CORONAVIRUS JHM INDUCED DEMYELINATING DISEASE: SPECIFIC DOMAINS ON THE E2-PROTEIN ARE ASSOCIATED WITH NEUROVIRULENCE

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INTRODUCTION

Infections of mice and rats with the coronavirus JHM are well established model systems to study mechanisms of virus induced demyelination[9,11,13-15,20-22], Massa et al., Dörries et al. this volume). Using virus strains originating from similar sources highly variable or even contradictory observations were made with regard to neurovirulence, type of disease and tropism for glial cells[2,3,12]. Despite of the highly advanced knowledge of virus structure at the molecular level the mechanisms of virus-host interactions important for pathogenesis are largely unknown. It had been shown previously, that intracerebral infection of Lewis rats can lead to encephalomyelitides associated with variable clinical symptoms and inflammatory demyelination. In most cases a rapidly progressing, fatal acute disease develops within 8-12 days p.i. (AE). Most interesting for studies of virus induced demyelination are animals, which show a slower onset of clinical signs (e.g. ataxic gait, paralysis) after incubation times of several weeks to months[13,14,15]. Such animals often survive, can suffer from clinical signs for months or sometimes develop a relapse[21]. This subacute demyelinating encephalomyelitis (SDE) is caused by a persistent virus

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infection of glia cells and neuropathologically characterised by lesions of primary demyelination in the white matter accompanied by infiltrations of lymphoid cells. Besides of host factors (age and genetics) the biological properties of the virus determine the outcome of infection. The results described in this chapter indicate, that defined regions of the peplomer protein E2 are a major determinant for the neurovirulence and pathogenetic potential of coronaviruses.

RESULTS

Pathogenicity and glia cell tropism of different JHM viruses

As summarized in Table 1, the original virus (obtained from Leslie Weiner), which was maintained by brain passages, caused different rates of AE and SDE. Selection of this virus by adaptation to tissue culture resulted in clones, which are highly virulent but cause only AE (JHM-Wt). Temperature sensitive mutants (e.g. JHM-Ts43) were selected, which can induce high rates of SDE. In contrast to JHM-Wt and JHM-Ts43, the virus shed from a persistently infected cell culture is not neurovirulent[1].

The major reason for the different biological behaviour could be related to a change in target cell specificity or in cytopathogenicity for brain cells. To answer this question, we infected mixed glial cell cultures derived from brains of newborn rats. Such cultures consist of astrocytes type I, microglia cells and oligodendrocytes. It had been shown recently, that rat brain macrophages and astrocytes are the major target for our JHM-virus isolates[12], Massa et al., this volume . JHM-Wt infection of such cultures leads to the development of large syncytia and destruction of the cells within two weeks. Oligodendrocytes display only antigen in association with syncytia formed by astrocytes. JHM-Ts43 induces also the formation of syncytia. However, the syncytia remain relatively small and a chronic infection with continuous release of infectious virus develops. By contrast, although JHM-Pi can infect astrocytes no

Table 1. Pathogenicity of JHM-Viruses for Lewis-rats.

DESIGNATION AND TYPE OF VIRUS	AGE OF RATS AT TIME OF INFECTION	
	8-12 Days	4-5 Weeks
JHM-Virus Original strain passaged in vivo	AE	AE and SDE
JHM-Wt Cell adapted wild type	AE	AE
JHM-Ts43 Temperature sensitive mutant	AE and SDE	SDE
JHM-Pi released by persistent infected cells	not virulent	not virulent

1) AE = Acute Encephalomyelitis
2) SDE = Subacute Demyelinating Encephalomyelitis

cytopathic changes are observed. The virus remains confined mainly to single cells. At high multiplicity of infection, infectious virus can be shedded for months without any obvious cytopathology. The fusion capacity for permanent tissue culture cell lines (DBT or Sac-) however is similar for all three types of virus. Therefore, specific differences in the capacity propagate and fuse glial cells is one important virological determinant for neurovirulence.

The peplomer E2 is obviously the protein responsible for cell fusion, binding of neutralising antibodies and attachment [7,8,10,16,18,19,23]. Some observations indicate that structural changes could be related to different biological properties [1,4,5,6,17]. Therefore, to characterise further the structural basis for virulence, we started to analyse antigenic binding sites (epitopes) related to regions of functional importance for fusion and neutralisation.

Characterisation of epitopes on the E2 peplomer protein

A collection of monoclonal antibodies (MAB) was obtained by fusion of lymphocytes from mice immunised with JHM-virus and the myeloma line P3X. The framework for definition of epitopes on the E2 protein was established by competition assays between biotinylated MAB's and unlabeled antibodies for binding sites on virus coated to the surface of microplates. The competition patterns obtained allowed to delineate at least six different epitopes[23]. We designated these epitopes as E2-A, E2-B, E2-C, E2-D, E2-E and E2-F. Since due to sterical reasons and differences in binding strengths the actual number of antigenic binding sites may be underestimated, additional methods were applied. A number of murine coronavirus strains was tested for their reactivity with anti-E2 MAB's with regard to binding, virus neutralisation and inhibition of cell fusion. Four different epitopes are located in regions which bind neutralising antibodies and are involved in cell fusion (E2-Aa, E2-Ab, E2-Ba, E2-Bb). MAB's against epitope E2-Aa are highly specific for JHM-virus, whereas MAB's against other epitopes show variable reaction patterns with other virus strains. Moreover, several selective reaction patterns are seen by immunoprecipitation and Western-blot analysis.

As shown by previous studies, the E2 protein of coronavirus JHM is derived from a primary polypeptide (p120), cotranslationally glycosylated (gp150), further processed to the gp200 of mature virus and probably partially cleaved to subunits of similar size (gp90). Furthermore, a virion glycoprotein (gp65) is detectable. The relation of gp65 to E2 has not yet been elucidated. Some MAB's react with all subunits of the E2 protein. Other MAB's react only with gp200 or with gp90 subunits (Fig. 1). Epitopes related to regions of biological functions are probably formed by the tertiary structure, since MAB's which neutralise and inhibit fusion do not bind to virus treated with detergent or proteolytic enzymes. A combination of all these results suggests, that the antigenic map of the E2 protein comprises more than nine epitopes.

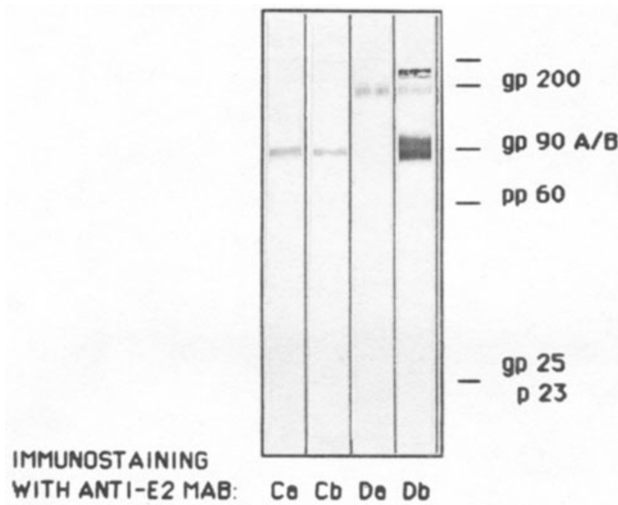

Fig. 1. Reactivity of different anti-E2 MABs with JHM-Wt virus.

Table 2. Differentiation of JHM-Wt, JHM-Ts43 and JHM-Pi with MABs against the E2-peplomer protein.

REACTIVITY OF MAB'S WITH JHM-VIRUSES OF DIFFERENT VIRULENCE

(NEUTRALISATION AND INHIBITION OF CELL FUSION)

DESIGNATION OF EPITOPE	JHM-WT		JHM-Ts43		JHM-Pi	
	NEUTR.	FUS.INH.	NEUTR.	FUS.INH.	NEUTR.	FUS.INH.
E2 - Aa	++++	++++	++++	++++	-	++++
E2 - Ab	+++	-	++	-	++++	-
E2 - Ba	++++	+++	+++	+++	-	-
E2 - Bb	++++	-	-	-	++	-
E2 -C to F	-	-	-	-	-	-

Structural and antigenic variability of the E2 peplomer protein

It is not possible to differentiate JHM-Wt, JHM-Ts43 and JHM-Pi with polyclonal antisera. However, as summarised in Table 2, these viruses can be distinguished by MAB's which neutralise or inhibit cell fusion. Two epitopes are associated with regions responsible for both fusion and binding of neutralising antibodies (E2-Aa and E2-Ab). For JHM-Pi, this region seems to be in a configuration, which allows to discriminate between fusion (Aa) and neutralisation (Ab). Differences are also recognisable for the epitopes Ba and Bb. Compared to JHM-Wt, JHM-Pi reveals more pronounced changes than JHM-Ts43.

Additional changes concerning epitopes which are not associtated with fusion and neutralisation are visible by differences in reaction patterns in immunoprecipitation and by immunostaining of Western-blots. The structural proteins of purified JHM-Wt, JHM-Ts43 and JHM-Pi were compared by polyacrylamide gel electrophoresis (PAGE). The electrophoretic mobility of gp200 do not differ between these viruses. By contrast, distinct differences are visible in the region of gp90 subunits. For JHM-Wt, only one band of gp90 is detectable by PAGE. For JHM-Ts43, two gp90 bands are clearly discriminatable. One protein migrates to the same position as shown for JHM-Wt, another protein migrates slower. JHM-Pi virus by contrast reveals one protein comigrating with gp90 of JHM-Wt and a slightly faster migrating protein. Furthermore, whereas both JHM-Wt and JHM-Ts43 contain gp65, this protein was not seen in purified JHM-Pi virus. Similar differences are detectable by PAGE-analysis of immunoprecipitates from infected cells (Fig. 2). In addition, the E2-precursor gp150 of JHM-Ts43 migrates slightly slower than the gp150 of JHM-Wt.

These viruses differ also in the reaction patterns of MAB's binding to epitopes not involved in fusion and neutralisation. As shown in Fig. 2, MAB's against epitope Aa precipitate from JHM-Wt lysates gp200, gp150 and gp90. With JHM-Ts43 lysates, a precipitation of gp200 is hardly detectable. However, it is conspicuous that anti Aa MAB's precipitate gp65 in addition to gp150, gp90A and B from JHM-Ts43 lysates. A MAB against epitope

F precipitates from JHM-Wt lysates gp150 and gp90, from JHM-Ts43 gp150, gp90B and again gp65. Distinct differences are also recognisable by Western-blot analysis. These observations indicate, that the E2 protein of isolates with different virulence may be changed by mutations, which affect the conformation of the peplomer. With regard to virus-host interactions, such changes could influence the accessability of binding sites for antibodies, the immunogenicity, the capacity for cell fusion and specific attachment to cell receptors.

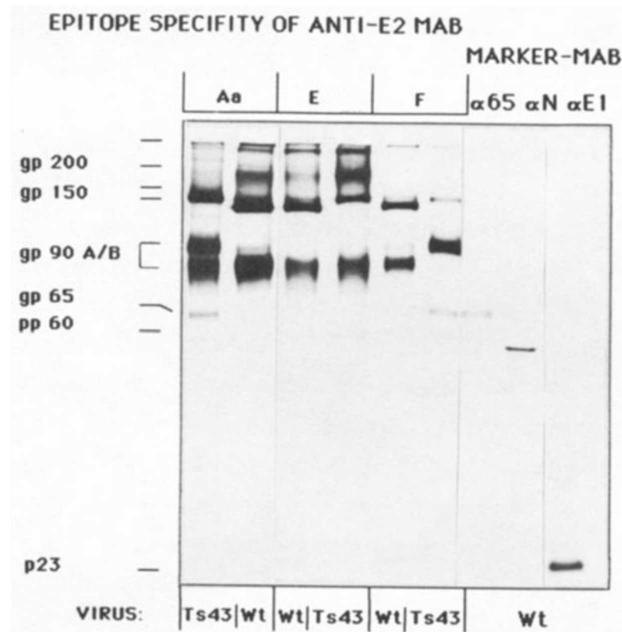

Fig. 2. Reactivity of different anti-E2 MABs with JHM-Wt and JHM-Ts43 virus. Immunoprecipitates of lysates from infected cells (labeled with ^{35}S-methionine) were analysed by PAGE.

In order to define more precisely relationships between structure and virulence, we selected variants from JHM-virus, which are changed in defined epitopes.

Selection of variants changed in defined E2-epitopes

Our approach for selection of variants is based on the observation, that the myeloma line P3X can be infected by JHM-virus. These cells had been used to immortalise lymphocytes in order to select our hybridoma lines[23]. Infected P3X cells started to release virus within 8 hours p.i. reaching titers of more than 10 000 pfu/ml. The cells continue to release virus for more than a week. Infection of hybridoma lines, which produce neutralising antibodies against E2, resulted after a delay of 2 days in release of virus to similar titers than in P3X cells. This virus population is not longer neutralisable by the MAB produced in the hybridoma line (Fig. 3). MAB's, however, which recognise other epitopes involved in neutralisation, still neutralise the JHM-variant. For example, virus selected from a hybridoma culture which produces MAB's against epitope Aa, can be neutralised by MAB's against epitopes Ab, Ba and Bb. The same virus variant however escaped neutralisation by MAB's against Aa. Moreover, by Elisa no specific binding of E2-Aa MAB's to this JHM-variant is detectable (Fig. 4). However, no reduction in binding of MAB's against other epitopes to that variant was seen. We selected four classes of JHM-variants, corresponding to the epitopes associated with neutralisation and cell fusion. In contrast to JHM-Ts43 or JHM-Pi, no structural differences were detectable by PAGE. Since only one definable epitope was probably changed by our selection procedure, only few mutations which affect precisely the conformation of E2 related to a specific antigenic binding site are likely to occur.

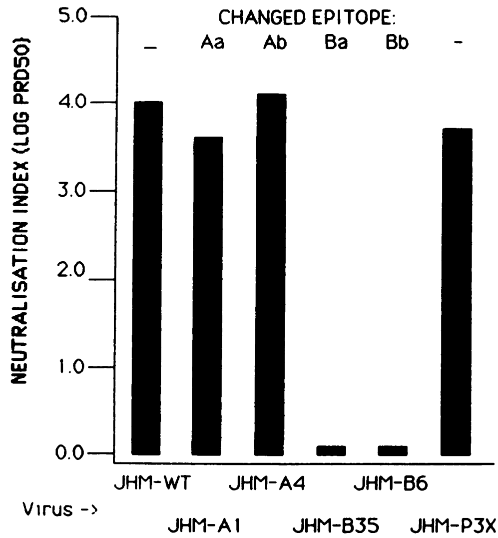

Fig. 3. Neutralisation of JHM-MAB variants with a MAB against epitope E2-Ba (antibody E2-B35)

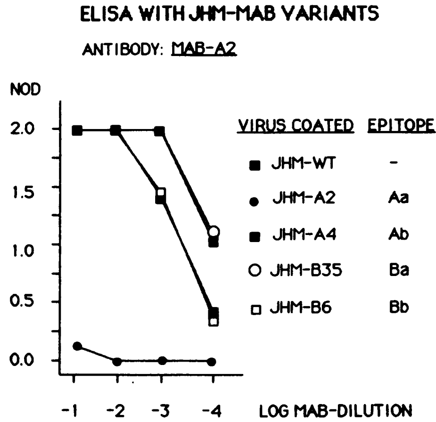

Fig. 4. Binding of a MAB against epitope Aa to JHM-MAB variants

Neurovirulence of JHM-MAB variants

The neurovirulence of these variants was tested by infection of Balb/c mice (Table 3). Groups of 8-10 female mice (7-8 weeks old) were infected by the intracerebral route with tenfold dilutions of each virus ranging from 10 000 to 0.1 PFU/animal. Both the JHM-Wt virus and JHM-virus recovered from P3X myeloma cells is highly virulent, the 50 % lethal dose is less than 0.1 PFU/mouse. Variants changed in epitope E2-Aa, E2-Ab and E2-Bb are of similar virulence. The mice developed an acute hepatitis and encephalomyelitis, leading to death within a few days after onset. By contrast, variants changed in epitope E2-Ba are less virulent and the mice displayed a strikingly different disease picture. The majority of mice develops within 3-5 days pronounced symptoms like ruffled fur, hunched position and paralysis of hindlegs. Most animals survive the acute phase and remain clinically diseased for several months. Such animals displayed widespread demyelinating lesions upon neuropathological investigation.

Table 3. Neurovirulence of JHM-MAB variants for Balb/c mice

VIRUS STRAIN	EPITOPE OF E2-PROTEIN CHANGED	LD50 (PFU/MOÜSE)	INCUBATION TIME (DAYS)	DISEASE
JHM-WT	------	<0,10	4-6	ACUTE HEPATITIS, ENCEPHALOMYELITS
JHM-P3X	----	<0,10	4-6	ACUTE HEPATITIS, ENCEPHALOMYELITS
JHM-A1	E2-Aa	0,07	3-9	ACUTE HEPATITIS, ENCEPHALOMYELITS
JHM-A4	E2-Ab	<0,10	3-9	ACUTE HEPATITIS, ENCEPHALOMYELITS
JHM-B35	**E2-Ba**	**>5600,00**	**3-17**	**CHRONICAL DISEASE**
JHM-B7	E2-Bb	0,21	3-7	ACUTE HEPATITIS, ENCEPHALOMYELITS

BALBc MICE (FEMALE, 7-9 WEEKS OLD) INFECTED BY THE INTRACEREBRAL ROUTE

SUMMARY AND CONCLUSIONS

Infections of rodents by murine coronaviruses can lead to chronic diseases of the central nervous system. These infections are interesting systems to study mechanisms which could be relevant for the pathogenesis of certain human diseases. One major factor influencing the outcome of infection is related to the virus.

To understand the virological basis for neurovirulence we compared JHM-virus isolates with different biological properties. JHM-Wt causes only acute disease, JHM-Ts43 a demyelinating encephalomyelitis and a virus shedded from persistently infected cells (JHM-Pi) is not virulent at all. The spread of these viruses in glial cell cultures reflects their different neurovirulence for animals. The peplomer E2 of these viruses reveals structural and antigenic differences. We characterised the epitopes of E2 with a panel of monoclonal antibodies. Four epitopes are associated with regions important for neutralisation, cell fusion and attachment. More than five epitopes are not related to such functions. Epitopes differ in their location and accessibility on the E2 protein subunits between JHM-Wt, JHM-Ts43 and JHM-Pi. To identify epitopes in regions important for pathogenesis, we performed animal studies with variants selected by monoclonal antibodies. Variants changed in a defined epitope (E2-Ba) induce in Balb/c mice a chronic disease. Variants changed in only one of the other three neutralisation epitopes induce acute disease.

These results support and extend the observation that the peplomer protein E2 is a major determinant for virulence and antigenic variability of coronaviruses [1,4,5,6,8,10,17,19,22,23]. Increasing evidence had been obtained that certain structural features of this protein are important for the cell tropism of the virus. Furthermore, this protein influences strongly the type and specificity of immune responses against viral and host antigens. The highly advanced knowledge on structure and replication of coronaviruses will be of great value to analyze further mechanisms leading to inflammatory

demyelinating diseases associated with a persistent virus infection.

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