The Peplomer Protein E2 of Coronavirus JHM as a Determinant of Neurovirulence: Definition of Critical Epitopes by Variant Analysis

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SUMMARY

We selected murine coronavirus JHM variants specifically changed in defined antigenic sites of the peplomer protein E2. Variants were isolated from the supernatants of monoclonal antibody hybridoma cell cultures which continued to secrete neutralizing antibodies after being infected with JHM. Comparative antigenic analysis and biological tests were performed in order to refine an operational epitope map and to characterize functional domains important for pathogenicity. The reaction patterns (neutralization, inhibition of cell fusion, immunofluorescence and binding in ELISA) between the variant viruses and the panel of monoclonal antibodies were very similar. Four groups of variants were characterized each of which revealed distinct changes affecting one defined antigenic site. These observations indicated that at least four independently mutable antigenic sites were associated with domains involved in cell fusion, neutralization and pathogenicity (E2-Aa, -Ab, -Ba and -Bb). JHM variants with alterations in the E2-Aa, -Ab or -Bb sites were similar to wild-type virus. These variants caused acute hepatitis and encephalomyelitis in mice. In contrast, JHM variants with changes in site E2-Ba had a strong propensity to induce chronic disease accompanied by demyelination persisting for several months.

INTRODUCTION

Persistent infection of rodents with the murine coronaviruses MHV-JHM or MHV-A59 can lead to the development of inflammatory demyelinating lesions in brain and spinal cord similar to those associated with some human neurological disorders. Such infections therefore provide experimental models to study pathogenetic mechanisms (Sörensen *et al.*, 1982; Wege *et al.*, 1982; Knobler *et al.*, 1982; Watanabe *et al.*, 1983; Lavi *et al.*, 1984; Wege *et al.*, 1984*a*; Beushausen & Dales, 1985; Massa *et al.*, 1986*a*, *b*; Suzumura *et al.*, 1986). Several virus and host factors affect the establishment of persistent infection and demyelination. The virulence of JHM virus is strongly influenced by different passage schedules, selection for temperature sensitivity or attenuation to persistent infection (Knobler *et al.*, 1982; Makino *et al.*, 1983; Wege *et al.*, 1983; Baybutt *et al.*, 1984; Taguchi *et al.*, 1985). Antigenic variation among murine coronaviruses is largely associated with the surface peplomer protein E2 (also designated S protein; Fleming *et al.*, 1983; Talbot & Buchmeier, 1985). Distinct antigenic and structural changes of E2 have been observed during persistent infection and it is probable that this protein plays a major role in the establishment of such infections (Makino *et al.*, 1983; Baybutt *et al.*, 1984; Wege *et al.*, 1987). In rat astrocytes those variants which differ from the original population by possessing a larger E2 precursor polypeptide are predominantly replicated (Taguchi *et al.*, 1985, 1986). The peplomer protein E2 is responsible for specific attachment to cells, for cell fusion and binding of neutralizing antibodies (Collins *et al.*, 1982; Wege *et al.*, 1984*b*; Sturman *et al.*, 1985; Laude *et al.*, 1986; Jimenez *et al.*, 1986). Therefore, structural features of E2 obviously have an important influence on the outcome of infection. This

conclusion is further supported by the observation that variants which escape neutralization by monoclonal antibodies (MAbs) can be of reduced neurovirulence (Dalziel et al., 1986; Fleming et al., 1986).

The peplomer protein E2 (M_r approximately 200000) is derived from a glycosylated precursor which is processed to subunits with a M_r of approximately 90000 (gp90B or S1 and gp90A or S2). The extent of cleavage is host cell-dependent and probably important for activation of cell fusion (Siddell et al., 1983; Sturman & Holmes, 1983; Sturman et al., 1985; Frana et al., 1985; Schmidt et al., 1987). Attempts to characterize with MAbs the topological relation of major antigenic sites to functions of E2 have shown that the domains responsible for fusion and neutralization are closely overlapping. Depending on the coronavirus strain, two to six antigenic sites have been characterized (Talbot et al., 1984; Wege et al., 1984b; Jimenez et al., 1986). For the murine coronavirus strain JHM used in the following studies, we delineated an epitope map by competitive immunoassays (Wege et al., 1984b). Only two antigenic sites associated with neutralization and fusion could be clearly defined by this technique, although biological tests indicated a more complex relationship. In addition, more than four antigenic sites are not related to these domains.

In this communication we report an approach for the generation of antibody-resistant JHM virus variants. A comparative antigenic analysis of these variants was performed in order to refine the epitope map of the E2 peplomer protein (Gerhard et al., 1981; Yewdell & Gerhard, 1981). Furthermore, we investigated the importance of defined antigenic sites in determining virus neurovirulence. Animal experiments revealed that antigenic modification of one site in particular correlated with the capacity to induce a chronic demyelinating disease.

METHODS

Virus and cells. Virus for density gradient purification was propagated in suspension cultures of Sac($-$) cells (Wege et al., 1979; Siddell et al., 1980). The reference wild-type JHM virus (JHM-wt) was propagated after three plaque passages on DBT cell monolayer cultures. Infectivity titres were determined by plaque assays on DBT cell cultures in which the primary medium was solidified with 1% Difco agar (Makino et al., 1983; Taguchi et al., 1985). The hybridoma cells were derived by fusion of spleen cells from mice immunized with JHM virus and the myeloma line P3-X63-Ag8 (P3X). These cells were cultured as previously described (Wege et al., 1984b).

Virus neutralization. Neutralization tests were performed as described by Wege et al., (1984b). Tenfold dilutions of hybridoma antibodies (generated as ascites in mice) were incubated with 150 to 200 p.f.u. of virus for 1 h at 4 °C. The amount of surviving infectious virus was measured by plaque assays on DBT cells and the antibody titre resulting in 50% reduction of plaques (PRD_{50}) was calculated. The frequency of neutralization-resistant variants was estimated by titrating the virus infectivity in the presence and absence of different MAbs (Gerhard et al., 1981). The frequency denotes the ratio between the titre in the presence of antibody and the titre in the absence of antibody.

ELISA. ELISAs were performed as described previously (Wege et al., 1984b) using density gradient-purified virus as an antigen. The net absorbance at 492 nm (net A_{492}) represents the difference between wells with antigen and wells without antigen. The MAbs were derived from hybridoma culture supernatants.

Immunofluorescence. Sac($-$) cells were infected in suspension with the virus variants to be tested as described previously (Wege et al., 1979) and grown on multispot slides (Dynatech Laboratories, Alexandria, Va., U.S.A.) until syncytia were visible. The cell cultures were fixed with cold acetone and, after incubation with MAbs, a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Dako, Copenhagen, Denmark) was used as second antibody.

Inhibition of cell fusion. DBT cells were cultured in 12-well Costar plates and infected with a dose of 30 to 50 p.f.u./well of the variant to be tested. Medium with 1% Difco agar was added 3 to 4 h post-infection (p.i.). In parallel assays, the appropriate MAbs were included in the agar overlay at a final dilution of 1/10 and 1/100 of ascites. The tests were evaluated by comparing the plaque diameter of infected cultures incubated with anti-E2 MAbs with controls which contained ascites from P3X cells or anti-nucleoprotein hybridomas.

Selection of JHM virus variants. Hybridoma cells (1×10^7) secreting anti-MHV JHM antibodies were infected with JHM-wt (0·5 to 1·0 p.f.u./cell). The adsorption (1 h, 37 °C) was terminated by rinsing the cells with RPMI medium. The cells were then cultured in suspension for up to 10 days, after which the culture supernatant was harvested by centrifugation and frozen at -70 °C. To measure the release of virus a sample of the hybridoma culture was taken each day and frozen after removing cells by centrifugation. The infectivity was determined in a microplaque assay without application of an agar overlay (Wege et al., 1984b) using DBT cells cultured in 96-well Costar plates. To monitor for JHM virus which may have escaped neutralization by the MAb secreted by the

hybridoma culture, 100 μl of each sample was incubated for 1 h at 4 °C prior to plaque assay with ascites fluid derived from the same hybridoma line which had been used for infection. The microcultures were stained after 20 to 24 h with May-Grünwald solution and the microplaques were counted.

To identify JHM virus variants, the virus in the supernatant obtained after termination of the culture (4 to 10 days p.i.) was neutralized to determine the PRD_{50} with different MAbs. In parallel assays, the same MAbs were incubated with wild-type virus or virus passaged in either P3X cells or hybridoma lines producing antibodies against the JHM matrix protein E1. To obtain virus stocks, virus variants isolated as plaques were plaque-purified a further three times in the presence of the selective MAb and then propagated on DBT cell monolayers. The name assigned to each JHM MAb variant virus was derived from the nomenclature used for the corresponding hybridoma antibody. For example, the variant designated JHM-A1 was released by the hybridoma line E2-A1.

Western blots, dot blots and immunostaining. The proteins of the purified virus were separated on 15% discontinuous SDS–polyacrylamide gels as previously described (Siddell *et al.*, 1980; Wege *et al.*, 1984*b*). Approximately 2 μg of protein was applied to each slot of the gel. The proteins were electroblotted with 25 mM-sodium dihydrogen phosphate buffer at pH 9 onto nitrocellulose sheets (Schleicher & Schüll, 0·45 μm) by electrophoresis using a Bio-Rad Trans-Blot cell (Towbin *et al.*, 1979). The transfer was performed in cold conditions at 25 V and 1000 mA for 4 h. Filters were washed with blocking buffer (50 mM-Tris–HCl, 200 mM-sodium chloride pH 7·4, 10% v/v horse serum) and then stained with MAb hybridoma culture supernatant followed by peroxidase-conjugated rabbit anti-mouse IgG (Dako) diluted in blocking buffer, for 16 h and 1 h respectively. Filters were then developed by the substrate α-chloronaphthol. Dot blots were performed on nitrocellulose sheets using a dot blot filtration device (Bio-Rad). The above described immunostaining technique was applied.

Animal experiments. Female BALB/c mice (7 to 9 weeks of age; Central Institute for Laboratory Animal Breeding, Hannover, F.R.G.) were infected by intracerebral inoculation of virus. The titre of neutralizing antibodies against coronaviruses was routinely monitored and was found to be below a dilution of 1 : 10 before infection. For determination of the LD_{50}, eight mice were infected for each dose, ranging from 10000 to 0·01 p.f.u. per animal. For histological examination (Nagashima *et al.*, 1978; Watanabe *et al.*, 1983), groups of mice were infected with graded doses below the LD_{50}. For isolation of infectious virus from brain, liver and spleen, homogenates were assayed by the plaque method on DBT cells in 12-well Costar plates.

RESULTS

Selection of JHM-MAb variants by infection of hybridoma lines

In order to select variants of JHM virus with mutations affecting defined domains of the peplomer protein, we infected neutralizing antibody-secreting hybridoma cell lines. As shown in Fig. 1, JHM virus infects and replicates in the myeloma cell line P3X at high titres. These myeloma cells had been used to generate the hybridoma lines for this study (Wege *et al.*, 1984*b*). Within 1 day p.i., the amount of virus detectable in the culture medium reached more than 10^5 p.f.u./ml. The release of infectious virus did not cease until termination of the culture (Fig. 1*a*). No cytopathic effect or decrease of cell numbers in comparison to uninfected cells was noted. Similar growth kinetics were observed in infected hybridoma cultures which produced antibodies against the matrix protein E1 (Fig. 1*b*). By contrast, a delay in release of infectious virus was noted if hybridoma lines were used which secreted virus-neutralizing anti-E2 MAbs. To obtain a representative collection of variants, we used nine different hybridoma lines. As shown by the examples in Fig. 1(*c*) to (*f*) the cultures finally produced amounts of infectious virus similar to the myeloma line P3X or the E1 hybridoma cells. To monitor for the appearance of neutralization-resistant variants during the incubation period, we treated a portion of each sample with ascites fluid containing the same MAb as secreted by the hybridoma culture (Fig. 1). For virus samples taken later than 4 days p.i., the infectivity was not significantly influenced by the attempt to neutralize the virus. For some hybridoma lines (e.g. E2-B7) the proportion of neutralization-resistant virus was variable within the first days p.i. The susceptibility to neutralization with the selecting MAb was determined by the plaque reduction assay when the culture was terminated (5 to 7 days p.i.). In all experiments performed, the released virus at this time was highly resistant to neutralization by the type of MAb contained in the hybridoma culture used for selection. The frequency of neutralization-resistant virus was estimated by determination of the neutralization index with each MAb for JHM-wt virus stocks and for virus released from the myeloma cell line P3X. The ratios were always less than 4×10^{-4}. In contrast, no significant titre differences were found for variant viruses and the complementary MAb. To
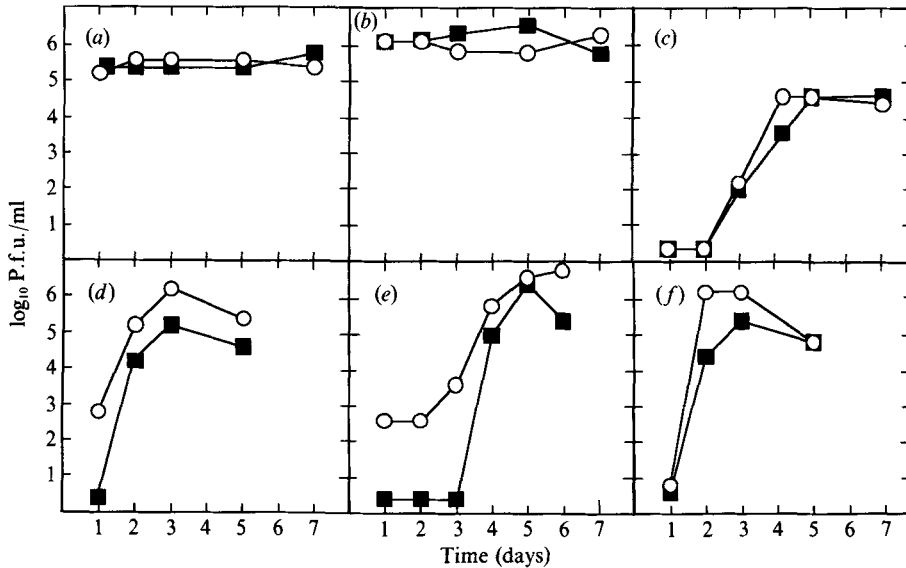
Fig. 1. Growth of JHM-wt virus in hybridoma cell cultures. The infection was started with 0·5 to 1·0 p.f.u./cell. ○, Direct determination of infectivity (plaque test); ■, determination of infectivity after incubation with the corresponding MAb for 1 h at 4 °C. (a) Myeloma P3X, IgG P3X; (b) hybridoma E1-A1, MAb E1-A1; (c) hybridoma E2-A1, MAb E2-A1; (d) hybridoma E2-A4, MAb E2-A4; (e) hybridoma E2-B7, MAb E2-B7; (f) hybridoma E2-B35, MAb E2-B35.

test the reproducibility of the approach described, we repeated the selection procedure with each hybridoma line two further times in independent experiments. Furthermore, virus stocks propagated from five different isolated plaques were tested by neutralization for each type of variant virus. The virus variants obtained always displayed the antigenicity described in the following sections.

Delineation of an epitope map by variant analysis

Nine different JHM MAb variants were obtained and classified by their reactivity with the panel of anti-E2 MAbs. For neutralization and cell fusion inhibition tests antibodies which define the antigenic sites E2-A and E2-B were used. Binding tests (ELISA and immuno-fluorescence) were also performed with MAbs which do not neutralize or inhibit cell fusion.

Neutralization

To confirm the site specificity of antigenic changes, each variant was incubated in parallel assays with a panel of neutralizing MAbs and the PRD_{50} was determined. Three examples are shown in Fig. 2. All variants escaped neutralization by the MAb present during their growth in the hybridoma culture. Moreover, most variants were also not neutralized by other MAbs that defined the same antigenic site as the selecting antibody. For example, the MAb E2-A1 did not neutralize JHM-A1 as well as the variants JHM-A3 and JHM-A26. The corresponding antibodies define the antigenic site E2-Aa. In contrast, JHM-A4 and JHM-A5 (antigenic site E2-Ab) or JHM-B6, JHM-B35 and JHM-B7 (antigenic sites E2-Ba and -Bb) were neutralized by MAb E2-A1 to a similar extent to JHM-wt. Two types of one-way reaction patterns were observed (Fig. 2 and Fig. 4a). Variant JHM-A2 escaped neutralization by MAb E2-A2, but was still strongly neutralized by the MAbs E2-A1, -A3 and -A26. On the other hand, JHM-B7 escaped neutralization not only by MAb E2-B7, but was also resistant to MAbs E2-B6 and -B35. However, MAb E2-B7 efficiently neutralized JHM-B6 and JHM-B35. The viruses JHM-wt, JHM-P3X and JHM-E1 (selected with a hybridoma secreting MAbs against glycoprotein E1) could not be distinguished by neutralization assays.
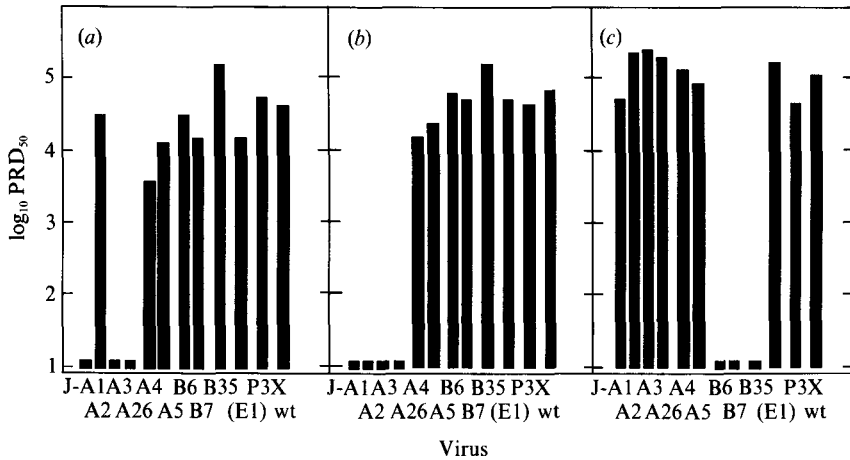
Fig. 2. Neutralization of JHM MAb variants with anti-E2 MAbs (plaque reduction test). (*a*) MAb E2-A1, (*b*) MAb E2-A2 and (*c*) MAb E2-B35.
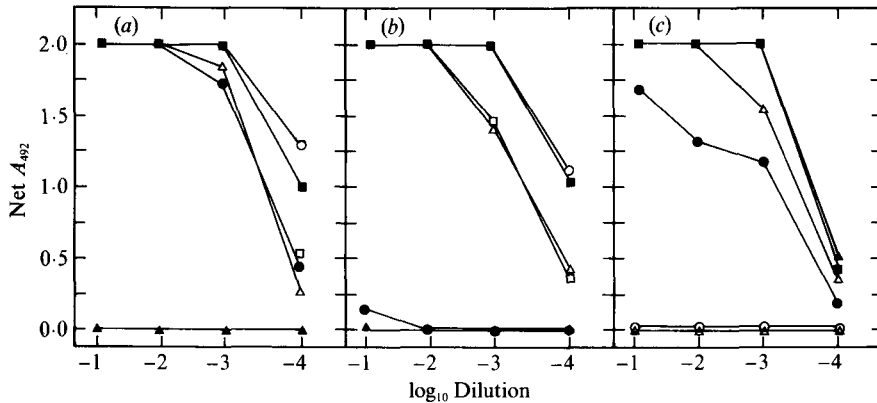

Fig. 3. Binding of anti-E2 MAbs to JHM MAb variants in ELISA. The viruses used were as follows with their changed epitopes in parentheses: ▲, JHM-E2-A1 (E2-Aa); ●, JHM-E2-A2 (E2-Aa); △, JHM-E2-A4 (E2-Ab); □, JHM-E2-B6 (E2-Ba); ○, JHM-E2-B35 (E2-Ba); ■, JHM-wt. (*a*) MAb E2-A1, (*b*) MAb E2-A2 and (*c*) MAb E2-B35.

ELISA, immunofluorescence and dot blot

Variants with antigenic changes affecting an epitope for neutralization may still bind the antibody (Blondel *et al.*, 1986). To test this possibility, we examined the ability of each MAb to bind to the JHM variants in ELISA (Fig. 3). The results correlated well with those obtained in the virus neutralization test (Fig. 2). Only MAbs which neutralized a given virus variant reacted with that variant in ELISA. Furthermore, the one-way reaction patterns as described for the neutralization assays were also observed. No complete correlation between binding and neutralization was obtained for reactions between variants JHM-A4 and A5 with their corresponding MAbs (Fig. 4). Although JHM-A4 was neutralized by MAb E2-A5, no significant binding was found in ELISA. Immunofluorescence tests on the whole confirmed the ELISA results. In addition, the binding of nine non-neutralizing MAbs (antigenic sites E2-C, E2-Da and -Db, E2-E and E2-F) to the JHM variants was compared with ELISA and immunofluorescence tests. In no case was a loss or change of binding relative to assays with JHM-wt observed. This suggests that in each variant the structural changes responsible for the loss of antibody binding are probably limited, affecting only the epitope against which the selective MAb was directed.
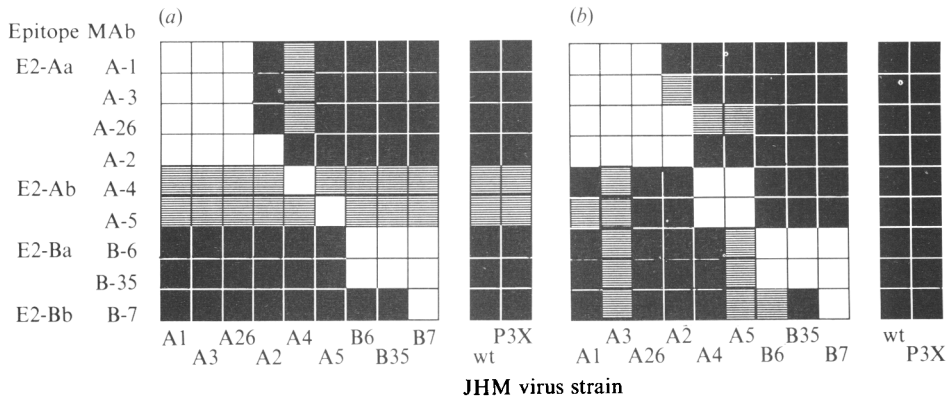
Fig. 4. Epitope maps delineated by variant analysis. (a) Summary of neutralization assays and (b) summary of ELISA binding. Open squares represent log $_{10}$ PRD$_{50}$ between 0 and 1 in (a) and net A_{492} between 0 and 0·5 in (b). The shaded squares represent log$_{10}$ PRD$_{50}$ between 1 and 3 in (a) and net A_{492} between 0·5 and 1·5 in (b). The filled squares represent log$_{10}$ PRD$_{50}$ greater than 3 in (a) and net A_{492} greater than 1·5 in (b).

A comparison of the antigenic maps derived by neutralization and ELISA of the variant collection with the panel of anti-E2 MAbs is displayed in Fig. 4. The patterns fall into essentially four different groups. As previously shown, we could only delineate two antigenic sites (E2-A and E2-B) by competitive immunoassays (Wege et al., 1984b). The antigenic sites E2-Aa, -Ab as well as the antigenic sites E2-Ba and -Bb are probably topographically closely associated.

Furthermore, all MAbs which reacted by immunostaining with JHM-wt virus recognized the variant viruses. The reactivity of those MAbs which defined at least five different SDS-resistant antigenic sites was quantified by dot blot analysis (data not shown). Despite the clear cut antigenic modifications demonstrated by MAbs that neutralized and inhibited fusion, no additional changes were found for other antigenic sites. Dot blot analysis with MAbs against viral nucleocapsid and matrix protein did not reveal other modfications.

Inhibition of variant virus-induced cell fusion by anti-E2 MAbs

Antibodies against antigenic E2-Aa are also able to inhibit JHM virus-induced cell fusion. The results supported the findings of the neutralization and ELISA tests. However, because not all neutralizing MAbs can inhibit cell fusion, it was not possible to extend and complement the epitope maps (Fig. 4) by fusion inhibition assays. No significant fusion inhibition was demonstrated with the MAbs A4 and A5 (antigenic site Ab) and A2 (antigenic site Aa) for any of the JHM variants. The MAbs B6, B7 and B35 were also able to inhibit cell fusion. Previously, we did not find any significant fusion inhibition with this class of MAbs (Wege et al., 1984b). This discrepancy might be due to the now more sensitive assay system and the significantly higher antibody titres of the MAbs used for this work. The results of cell fusion inhibition correlated well with those obtained by virus neutralization tests. Therefore, domains responsible for cell fusion are probably closely related to the binding sites for virus-neutralizing antibodies.

Comparison of structural proteins

JHM virus populations released during persistent infections or during passage in neural cells can be differentiated from the JHM-wt E2 protein by the migration pattern in polyacrylamide gel electrophoresis (Taguchi et al., 1985, 1986). To determine whether or not the antigenic changes of our JHM MAb variants were related to extensive structural modifications, each JHM variant was propagated in Sac(−) suspension cultures, purified by density gradient centrifugation and analysed by Western blotting. No differences in electrophoretic behaviour of the peplomer E2 protein and the nucleocapsid N or the matrix protein E1 were detected.

Table 1. *Neurovirulence of JHM MAb variants*

Virus	Epitope of E2 protein changed	LD_{50} (p.f.u./mouse)	Incubation time (days)	Disease
JHM-wt	–	<0·10	4–6	Acute hepatitis, encephalomyelitis
JHM-P3X	–	<0·10	4–6	Acute hepatitis, encephalomyelitis
JHM-A1	E2-Aa	0·07	3–9	Acute hepatitis, encephalomyelitis
JHM-A4	E2-Ab	<0·10	3–9	Acute hepatitis, encephalomyelitis
JHM-B35	E2-Ba	>5600	3–17	Chronic disease
JHM-B7	E2-Bb	0·21	3–7	Acute hepatitis, encephalomyelitis

Pathogenicity

To compare the neurovirulence of our variant virus collection, we used mice instead of rats for the following reasons. More than 10000 p.f.u. of JHM-wt virus are necessary to induce a demyelinating disease in rats, but a dose of less than 0·1 p.f.u. causes disease in mice. Therefore, in the rat system highly virulent revertants to the wild-type phenotype might obscure a change in pathogenicity characteristic for the variant virus. Furthermore, the infectious titres of several virus stocks were not high enough to test all variants with the same range of doses.

Female BALB/c mice were infected by intracerebral inoculation of graded doses of JHM-wt, JHM-P3X and each JHM MAb variant as described in Methods. As summarized in Table 1, for JHM-wt and JHM-P3X the LD_{50} was less than 0·1 p.f.u. and the mice died rapidly within less than a week. JHM MAb variants changed in antigenic sites E2-Aa and E2-Ab were of similar virulence. Variants changed in antigenic site E2-Bb were slightly less virulent but induced similar symptoms to JHM-wt virus. In most cases, the severity of symptoms progressed rapidly and the mice died within a few days after the onset of disease. In contrast, variants changed in antigenic site E2-Ba were significantly less virulent. Although animals developed clinical symptoms (ruffled fur, hunched position) within a short incubation time, the further course of disease differed strikingly from mice infected with JHM-wt, JHM-P3X or other JHM MAb variants. Partial paralysis of the hind legs and ruffled fur persisted for periods up to several months and was followed by complete recovery. For neuropathological investigation, groups of mice which survived the infection were dissected 1 to 4 months p.i. and examined. Ten of 12 investigated mice contained demyelinating lesions in brain and spinal cord (Fig. 5). Neurons were often conserved. In contrast, survivors of infections with JHM-P3X revealed no conspicuous pathological changes.

The growth of the viruses JHM-P3X and JHM-B35 was compared in brain, spleen and liver of infected mice over a period of 20 days. For each time point, three mice were dissected. The highest amount of infectious virus was recovered 4 to 5 days p.i. from brain tissue and the amounts were similar for both viruses. Only a slight difference was found in the time course. Virus JHM-P3X could only be isolated from infected mice for 6 days following infection. In contrast, the amount of infectious JHM-B35 virus declined more slowly. At 15 days p.i. infectious virus was no longer detected.

DISCUSSION

Hybridoma cultures which produce neutralizing antibodies against the E2 peplomer protein were infected with JHM. Virus recovered from the culture supernatants was found to be resistant to neutralization by the antibody of the infected hybridoma cells. A collection of antibody-resistant JHM variants was produced and analysed with the panel of anti-E2 MAbs. The results indicated that at least four mutable antigenic sites were associated with domains important in the biological functions of the E2 protein. Furthermore, the property to cause a chronic demyelinating disease was associated with the selective change of a particular antigenic site designated E2-Ba.

The selection process for JHM virus changed in a particular epitope must occur within the first growth cycles of the virus because for 2 to 3 days p.i. the culture supernatants did not contain significant amounts of infectious virus (Fig. 1). No delay in virus release was observed if
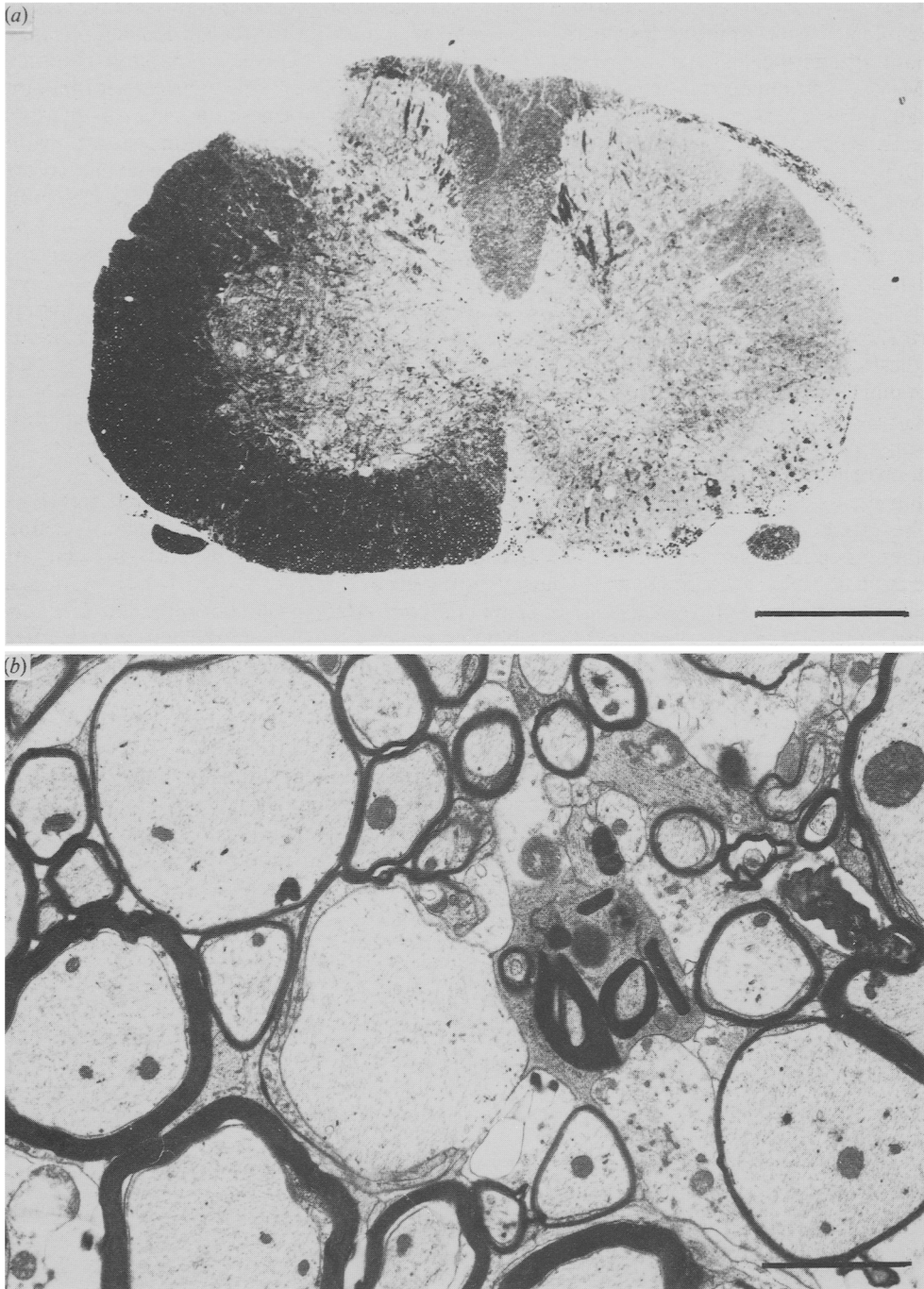
Fig. 5. Cross-section from the spinal cord of a mouse dissected 3·5 months after infection with variant JHM-B35. (a) Embedded in Araldite, stained with toluidine blue. The right half of the spinal cord is almost completely demyelinated with the exception of the posterior column and the spinal roots. Bar marker represents 40 μm. (b) Electron micrograph of the same preparation. New myelin sheaths have been formed around naked axons. Fresh remyelination indicated by incomplete myelin ring on the large axon in the centre of the figure. Macrophages containing myelin debris are still present. Embedded in Araldite, stained with uranyl acetate and lead citrate. Bar marker represents 1 μm.

the myeloma line P3X or anti-matrix as well as anti-nucleocapsid protein hybridoma cultures were infected. Several mechanisms are conceivable which favour the replication and release of variant viruses specifically changed in the site of a particular epitope. The rapid evolution of JHM MAb variants could be due to displacement of the wild-type population with mutants already present in the virus stocks. The stock cloned by plaque passages probably still consists of a heterogeneous RNA genome population differing by only a few mutations in a dynamic equilibrium (Domingo *et al.*, 1985). Furthermore, specific single site mutations which have an impact on the tertiary structure of E2 could be favoured within the first growth cycles in the presence of specific antibodies.

The first site for replication and release of infectious virus might be a small fraction of cells which do not synthesize antibodies. Spread of virus that displays the wild-type phenotype would be efficiently impeded by neutralization with antibodies in the culture supernatant. Furthermore, an abortive infection might occur in the presence of neutralizing antibodies synthesized within the same cell. Similar to the processing of E2 protein, newly translated immunoglobulin chains are released into the endoplasmic reticulum. From this site maturation proceeds by glycosylation, cleavage to subunits, transport to the Golgi apparatus and secretion. During virus replication, either the transport or the maturation of E2 protein displaying the epitope recognized by MAbs synthesized in the same cell might be suppressed. Studies on the maturation of the vesicular stomatitis virus (VSV) G glycoprotein indicated that intracellular applied antibodies can bind and influence viral protein synthesis. Antibodies against defined synthetic peptide fragments of VSV G glycoprotein were introduced by microinjection and efficiently inhibited the transport of newly synthesized G protein to the Golgi apparatus (Arnheiter *et al.*, 1984). Finally, virus assembly and release could be impaired. It is also conceivable that antigenic modulation by binding of certain MAbs on the cell surface affects virus synthesis at the transcriptional and translational levels. Such mechanisms have been observed for persistent measles virus infections (Fujinami *et al.*, 1984).

The domains of E2 peplomer involved in biological functions carry epitopes that are largely dependent on the conformation of the protein and may be formed from discontinuous stretches of amino acid sequences (Barlow *et al.*, 1986). The E2 protein antigenic sites involved in neutralization and fusion are probably shaped during maturation in the Golgi apparatus (Repp *et al.*, 1985). JHM MAb variants grown in hybridoma lines differ probably by only a few mutations affecting critical sites for the formation of defined epitopes. The resistance to neutralization and loss of fusion inhibition for a defined MAb correlates with a lack of binding to the epitope in question as indicated by ELISA. However, MAbs against other epitopes including those probably defined by the primary amino acid sequence still bind to the variant. Furthermore, in contrast to the observations with viruses re-isolated from persistent infections or from rat brain cells (Makino *et al.*, 1983; Taguchi *et al.*, 1985, 1986; Wege *et al.*, 1987), no obvious change of migration in polyacrylamide gel electrophoresis was noticeable. The mutations responsible for the antigenic changes of the JHM MAb variants are remarkably stable. JHM virus re-isolated from mice 2 weeks p.i. or passaged four times through brains of suckling mice did not revert to the wild-type phenotype. Since the complete sequence of the JHM virus E2 gene is now available (Schmidt *et al.*, 1987), the critical mutations differentiating such variants from the wild-type virus can be analysed. Such studies might help in learning more of the relations between structure and function. Antigenic sites of E2 protein associated with domains of biological importance are probably largely conformation-dependent (Talbot *et al.*, 1984). Therefore, mutations located in very different regions of the gene could be of importance for the accessibility and conformation of binding sites.

The framework for the delineation of an epitope map with our panel of anti-E2 MAbs was obtained by competitive immunoassays (Wege *et al.*, 1984*b*). Due to different binding properties of MAbs and steric effects this approach could still underestimate the actual number of antigenic sites. The patterns obtained by outlining the results of neutralization, inhibition of cell fusion, immunofluorescence and binding in ELISA for all combinations of variants and MAbs are remarkably similar (Fig. 4). Four different groups of variants changed selectively in antigenic sites associated with neutralization and cell fusion were obtained. With additional

observations (e.g. binding patterns with different murine coronavirus strains and mutants of JHM virus, reactivity in immunoprecipitation and Western blots; Wege *et al.*, 1987 and unpublished results), these reaction patterns indicate that four different antigenic sites are located in domains of the E2 protein important for cell fusion and neutralization. A final correlation of our interpretation with the epitope maps delineated with other panels of anti-E2 MAbs and JHM viruses should be possible by studies combining representative MAbs and JHM viruses.

It has been shown recently that JHM virus variants selected by their lack of neutralization with E2 MAbs reveal specific alterations of the E2 protein and can be altered in pathogenicity (Dalziel *et al.*, 1986; Fleming *et al.*, 1986). Our findings obtained with a different system complement and extend these studies. Variants selected by MAbs against epitopes in sites E2-Aa, E2-Ab and E2-Bb are as virulent as the JHM-wt virus and induce a rapidly progressing acute hepato-encephalomyelitis. Distinct results were obtained with variants changed in antigenic site E2-Ba (Table 1 and Fig. 4). These variants are significantly less virulent, but have a strong propensity to induce a chronic demyelinating disease associated with a persistent virus infection of the central nervous system. This changed biological behaviour is not simply caused by a loss of neurotropism, since the virus titres in brain and peripheral organs reached within a few days p.i. do not differ between the highly virulent JHM-P3X and the variant virus. Active demyelinated lesions are still prominent several months p.i. in most of the surviving mice. Further studies of such infections may give additional insights on the virus–host interactions leading to persistence and demyelination.

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