialysed against 20 mM-Tris–HCl pH 8. The proteins were purified by affinity chromatography with DEAE-Affi-Gel Blue (Bio-Rad) to obtain purified IgG. The bound globulin fraction was eluted by a linear sodium chloride gradient (50 mM to 500 mM). The peak fraction was again concentrated by ammonium sulphate precipitation, dialysed against 0·1 M-sodium carbonate buffer (pH 7·6) and adjusted to a protein concentration of 3 mg/ml. The globulins were biotinylated with biotinyl-*N*-hydroxysuccinimide ester (BHSE, E-Y Laboratories, Medac, Hamburg, F.R.G.) as described by Guesdon *et al.* (1979). The optimal concentration giving biotinylated probes with strong self-competition in the competitive immuno-

assay varied for each hybridoma antibody between 50 and 150 μg BHSE/mg protein. The reaction mixture was incubated for 3 h at room temperature and terminated by extensive dialysis against phosphate-buffered saline. For stabilization, 50% normal rabbit serum was added before storage at −20 °C.

Enzyme-linked immunoassay (ELISA). The assay was performed as described previously (Dörries & ter Meulen, 1983; Wege *et al.*, 1984). Density gradient-purified virus in 0·05 M-sodium carbonate buffer pH 9·6 was coated at 4 °C in wells of flat-bottomed microtitre plates (Dynatech, Nürtingen, F.R.G.) by incubation overnight. The optimal virus antigen concentration was determined by chequerboard titrations with a mixture of ten anti-E2 hybridoma antibodies (tissue culture supernatant) and corresponded to a range of 0·2 to 1 μg of protein/well. Negative controls consisted of culture medium from P3-X63-Ag8 cells and monoclonal antibodies against measles virus. In tests where ascites was used, an anti-measles virus ascites fluid was included. The specificity of the assay was also determined using antigen prepared from uninfected cells as described for the radioimmunoassay. For detection of virus-bound antibody, peroxidase-labelled rabbit anti-mouse IgG (Dakopatts, Hamburg, F.R.G.) was used. Antibodies were diluted in 0·05 M-Tris–HCl pH 7·4 with 0·35 M-sodium chloride, 0·1% Tween-20, 0·0005% phenol red and 5% rabbit serum. The same buffer was used without serum for washing. The substrate for the enzyme reaction was 0·5 mg/ml *o*-phenylenediamine in 0·1 M-citrate buffer pH 5. The tests were evaluated with a multichannel photometer (Flow Laboratories) connected to a computer system (Mini-minc; Digital) programmed to calculate the net absorbance at 492 nm (NA, A_{492} of wells with antigen minus A_{492} of wells without antigen). A regression line was generated from the linear part of the titration curve (twofold dilutions). The ELISA titre was estimated as the next lower dilution step nearest to the point at which the regression line crossed the x-axis. Negative antibody controls consisted of P3-X63-Ag8 culture supernatant or anti-measles ascites diluted 1 : 10.

The ELISA for competitive immunoassays was based on competition between labelled (biotinylated) and unlabelled hybridoma antibodies for antigen-binding sites. The limiting antigen concentration (gradient-purified virus) was determined by chequerboard titrations with a mixture of biotinylated anti-E2 hybridoma antibodies diluted 1 : 1000. Competitor antibodies (ascites fluid clarified by centrifugation) were applied in tenfold dilutions and incubated for 1 h at 37 °C. The microplates were washed and biotinylated hybridoma antibody was added at a dilution that resulted in a NA of 1·2 to 1·9 in a non-competitive assay. Avidin coupled to horseradish peroxidase (E-Y Laboratories) was used to measure bound biotinylated mouse globulin.

To define epitope groups, competitions were considered as positive only if they occurred symmetrically when each antibody was used in turn both as competitor and in turn as biotinylated probe against the complete set of specimens. The degree of competition was evaluated relative to the self-competition curve and rated as strong (+ + + +), significant (+ +) or negative (−). The result was rated as strong competition if the first three $\log_{10}$ dilutions caused more than 70% reduction of the NA compared to the biotinylated probe without competitor (e.g. D12 with D13, Fig. 2*d*). The curve represented significant competition if the first three $\log_{10}$ dilutions caused a 40 to 60% reduction of the NA relative to the self-competition of the biotinylated probe (e.g. E17 with E16, Fig. 2*e*). Results were considered negative if the reduction of the NA was not more than 40% and extended for less than three $\log_{10}$ dilution steps. Weak or doubtful competitions were repeated by mixing the biotinylated probe and competitor dilutions in a test tube before titration on the virus-coated plate.

Labelling of intracellular proteins and immunoprecipitation. Monolayers of Sac(−) cells in 5 cm tissue culture dishes were infected with an m.o.i. of 0·01 p.f.u./cell and incubated at 37 °C. About 12 to 16 h later, when 50 to 75% of the cells had formed syncytia, the culture medium (Eagle's MEM with 5% heat-inactivated foetal calf serum) was replaced by MEM without methionine or serum. For labelling, 100 μCi [^{35}S]methionine (Amersham) was added 20 min later. After a labelling period of 45 min the incorporation was terminated by washing with ice-cold MEM and lysates were prepared according to Stephenson & ter Meulen (1979). The lysis buffer was made of 10 mM-Tris–HCl pH 7·4, 150 mM-sodium chloride, 1 mM-EDTA, 1% NP40, 500 units aprotinin/ml and 0·2 mg phenylmethylsulphonyl fluoride/ml. Immunoprecipitations were performed by a modified version of the method of Siddell *et al.* (1981). For each hybridoma cell line, mouse ascites fluid and concentrated culture supernatant was tested in several dilutions to identify the specificity of the antibody. After 2 h at 4 °C, immune complexes were precipitated with Immuno-Precipitin (formalin-fixed *Staphylococcus aureus* cells; Bethesda Research Laboratories).

To collect hybridoma antibodies that did not bind to Protein A, rabbit anti-mouse IgG (Dakopatts) was added, and the complexes were precipitated by overnight incubation at 4 °C. The precipitates were washed once with 0·1 M-Tris–HCl pH 9 with 0·5 M-lithium chloride, once with the same buffer at pH 7·2 and once at pH 7·2 without lithium chloride. The proteins were dissociated by incubation for 10 min at 37 °C in electrophoresis sample buffer (Siddell *et al.*, 1980) and analysed on a 15% discontinuous SDS–polyacrylamide gel (Laemmli, 1970) with a bisacrylamide to acrylamide ratio of 1 : 173·5 for the separating gel. Fluorography was performed according to Bonner & Laskey (1974).

Neutralization assay. Hybridoma antibodies in tenfold dilutions were incubated with 150 to 200 p.f.u. of virus for 1 h at 4 °C. Ascites fluids were inactivated for 30 min at 56 °C before mixing with the virus. Virus controls

consisted either of anti-measles ascites (diluted 1 : 10), concentrated P3-X63-Ag8 culture supernatant and supernatant from a non-neutralizing hybridoma culture, depending on the source of antibody to be tested. The amount of infectious virus was measured by titration on monolayers of Sac(−) cells in 24-well Costar plates. Microplaques were stained with Giemsa 20 to 24 h later. The antibody dilution resulting in 50% reduction of plaques (PRD_{50}) was calculated. To compare the strength of neutralization between hybridoma antibodies that differed in ELISA titres, we pre-diluted the samples accordingly.

Binding of antibodies to the surface of infected cells. Sac(−) cells were cultured in suspension (Siddell *et al.*, 1980) and infected with JHM virus at an m.o.i. of 2 p.f.u./cell. Six h after infection, the cells were collected by centrifugation and incubated on ice in aliquots of 200 µl containing 2×10^5 cells with hybridoma antibodies diluted by 1 : 10 to 1 : 100. The medium used for incubation and washing consisted of MEM with 5% foetal calf serum and 10 mM-sodium azide. After 30 min at 4 °C, cells were washed three times and stained for 30 min at 4 °C with fluorescein isothiocyanate-labelled rabbit anti-mouse globulins (Dakopatts). After washing twice, the immune fluorescence was immediately scored in a u.v. microscope. Both ascites fluid and concentrated culture medium containing hybridoma antibodies were compared. Controls consisted of ascites fluid and concentrated culture fluid from an anti-nucleocapsid hybridoma line. The intensity of fluorescence was rated as brilliant (+ + + +), significant (+ +) or insignificant (−).

Inhibition of cell fusion. Monolayer cultures of Sac(−) cells in 48-well cluster plates (Costar) were infected with JHM virus with about 30 p.f.u./well and dilutions of ascites fluid (1 : 10 to 1 : 1000) containing hybridoma antibodies were added 3 h later. The cell cultures were stained with May–Grünwald and Giemsa 16 to 20 h later. The number and size of syncytia were judged microscopically and compared to the virus controls containing ascites fluid from an anti-nucleocapsid hybridoma line.

Protection of rats against acute encephalomyelitis. Suckling rats (5 to 8 days old) were infected by intracerebral injection of 30 µl JHM virus (1×10^5 p.f.u., 5·2 LD_{50}). One day later, 50 µl of ascites fluid containing hybridoma antibodies was administered intraperitoneally. At least five infected control animals from the same litter were included, and at least two litters were infected for testing each antibody. The control rats received ascites fluid with antibodies against the nucleocapsid protein. All control rats died within 6 to 12 days. The antibody was designated as protective if at least 50% of the rats survived and did not develop encephalomyelitis.

RESULTS

Production and specificities of antibody-producing hybridoma lines

Spleen cells from two hyperimmunized mice were fused in independent experiments. Detectable amounts of hybridoma antibodies against JHM virus were produced by 255 out of 383 cultures (67%). More than 40% of these lines were directed against the E2 protein, about 10% recognized other JHM structural proteins and for 11% of the cultures the specificity could not be determined although these antibodies reacted by immunofluorescence and gave high ELISA titres. Finally, we selected 22 clones secreting antibody against E2 for the studies described here. All these clones are of the IgG class and bind to Protein A.

Patterns of immunoprecipitation obtained with anti-E2 hybridoma antibodies

The specificity of each antiviral hybridoma antibody was identified by immunoprecipitation of [^{35}S]methionine-labelled lysates from JHM-infected cells. As shown in Fig. 1 and summarized in Table 2, three different patterns were observed with antibodies against E2. One group of antibodies precipitated gp170, gp150 and gp98. A second group of antibodies precipitated gp150 and gp98, and the third group precipitated only the intracellular form of E2, namely gp150. The different patterns of reactivity were not due to variations in the specific concentration of each antibody because the same type of pattern was observed for each hybridoma at different dilutions of either ascites fluid or supernatant. High concentrations of hybridoma antibodies belonging to the third group precipitated an increased amount of gp150 but still did not precipitate gp98 or gp170. These proteins were also not visible after the fluorographs had been exposed for longer. Culture medium from P3-X63-Ag8 cells sometimes gave faint non-specific precipitation of pp60 and p23. Similar background was also found with anti-measles virus hybridoma antibodies from cultures or ascites.

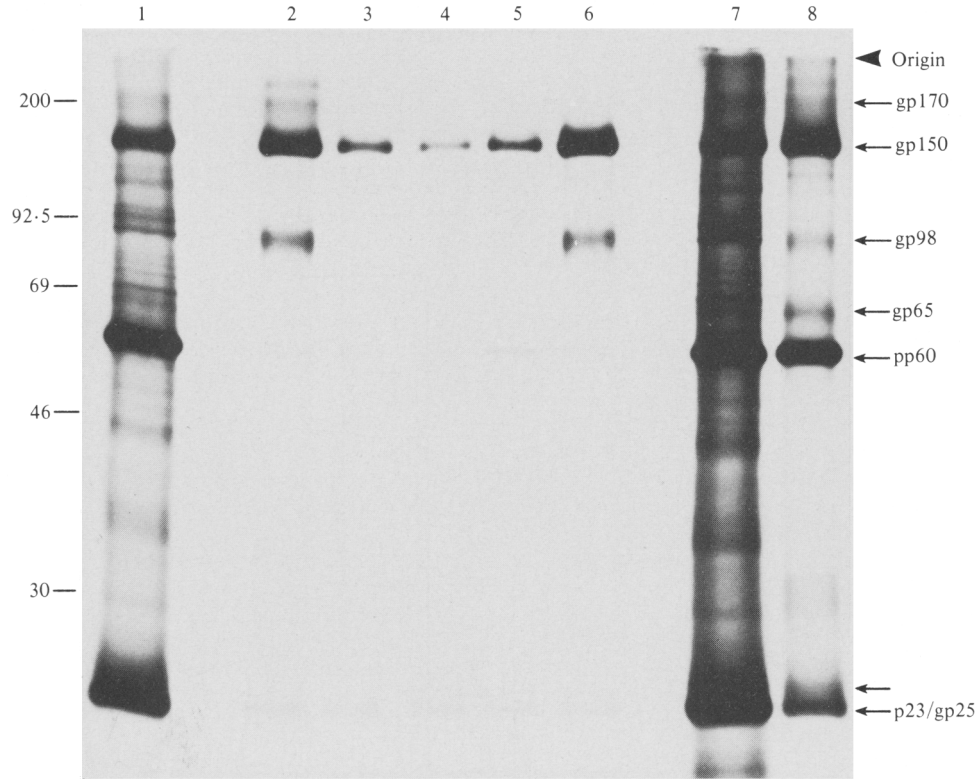

Fig. 1. Immunoprecipitation of JHM virus proteins with anti-E2 hybridoma antibodies from ^{35}S-labelled lysates of infected cells. Concentrated culture supernatants of the hybridomas A3 (lane 2), C11 (lane 3), D15 (lane 4), E16 (lane 5) and B7 (lane 6) were used. Lysates without immunoprecipitation (lanes 1 and 7) show gp150, pp60 and p23/gp25. Immunoprecipitation with anti-JHM mouse serum (lane 8) displays additionally the virion protein gp65 and derivatives of gp150, namely gp98 and gp170, which can form aggregates of high mol. wt. ^{14}C marker proteins (CFA.626, Amersham Buchler) were used to indicate molecular weights (left side, $\times 10^{-3}$). The precursor–product relationships and the identity of intracellular viral proteins with structural proteins of virions were previously described (Siddell *et al.*, 1981; Siddell, 1982).

Delineation of epitopes by competitive immunoassays

As a basis for a further characterization of the peplomer protein E2, we defined major epitope groups of this protein. The purified immunoglobulin fraction from ascites fluid of each hybridoma line was labelled with biotin and competitive immunoassays were performed on plates coated with gradient-purified JHM virus. Each antibody used as a competitor was also tested in turn as a biotinylated probe against the panel of antibodies shown in Table 1. The competition curves shown in Fig. 2(*a*) to (*f*) display the net absorbance values at 492 nm versus the dilution of the competing antibody.

As representatively shown in Fig. 2 and summarized in Table 1, six groups of anti-E2 antibodies were clearly delineated by competitive immunoassays. The corresponding epitope groups of the E2 protein were designated with the letters A, B, C, D, E and F. The final classification of the antibody is also indicated by that letter (e.g. anti-E2-A1 denotes a hybridoma antibody that recognizes the epitope A). A one-way competition was observed with some hybridoma antibodies from group A to B and from group D to E (Table 1). All group A antibodies competed with the biotinylated antibody B6 (Fig. 2*b*). Antibody A5 competed with antibody B7. Similarly, E16 was competed by D13, D14 and D15, or E17 was competed by D15.

Table 1. *Summary of competitive immunoassays*

Biotinylated antibody	Competitor antibody																	
	E2-A1	E2-A2	E2-A3	E2-A4	E2-A5	E2-B6	E2-B7	E2-C8	E2-C9	E2-C10	E2-C11	E2-D12	E2-D13	E2-D14	E2-D15	E2-E16	E2-E17	E2-F18
E2-A1	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–
E2-A2	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–
E2-A3	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–
E2-A4	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–
E2-A5	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–	–	–
E2-B6	++++	++++	++++	++++	++++	++++	++++	–	–	–	–	–	–	–	–	–	–	–
E2-B7	–	–	–	–	++	++	++++	–	–	–	–	–	–	–	–	–	–	–
E2-C8	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–
E2-C9	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–
E2-C10	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–
E2-C11	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–	–	–	–	–
E2-D12	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–
E2-D13	–	–	–	–	–	–	–	–	–	–	–	++++	++++	++++	++++	–	–	–
E2-D14	–	–	–	–	–	–	–	–	–	–	–	++	++	++++	++++	–	–	–
E2-D15	–	–	–	–	–	–	–	–	–	–	–	++	++	++++	++++	–	–	–
E2-E16	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++	++++	++	–
E2-E17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++	++	++++	–
E2-F18	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	++++

Since the above-mentioned competitions did not occur symmetrically if the biotinylated probe was used in turn as a competitor, we do not interpret these results as an indication for overlapping epitopes. The competitions within groups D and E also showed some heterogeneity in strength. The competition of biotinylated B7 by B6 was weaker than biotinylated B6 by B7. The same result was observed for D14 and D15 with D12 and D13.

Finally, four hybridoma antibodies which are listed in Tables 2 and 3 (X19, X20, X21, X22) did not give self-competition after biotinylation despite the use of a wide range of concentrations and protein to biotin ratios and, therefore, could not be classified.

Correlation of biological activities of hybridoma antibodies with epitope groups

Correlation of the reactivity of hybridoma antibodies in the immunoprecipitation reaction and competitive immunoassay with the capacity to neutralize JHM virus, to inhibit cell fusion and to protect JHM-infected rats against fatal disease gave the results which are summarized in Table 3. We found that the antibodies that precipitate gp150 and also the structural virion protein gp98 (epitope B), and the antibodies precipitating all three glycoproteins (epitope A), revealed neutralizing activity directed against JHM virus. However, all antibodies that immunoprecipitated only the intracellular gp150 (epitopes C, D, E, F) did not neutralize JHM virus significantly. The neutralizing titres obtained with ascites fluid are summarized in Table 2. To compare the neutralizing activity, ascites fluids were diluted to the same ELISA titre before performing the neutralization test. It can be seen that antibodies that bound to epitope A neutralized JHM virus significantly more efficiently than antibodies against epitope B. Furthermore, antibodies A1, A2 and A3 neutralized to a higher titre than antibodies A4 or A5.

Only some of the antibodies that define epitope A, namely A1, A2 and A3 could significantly inhibit the fusion of infected cells. The same antibodies protected JHM-infected suckling rats against fatal acute encephalomyelitis. In contrast, antibodies A4 and A5 which defined the same epitope group by competition immunoassay, did not significantly inhibit cell fusion and could not protect infected animals. Therefore, comparison of biological functions allows a subdivision of this epitope group (designated a and b in Table 3). Furthermore, the antibodies that define epitope B neutralized JHM virus but did not inhibit cell fusion and were not protective. Antibodies that define epitope groups C, D, E and F as well as those which could not be classified by competitive immunoassays bound to purified virus in the ELISA, but revealed no significant biological activity in the parameters tested. Nevertheless, several antibodies that recognized epitope D (D14, D15) and E (E16) bound to the surface of infected cells. Other antibodies against epitopes D and E gave no significant surface staining. Therefore, these epitopes may be extensive enough to comprise regions which differ in their accessibility at the cell surface. However, we could not subdivide these epitopes by competition assays or biological activities.

DISCUSSION

In this study hybridoma antibodies against JHM virus were used for a topographical and functional analysis of different epitopes on the viral peplomer protein E2. We observed three different patterns when immunoprecipitated lysates of JHM-infected cells were analysed by gel electrophoresis (Fig. 1, Table 2). One group of hybridoma antibodies precipitated gp170, gp150 and gp98, another group precipitated gp150 together with gp98 and a third group precipitated gp150 only. Conformations of epitopes on gp150, the intracellular form of E2, are obviously not changed during further processing to the mature peplomer of the virion, which consists of gp170 and gp98. Purified virus, which was used as an antigen for immunoassays, does not reveal gp150 under the conditions of electrophoresis. Hybridoma antibodies that precipitate gp150 only, probably react with antigenic binding sites determined by the primary amino acid sequence. These binding sites may be accessible due to the alkaline pH conditions of the ELISA coating buffer but not in the conformation of gp98 and gp170 taken up in cell lysates. Antibodies that reveal different patterns of immunoprecipitation probably react with topographically distinct binding sites and recognize different maturation stages of E2. Further studies with hybridoma antibodies may help to clarify pathways leading to the formation of peplomers of JHM virus.

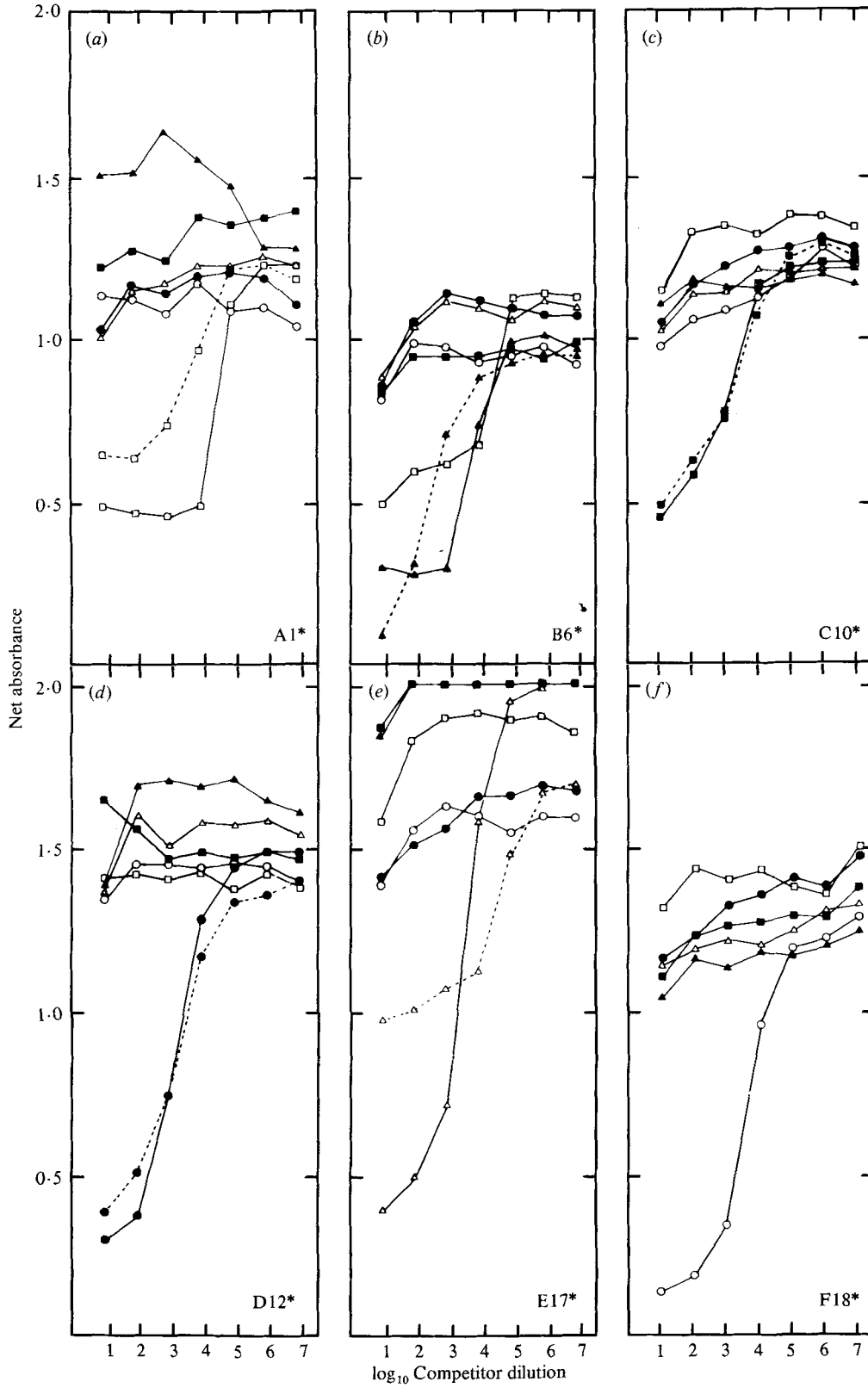
(a)
(b)
(c)
(d)
(e)
(f)
A1*
B6*
C10*
D12*
E17*
F18*
Net absorbance
2·0
1·5
1·0
0·5
1 2 3 4 5 6 7
log10 Competitor dilution

Table 2. *Correlation of immunoprecipitation with the capacity to neutralize JHM virus*

Designation of hybridoma antibody	ELISA titre $\times 10^2$*	Neutralization titre (PRD_{50})†	Pattern of immunoprecipitation‡
E2-A1	4096	300000	gp98, gp150, gp170
E2-A2	4096	210000	
E2-A3	1024	12000	
E2-A4	512	250	
E2-A5	256	2100	
E2-B6	1024	36	gp98, gp150
E2-B7	512	76	
E2-C8	512	<20	gp150
E2-C9	256	<20	
E2-C10	1024	<20	
E2-C11	512	<20	
E2-D12	256	<20	
E2-D13	512	<20	
E2-D14	1024	<20	
E2-D15	2048	<20	
E2-E16	4096	<20	
E2-E17	512	<20	
E2-F18	512	<20	
E2-X19	128	<20	
E2-X20	256	<20	
E2-X21	512	<20	
E2-X22	512	<20	

* The values are shown for ascites fluid which was used for the competitive immunoassays.

† The hybridoma antibodies were prediluted according to their ELISA titre to obtain the same specific antibody concentration. Antibodies that did not neutralize were tested without predilution.

‡ Immunoprecipitations were done with ascites fluid and concentrated tissue culture fluid.

Table 3. *Correlation of epitope groups delineated by competitive immunoassays with biological properties of hybridoma antibodies directed against the peplomer protein E2*

Designation of Epitope		Antibody	Pattern of immunoprecipitation	Binding to surface of infected cells	Inhibition of cell fusion	Neutralization of JHM virus	Protection *in vivo*
A	a	A1, A2, A3	gp98, gp150, gp170	++++	++++	++++	++++
	b	A4, A5		++	−	++	−
B		B6, B7	gp98, gp150	++++	−	+	−
C		C8, C9	gp150	−	−	−	−
D		D12, D13	gp150	−	−	−	−
		D14, D15	gp150	++	−	−	−
E		E16	gp150	++	−	−	−
		E17	gp150	−	−	−	−
F		F18	gp150	−	−	−	−
X		X19, X20, X21, X22	gp150	−	−	−	−

Fig. 2. Competitive immunoassays with anti-E2 antibodies. Microtitre plates coated with purified virus and incubated with tenfold dilutions of competitor antibody. The values are displayed as net absorbance (NA) and the competitions were evaluated relative to the self-competition curve as described in the text. Competitions are shown in each panel (*a* to *f*) representatively for one biotinylated antibody to delineate epitopes A to F (Table 1). □——□, A1; □---□, A3; ▲——▲, B6; ▲---▲, B7; ■——■, C10; ■---■, C11; ●——●, D12; ●---●, D13; ○——○, F18; △——△, E17; △---△, E16. The biotinylated antibody used is denoted by an asterisk.

Competitive immunoassays with these antibodies resulted in a classification of at least six binding groups which allowed the delineation of corresponding epitopes on the peplomer protein E2 (Fig. 2, Table 1). The definition of epitopes by competitive immunoassays is based on the assumption that attachment of a hybridoma antibody to a specific site hinders the binding of another antibody recognizing the same or an overlapping site (Stone & Nowinski, 1980; Yewdell & Gerhard, 1982; Kimura-Kuroda & Yasui, 1983). Positive competition indicates at least a proximity of the binding sites on the surface of the antigen molecule, although the final topographical relationship in terms of structure depends strongly on the three-dimensional configuration of the protein. To avoid misinterpretations due to different avidities and specific antibody titres, we considered competition to be meaningful only when it was extended over a range of several $\log_{10}$ dilutions and also occurred when the competitor antibody was used in turn as a biotinylated probe. One-way reactions may indicate that the biotinylation of these hybridoma antibodies slightly changed the conformation of the antigen-combining site. This change could allow a weak binding of these antibodies to a related epitope. Therefore, the epitopes A and B or D and E respectively, comprise closely related binding sites, although a definitive overlapping of these epitopes was not found.

The delineation of epitopes on the peplomer proteins provided the basis for a study of functionally active regions of E2 of the mature virion (Table 3). Previous studies with hybridoma antibodies indicated that the peplomer glycoprotein E2 is important for attachment, cell fusion and induction of neutralizing antibodies (Collins *et al.*, 1982). However, the location of biologically active sites on the peplomer protein has not been deduced. The most important regions of the mature E2 protein associated with biological functions are located within epitopes A and B. In our study epitope A comprises binding sites of different biological importance for fusion and neutralization. The data indicate that the fusion activity is associated with epitope A on the gp170/gp98 complex. Additional binding sites for neutralizing antibodies within epitope A are strongly overlapping, since we could subdivide this epitope group by biological tests but not by competition assays. For the A59 virus, proteolytic cleavage of E2 probably leads to subunits of similar size (Sturman & Holmes, 1983). It is therefore conceivable that cleavage of gp170 results in a fraction of gp98 protein molecules which carry at least parts of epitope A and are the active component for fusion. Activation of a fusion protein by cleavage is an important mechanism for paramyxoviruses (Scheid & Choppin, 1977). Storz *et al.* (1981) showed that trypsin enhances the fusion activity of bovine coronaviruses. It is not yet clear whether this effect is related to a similar cleavage process which is operative for all coronaviruses. Talbot *et al.* (1984) and Buchmeier *et al.* (1984) showed recently that their panel of anti-E2 antibodies allowed the delineation of three epitopes. These epitopes probably correspond partially to epitopes A and B. However, these correlations are only tentative. Processing and glycosylation of E2 may be strongly influenced by the host cell and result in a slightly different configuration of epitopes.

The capacity of the antibodies to protect animals against the development of encephalomyelitis by a single injection seems to be associated with their capacity to inhibit cell fusion. The capacity to neutralize infectious virus *in vitro* is not sufficient for protection *in vivo*, as demonstrated by antibodies A4, A5 and B6, B7. As with infections by paramyxoviruses, protection against disease required neutralization of extracellular virus as well as inhibition of cell fusion to prevent spread of virus (Merz *et al.*, 1980).

At least four epitopes were defined by antibodies that bind to purified virus in ELISA and immunoprecipitate only gp150 (epitopes C, D, E, F). These antibodies probably recognize epitopes on the gp150 which are conserved during further processing. Although at present no significant biological function of the mature virion can be associated with these epitopes, they can be clearly discriminated by competition assays. Finally, we obtained hybridoma antibodies which could not be classified because the biotinylated probes revealed no self-competition (numbers X19 to X22 in Table 2). It is conceivable that biotin is coupled to the antigen-combining site and thus hinders the binding of the antibody or that biotinylation changes indirectly the configuration of the combining site.

Recently, Fleming *et al.* (1983) showed that closely related murine coronavirus strains differ

particularly in the antigenic sites recognized on the E2 protein. We could show that an avirulent variant of JHM virus released from a persistently infected cell line is not neutralized by some of the antibodies which now define epitope A (Baybutt *et al.*, 1984). These findings indicate that hybridoma antibodies will be useful in defining further the biologically active regions of the viral proteins and their role in the pathogenesis of disease.

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