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Antigenic Determinants of Measles Virus Hemagglutinin Associated with Neurovirulence

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The biological activity of monoclonal antibodies specific for the hemagglutinin protein of measles virus strain CAM recognizing six epitope groups according to their binding properties to measles virus strain CAM/R401 was investigated in vivo in our rat model of measles encephalitis. When injected intraperitoneally into measles virus-infected suckling rats, some monoclonal antibodies modified the disease process and prevented the necrotizing encephalopathy seen in untreated animals. The analysis of measles virus brain isolates revealed emergence of variants that resisted neutralization with the passively transferred selecting monoclonal antibody but not with other monoclonal antibodies. Monoclonal antibody escape mutants were also isolated in vitro, and their neurovirulence varied in the animal model. Sequence data from the hemagglutinin gene of measles virus localize a major antigenic surface determinant of the hemagglutinin protein between amino acid residues 368 and 396, which may be functionally important for neurovirulence. The data indicate that the interaction of antibodies with the measles virus H protein plays an important role in the selection of neurovirulent variants. These variants have biological properties different from those of the parent CAM virus.

Measles virus (MV) is an important human pathogen that leads to an acute infection which is still responsible for about 2 million deaths per year in developing countries. Besides acute measles, MV induces two fatal diseases of the central nervous system (CNS), subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis. These diseases develop as a result of persistent infections characterized by restriction of viral envelope gene expression as a consequence of transcriptional and translational alterations (27, 33). SSPE develops in the presence of high-titered antimeasles antibodies in the cerebrospinal fluid and in serum which are apparently not able to eliminate MV in the brain. The extensive molecular sequencing of CNS-derived disease-associated MV genomes has demonstrated a surprisingly high frequence of nucleotide variations (4, 6, 31), and it has become apparent that clonally distinct lineages of MV are present simultaneously in SSPE at the end stage of the long-lasting disease process (2). Information on the biological significance of this observation for the course of infection, for disease development, and for its pathogenesis is lacking.

While much knowledge has been accumulated about the molecular organization of MV, its biological properties, in particular the molecular biology of its virulence and tropism, are not well understood. It has been shown in other virus-host systems that structural determinants on the major surface glycoprotein strongly influence viral tissue tropism and pathogenesis (22). Therefore, to define the role of the hemagglutinin (H) protein for MV interaction with brain tissue, monoclonal antibodies (MAbs) against the H protein that neutralize the infectivity of MV were tested in our rat model of experimental measles infection. The biological effects of the MAbs on the selection of neurotropic variants and on the development of MV encephalomyelitis and the neutralization profiles of viral reisolates from brain material of infected and MAb-treated animals are described. The data obtained show a close correlation between in vivo- and in vitro-selected mutants. The results indicate that a selection of viral variants with neurovirulent properties occurs in infected animals in the presence of neutralizing MAbs. We also report the molecular characterization of a major antigenic site on the H protein and discuss its possible role in neurovirulence.

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MATERIALS AND METHODS

Tissue culture, viruses, generation of MAbs, and serological tests. Conditions for growth of cells and the selection of MAbs have been reported previously (32). Immunoglobulin (Ig) isotypes were determined by enzyme immunoassay (EIA) as instructed by the manufacturer (Medac, Hamburg, Germany). The CAM/R40 strain of MV, originally obtained from K. Yamanouchi, Tokyo, Japan (15), was passaged by intracerebral (i.c.) inoculation in newborn Lewis rats. Stock CAM/RB virus (2×10^5 to 4×10^5 50% tissue culture infective doses per ml) was prepared as 25% brain homogenate from moribund rats in phosphate-buffered saline and used for all in vivo experiments because of the excellent reproducibility of disease course and duration as well as induced mortality (19). For the selection of MAb escape mutants in vitro and the preparation of antigen, CAM/RB was passaged once on Vero cells (multiplicity of infection of 0.001). The titer of the resultant CAM/R401 was 8×10^6 to 15×10^6 PFU/ml.

MV antigen for serological assays was prepared by density gradient purification from infected Vero cells as described previously (17). In the EIA, 1 μg of protein was coated onto flat-bottom microtiter plates and incubated with H-specific MAbs followed by phosphatase-coupled anti-mouse Ig and *o*-phenylenediamine as the substrate. Determination of hemagglutination-inhibiting (HI) activities, immunoprecipitation of in vivo-radiolabeled MV protein, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (SDS-PAGE), Western immunoblotting, and immunostaining were performed as described previously (3, 19, 23). For the determination of MV neutralizing titers (NT titers), 100 PFU of virus was incubated

(1 h at 37°C) with twofold dilutions of tissue culture supernatants containing MAbs against H protein. Then the virus-antibody mixtures were incubated (1 h, 37°C, 5% CO_2) with semiconfluent monolayers of Vero cells, and monolayers were overlaid with 0.75% agar. The number of viral plaques was determined after 6 days following staining with neutral red. The NT titer of an antibody was determined as the dilution that neutralized more than 50% of the input virus (i.e., reduced the number of plaques to fewer than 50).

Competitive binding EIA. Mouse Ig of the MAbs was purified from hybridoma culture supernatant by either hydroxylapatite column purification or ammonium sulfate precipitation. The purified Ig fractions (3 mg/ml) were coupled to biotin with biotinyl-*N*-hydroxylsuccinimide ester (BHSE; Pierce) as instructed by the distributor. Optimal self-competition by biotinylated antibodies was achieved with a BSHE/IgG ratio of 200 mg of BSHE per mg of IgG. For competition EIA, density gradient-purified CAM/R401 was coated onto round-bottom microtiter plates (Nunc) (1 μg of viral protein per well). The respective competitor antibody was applied in triplicate 10-fold dilutions and incubated for 1 h at 37°C. Following extensive washes, the biotinylated Ig was added at a dilution that yielded an absorbance of 1.5 ± 0.2 (100% relative A_{492}) as determined in a noncompetitive assay. After the final incubation with horseradish peroxidase coupled to avidin, *o*-phenylenediamine was used as the substrate. The criterion for overlapping antigen-binding sites (competition) was a reciprocal decrease of absorbance over several logs of antibody dilution. Each antibody was used both as a competitor as well as in the biotinylated form.

Isolation of viral escape mutants by using MAbs. For the isolation of MAb escape mutants, 10-fold dilutions (10^{-1} to 10^{-6}) of CAM/R401 were incubated with heat-inactivated (30 min at 56°C) virus-neutralizing H-specific ascites fluid (diluted 1:10; 45 min at 37°C) before infection of Vero cells. After 120 min, the virus-antibody dilution was removed and the infected cells were overlaid with 0.75% agar. Viral plaques were isolated after 5 to 6 days and seeded on 10^6 Vero cells in the presence of the respective anti-H ascites fluid (diluted 1:10). After two additional selection cycles, mutants remained stable in the absence of the respective antibody (i.e., they retained their property of being no longer neutralizable with the selecting antibody), and stocks were prepared and stored at −70°C for further use.

Assessment of neurovirulence. Suckling (1- to 2-day-old) or weanling (3- to 4-week-old) Lewis rats, purchased from the Zentralinstitut für Versuchstiere, Hannover, Germany, were infected by i.c. inoculation with CAM/RB (clarified supernatant, 10^4 PFU in 25 μl), with Vero cell-derived CAM/R401, or with MV escape variant CAM-L77, CAM-NC32, CAM-K29, or CAM-K71 (dose range, 0.5×10^6 to 1.6×10^6 PFU). In some experiments, suckling rats were injected intraperitoneally (i.p.), 4 to 8 h following i.c. infection, with 0.1 to 0.3 mg of Ig purified from hybridoma cultures equivalent to 5,000 MV neutralizing units (18). Animals were observed for the occurrence of signs of encephalitis: hunched position, abnormal gait, ataxia, paresis, and convulsions. Some rats which did not induce overt disease were sacrificed for virological studies at 5 to 7 days (suckling) or 10 to 12 days (weanling rats) postinfection. Isolation of virus was attempted by cocultivation of the MV-permissive Vero cells with fresh brain material, and viral titers in CNS material were determined. Additionally, representative frozen brain sections were screened for the presence of viral antigen and neuropathological abnormalities as described previously (17–19). Brain homogenate from newborn rats infected with mutant virus was used directly for infection of further litters. Three to four successive blind passages into newborn animals were done consecutively, after which we attempted to isolate virus from or demonstrate viral antigen in their brain tissues. This procedure was repeated before a mutant was considered nonneurovirulent.

cDNA cloning and nucleotide sequencing of escape mutant H genes. cDNA was produced by using synthetic oligonucleotide primers which were complementary in sequence to the 3′ and 5′ ends of MV H-gene mRNA (26). First-strand cDNA-RNA hybrid molecules or double-stranded cDNA species were amplified in vitro by using *Taq* polymerase (Promega) for 30 3-min cycles of primer extension at 72°C in the presence of both forward and reverse H-gene primers (each at a final concentration of 10^{-6} M). The amplified reaction mixture was resolved by agarose gel electrophoresis, and the band of amplified MV H-gene cDNA was isolated on a DEAE membrane. The 5′ termini of the DNA molecules were then phosphorylated with [γ-^{32}P]ATP, and the 3′ termini were digested (resected) with T4 DNA polymerase to regenerate the *Xma*I and *Acc*I restriction site overhangs which had been engineered into the gene-specific oligonucleotide primers (26). Bluescript SK+ plasmid DNA (Stratagene) was digested successively with the enzymes *Xma*I (New England Biolabs) and *Acc*I (Amersham), and the 42-bp *Xma*I-*Acc*I region of the plasmid's polylinker was removed by agarose gel electrophoresis of the digested DNA. The ends of the linearized plasmid were then treated with calf intestinal phosphatase at 0.05 U/pmol of 5′ ends. Resected DNA (1 to 20 fmol) was mixed with 5 fmol (10 ng) of the prepared Bluescript plasmid vector, and ligation was carried out according to standard protocols. The ligated mixture was then used to transform commercially available *Escherichia coli* DH5α competent cells (Bethesda Research Laboratories) as instructed by the supplier. Transformant colonies were analyzed by in situ hybridization (11) with MV H-gene-specific random-primed DNA probes which had been labeled with [α-^{32}P]dCTP (9). Colonies which were found to contain plasmids bearing full-length H-gene inserts or, in some cases, gene fragments were used for sequence analysis. MV H-gene sequences were determined by the dideoxynucleotide (chain termination) technique (25), using synthetic oligonucleotide primers (17- to 19-mers) corresponding to MV H-gene internal sequences in both strands. The primers were between 100 and 300 bp distant from each other and allowed overlapping sequences to be determined in both positive and negative strands of the H gene.

RESULTS

Characterization of MAbs by in vitro assays. All MAbs used in this study were generated against the Edmonston (EDM) strain of MV, were of either the IgG2a (K89, K17, NC26, NC32, K83, DW15, L77, and K4) or IgG3 (K29, K53, and K71) isotype, and, except for MAb K4, reacted also with CAM/R401. The MAbs recognized conformational epitopes on the H protein, as they failed to bind in Western blots to MV-infected cell lysates. The specificity was proven by their ability to inhibit the hemagglutinating activity of MV (HI titer range of ascites fluid, $1:2^6$ to $1:2^{12}$) and to immunoprecipitate the in vivo-radiolabeled viral H protein, which appears as a broad band of approximately 75 to 80 kDa. All MAbs except K4 neutralized CAM/R401 in a plaque reduction assay (NT titers of 1:80 to 1:1,280). Because K4 did not react with the H protein of strain CAM in any test, this MAb was used as a negative control in the ensuing experiments. A competitive binding EIA demonstrated different patterns of reactivity against CAM. Three of the antibodies (L77, K29, and K17) did not compete

TABLE 1. Competitive EIA

Competitor antibody	Antigenic site	Reduction of A_{492} with given biotinylated antibody (IgG)[a]								
		K17	K29	K53	K71	NC26	NC32	K83	DW15	L77
K89	A	−	−	−	−	+	−	−	−	−
K17	B	++	−	−	−	+	−	−	−	−
K29	C	−	+++	−	−	−	−	−	−	−
K53	D	−	−	+++	++	+++	++	−	−	+
K71	D	−	−	++	+++	+++	+	−	−	−
NC26	D/E	−	−	++	++	+++	++	+++	+++	−
NC32	D/E	−	+	+++	+	+++	++	+++	+++	−
K83	E	−	−	−	−	+++	++	+++	+++	−
DW15	E	−	−	−	−	+++	+++	+++	+++	−
L77	F	−	−	+	−	−	−	−	−	+++

[a] −, <40% competition; +, 40 to 55% competition; ++, 56 to 70% competition; +++, >70% competition.

with each other or with other MAbs (e.g., NC32, K83, and K71). The latter three did partially compete with each other and probably interact with closely spaced antigenic sites. Largely identical results were obtained when MV EDM was used instead of CAM/R401 as the antigen. These data allow the MAbs to be assigned to six different binding groups (Table 1). MAb K89 may form a further group because the other MAbs except NC26 did not compete for binding to MV antigen.

Biological effects of MAbs on MV infection. The effects of MAbs on the virus-host interaction were analyzed in vivo in our animal model. Intracerebral infection of suckling Lewis rats with CAM/RB or CAM/R401 led after 5 to 6 days to a fulminant fatal neurological disease as described previously (19). MV was regularly isolatable from the CNS. There was no correlation between HI or NT titers of the various MAbs and their effects in vivo. The i.p. injection of MAbs K71, K53, and K4 into 1- to 2-day-old Lewis rats 4 to 8 h following the i.c. infection had no effect on the disease process (Table 2). All animals receiving these MAbs died from acute necrotizing encephalopathy (NE) within 5 to 8 days. Similarly, the transfer of MAb K29 or K89 had no effect on the disease course in most rats. Only 3 of 20 animals survived beyond 8 days postinfection but eventually died during the ensuing 3 days from acute encephalitis (AE), which differs from NE by the occurrence of mononuclear (lymphocytic) cell infiltrates. The effect of MAb DW15 was intermediate; 50% of the recipient animals died from NE and 50% developed AE between days 10 and 13 postinfection. However, MAbs K83, K17, L77, and NC32 modulated the course of the experimental measles infection. Some recipients of these antibodies survived much longer than controls, developing antibody-dependent acute or subacute encephalitis (AD-AE or AD-SAME). No rat died from NE, 55% developed AD-AE, and 36% developed AD-SAME after 4 to 7 weeks. Five rats survived without clinical disease through 11 weeks. Convulsions beyond 3 weeks following infection were rare, and some animals developed paralysis of limbs without further disease progression. Histopathologically, in many of the recipients of disease-modulating MAbs, perivascular inflammatory infiltrates prevailed and became the prominent features. These changes were indistinguishable from the

TABLE 2. Antibody-dependent modulation of measles encephalitis

Antibody treatment[a]	Incubation period (days)	Neuropathology	Incidence (no. diseased/total) [%]	Virus isolated ($\log_{10}$ PFU)/g of brain wt)[b]
Anti-N, P, M[c]	5–7	NE	19/19 (100)	4.5
Anti-H				
K83	8–10	AD-AE	4/16 (25)	3.0
	28–44	AD-SAME	10/16 (63)	0[d]
L77	8–12	AD-AE	10/16 (63)	2.1
	18–30	AD-SAME	4/16 (25)	0
NC32	8–13	AD-AE	11/15 (73)	2.6
	20–32	AD-SAME	4/15 (27)	0
K17	10–16	AD-AE	8/12 (67)	2.7
	26–36	AD-SAME	3/12 (25)	0
DW15	5–8	NE	4/9 (45)	3.8
	10–13	AD-AE	5/9 (55)	3.2
K29	5–7	NE	8/10 (80)	4.4
	9–11	AD-AE	2/10 (20)	3.0
K89	5–8	NE	9/10 (90)	4.6
	10	AD-AE	1/10 (10)	3.5
K71	6–7	NE	10/10 (100)	4.1
K53	5–8	NE	9/9 (100)	4.5
K4	5–6	NE	6/6 (100)	4.2

[a] One- to two-day-old rats were injected i.p. with 0.1 to 0.3 mg (equivalent to 5,000 MV neutralizing units) of Ig within 6 h after i.c. infection with 10^4 PFU of CAM/RB.

[b] Mean titer of two to eight individual rat brains determined when animals were moribund (6 to 16 days postinfection).

[c] Data from reference 18.

[d] From one animal, $10^{2.2}$ PFU was isolated 35 days p.i.

TABLE 3. Neutralization profile of viral reisolates from antibody-treated rats[a]

MAb transferred	Isolates from disease type[b] (no. tested)	Mean reciprocal NT titer[c]						
		K17	K29	K71	K83	DW15	NC32	L77
None (untreated)	NE (6)	320	320	640	160	160	160	2,560
K29	NE (3)	270	130	530	270	210	160	1,280
K71	NE (2)	120	240	80	160	160	160	1,280
DW15	NE (2)	320	320	480	60	30	240	1,920
K17	AD-AE (4)	≤10	280	ND	200	ND	ND	2,560
K29	AD-AE (1)	160	10	640	160	160	ND	2,560
K83	AD-AE (5)[d]	180	320	350	<10	≤10	160	2,400
DW15	AD-AE (3)	270	430	ND	≤10	≤10	ND	2,990
NC32	AD-AE (4)	300	360	ND	160	ND	<10	2,700
L77	AD-AE (4)	160	300	420	200	200	240	<10

[a] Rats were infected at the age of 1 to 2 days with CAM/RB, and MAbs were passively transferred i.p. 4 to 6 h later.
[b] Virus isolates were obtained from different rats 7 to 8 (NE) or 9 to 14 days (AD-AE) after infection.
[c] The titer represents 50% neutralization of 100 PFU. Values thus indicate how well a MAb neutralizes a given isolate; i.e., values of 10 or below indicate no neutralization. NT titers against CAM/RB were identical to those of reisolates from untreated animals. ND, not determined.
[d] Includes one isolate from an animal with AD-SAME.

subacute measles encephalitis (SAME) that is observed in up to 20% of 3-week-old nontreated rats.

Characteristics of in vivo-generated MAb variants. Infectious MV could be reisolated from rats that developed NE or AE. The titers of reisolates varied depending on the incubation period. In all animals tested before day 8 postinfection, levels of 10^4 to $10^{4.5}$ PFU/g of brain weight were reached irrespective of antibody treatment. From recipients that developed AD-AE, viral titers isolated from CNS material were 1 to 3 orders of magnitude lower than titers from control rats, whereas no MV could be recovered from rats with AD-SAME (Table 2).

To further characterize the virus-host relationship in the presence of neutralizing antibodies, neutralization profiles of the viral reisolates from rats with AD-AE (n = 20) as well as nonmodulated encephalopathy (n = 13) were determined (Table 3). Isolates from animals with NE could be neutralized with all MAbs, although the selecting antibody that was passively transferred to the respective animal neutralized its reisolate somewhat less well than the other MAbs did. Interestingly, the reisolates from animals with AD-AE could not be neutralized in vitro by the selecting MAb, and in the cases of DW15 and K83, the isolates were only weakly neutralized by the competing antibody. All noncompeting MAbs (Table 1) retained the ability to neutralize the reisolates, and their NT titers for the reisolates were similar to those for CAM/RB wild-type virus (Table 3). The results of immunoprecipitation experiments with in vivo-radiolabeled viral proteins are in good agreement with the neutralization profiles. For example, the H protein of the reisolate from an animal treated with MAb NC32 was not reactive with NC32 or NC26, while the other MAbs precipitated the H protein of this particular reisolate, and the H protein of reisolates from K83-treated animals did not precipitate with the competitive MAbs K83 and DW15 but precipitated with all others (Fig. 1). Analogous data were obtained with other reisolates in that the transferred

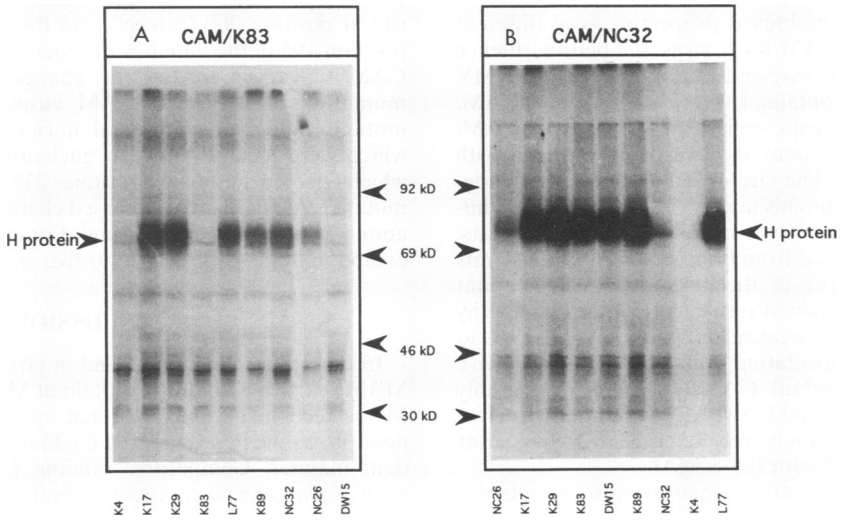

FIG. 1. Radioimmunoprecipitation of MV isolated from animals treated with MAb K83 (A) or MAb NC32 (B). Vero cells were infected with the isolates in the presence of [^{35}S]methionine for 4 h. Lysates were incubated with nine different H-reactive MAbs. The bands visible in lane K4 indicate background precipitates or nonspecific cellular proteins, since MAb K4 does not interact with the H protein of MV CAM strains.

TABLE 4. In vivo characteristics of MV MAb escape variants

Mutant virus	Dose (log_{10})	Incubation period[a] (days [mean day of onset])	Incidence[b] (% [no. dead/total])	MV isolation from brain		Histopathology[c]	
				Frequency[d]	Titer[e] (log_{10})	Type	Viral antigen[f]
CAM/RB	4.0	8–12 (9.3)	95 (19/20)	+++	4.6	AE	Abundant
CAM/R401	6.3	9–17 (11.9)	84 (16/19)	++	3.9	AE	Abundant
CAM/L77	5.7	8–16 (10.7)	75 (12/16)	++	3.8	AE	Abundant
CAM/NC32	5.3	10–16 (11.8)	83 (10/12)	+++	4.5	AE	Abundant
CAM/K83	5.3	10–34 (20.8)	57 (8/14)	++	2.6	AE, SAME	Abundant
CAM/K17	3.7	13–18 (16.3)	30 (3/10)	+	3.3	AE, focal changes	Scattered neurones
CAM/K29	5.9	NA[g]	0 (0/15)	−	NA	Focal changes	Scattered neurones
CAM/K71	6.4	NA	0 (0/17)	−	NA	No changes	None

[a] Interval between infection and onset of clinical signs.
[b] Weanling rats were infected at age 21 to 24 days. Data represent incidence of fatal clinical signs of encephalitis.
[c] Rats were examined when moribund or within 7 days of disease onset.
[d] Frequency of viral isolation by cocultivation 10 to 12 days postinfection. +++, >75%; ++, 50 to 75%; +, 25 to 50%; −, <5%.
[e] Mean MV titer per gram of brain weight of successful isolations from three to six animals.
[f] Viral antigen was detected within lesions and in apparently normal brain tissue by indirect immunofluorescence using a cocktail of N and H MAbs.
[g] NA, not applicable. In these animals, neuropathology was determined 18 to 21 days after infection.

modulating as well as competing MAbs did not precipitate the respective reisolate.

Isolation of MAb escape variants in vitro. To start with a defined population of cloned virus, escape variants were selected in vitro from MV CAM/R401, using the neutralizing H-specific MAbs. The variants were stable after three Vero cell passages in the presence of the respective selecting antibody. Mutant viral protein was prepared for EIA as well as HI tests to assess binding of MAbs other than those used for selection. In each case, the selecting MAbs and antibodies competing for the same epitope were no longer able to interact with the appropriate escape mutant, while the other MAbs were still binding, inhibited the hemagglutinating activity of mutant virus, and neutralized its infectivity. Thus the results of these tests correlated well with the competitive binding studies (Table 1). Furthermore, immunoprecipitation experiments indicated that the selecting MAbs were incapable of precipitating the escape mutants in all cases. The virus stocks generated were used for infection of rats and for the generation of viral RNAs for cDNA cloning and sequence determination.

Neurovirulence of in vitro-isolated MAb escape variants. To determine whether their biological properties were different from those of the parent CAM/R401 virus, the neurovirulence of the escape mutants was assessed in the rat model for MV encephalitis. The results obtained with CAM/RB and CAM/R401 were similar; also, escape variants CAM/L77 and CAM/NC32 were highly neurovirulent and caused encephalitis with similar kinetics (Table 4). The virological and neuropathological properties were indistinguishable. The incidence of clinically overt encephalitis was lower in CAM/K83-infected rats, and infectious MV reisolated from brain material of these rats displayed the characteristics of the parent inoculum variant virus, as the reisolates remained resistant to neutralization by the selecting MAb but were neutralized by the other MAbs to a similar degree as the inoculating mutant (data not shown). The neurovirulence of mutant CAM/K17 was considerably lower, and both CAM/K29 and CAM/K71 were nonneurovirulent (Table 4). Virus could not be reisolated 12 to 14 days after infection from rats treated with these MAbs.

To determine whether eventually neurotropic variants develop, consecutive blind passages through suckling rat brain of mutants CAM/K71 and CAM/K29 were done. The pattern and incidence of disease and viral titers isolated from clinically well animals at day 5 after infection did not change. No virus could be isolated from such rats 12 to 14 days after infection, thus providing no evidence for development of neurovirulent variants originating from either CAM/K71 or CAM/K29 (data not shown). On the other hand, reisolates from variant CAM/L77-, CAM/NC32-, or CAM/K83-infected rats were highly neurovirulent and remained stable in vivo.

Localization of mutations by nucleotide sequencing. To localize the mutations in the primary sequence of the H protein which were responsible for the abolition of MAb binding, the nucleotide sequences of the H genes of CAM/R401 and the escape mutants were determined. The data indicate that of the 14 nucleotide changes between the published EDM strain sequence (1) and CAM, 12 are expressed. This might reflect the adaptation of the CAM strain to the rat brain. A nucleotide change from A to G at position 619 leads to loss of a glycosylation site in the CAM H protein. This site is used in EDM, as indicated by size difference of the CAM and EDM H proteins in SDS-PAGE (data not shown). Table 5 shows the differences between the CAM H-gene sequence and those of various MAb escape mutants. All the mutants share the mutations that lead to the regeneration of the glycosylation site at position 200 (S to N) and the change from G to R at position 195 in the amino acid sequence. In the escape mutant CAM/K29, these are the only changes observed between the mutant and the parent CAM virus. The three remaining mutants contained additional nucleotide sequence changes, which were located between nucleotides 1150 and 1203 and which affected amino acid residues 377 to 395 in the protein. In mutant CAM/L77, two expressed changes affecting consecutive amino acid residues were found. For each of the wild-type and escape mutant stocks, three to five clones were sequenced.

DISCUSSION

In this study, the in vitro and in vivo activities of a panel of MAbs reactive with the H protein of MV were determined. All MAbs recognize conformational epitopes and all except K4 neutralized the neuroadapted CAM in a complement-independent manner. Competitive binding EIAs revealed different profiles with partial competition between some of the antibodies, suggesting that six partially overlapping sites with seven epitopes are recognized by the MAbs on the H protein.

It is generally still accepted that the monotypic status of MV is biologically not changed by the existence of variants even

TABLE 5. Comparison of sequences between CAM and MAb escape mutants

Mutant virus	Sequence change(s) induced in mutant virus[a]		Neurovirulence of mutants[b]	Disease-modulating effect of selecting antibody[c]
	Nucleotide(s)	Amino acids		
CAM/L77	603, 619, 1150, 1153	195 G→R, 200 S→N, 377 R→Q, 378 M→K	Yes	Yes
CAM/NC32	603, 619, 1182	195 G→R, 200 S→N, 388 G→S	Yes	Yes
CAM/K83	ND[d]	ND	Yes	Yes
CAM/K17	ND	ND	Weak	Yes
CAM/K29	603, 619	195 G→R, 200 S→N	No	No
CAM/K71	603, 619, 1203	195 G→R, 200 S→N, 395 E→K	No	No

[a] With the exception of the mutations at positions 603 and 619, all differences between EDM and CAM/RB are also present in the MAb escape mutants derived from CAM/R401.
[b] See table 4.
[c] See table 2.
[d] ND, not determined.

with rather extensive epitope changes. In relation to both measles epidemics occurring in highly immunized populations and CNS disease processes associated with MV, the extent of antigenic variation is of great importance in understanding MV tropism and vaccine failure. It is clear that the presence of neutralizing antibodies in the blood has a major impact on the ability of virus to spread in the infected organism. One way that MV might evade immune surveillance would be that lymphotropic MV is carried into the brain by invading infected peripheral blood cells similar to the way in which the closely related canine distemper virus infects the dog brain (30). It is also conceivable that a selection of variants which adopt new biological properties such as the ability to persist in the brain takes place under the pressure of neutralizing antibodies. To determine whether this process occurs in vivo, experiments using the rodent-neuroadapted CAM strain were carried out in the rat model of measles encephalitis.

The passive transfer of MAbs into i.c. MV-infected rats showed that modulating MAbs, particularly L77, NC32, K17, and K83, suppressed acute clinical and histopathological signs of infection, changed the clinical course to AD-SAME, and caused a reduction in the amount of infectious virus that could be reisolated from brain tissue. The reisolates have the characteristics of escape variants that obviously evolved from CAM/RB in vivo in the presence of the transferred neutralizing MAbs. These results confirm and extend previous observations obtained in vivo and in vitro (5, 18, 28, 36).

Antibodies directed against viral envelope proteins have been shown to act at different levels. Clinical and experimental studies provide evidence for a decreased viral load due to neutralization of extracellular virus. Antigenic modulation by shedding and/or internalization of viral proteins has been proposed to support the establishment of persistent infection (5, 10), and transcriptional attenuation of envelope genes due to binding of certain H-reactive MAbs to infected cells was shown to result in restricted virion formation and release (18, 28). An inositol phospholipid signal generated by the binding of antibody to MV H protein was suggested to play a role in the down-regulation of viral antigens (35). All of these factors may cooperate and result in modulation of disease course and pathology. Also in a clinical situation, variants that are particularly adapted to grow and survive in the CNS may be selected by the humoral immune response, leading to escape of some virus particles from immune surveillance. This interpretation is supported by the observation of an unbalanced primary immune response to individual epitopes on parainfluenza type 3 viral glycoproteins in reinfections of infants and children (7, 13).

If this phenomenon also occurs in natural MV infections, the selection of virus variants could provide the basis for establishment of viral persistence in brain cells and finally result in the development of SSPE. In an attempt to corroborate this hypothesis, we correlated the neurovirulence of in vitro-selected MAb escape mutants of CAM with sequence data of the H gene. Some escape mutants were completely nonneurovirulent, and others displayed high or intermediate levels of neurovirulence. Sequence data reveal single amino acid changes as a result of MAb escape and localize a surface domain potentially important for neurovirulence at residues 368 to 396. The idea that a single amino acid change is responsible for the alteration in neurovirulence is not difficult to accept, since it was recently demonstrated that a single change in the envelope protein of lymphocytic choriomeningitis virus (L to F) changed the tropism of the virus from the lymphocytes to the CNS of infected mice (21). Furthermore, in rabies virus and mumps virus, single changes induced by MAb selection have been shown to alter the neurovirulence properties of these viruses (16, 29).

The results do not support the assumption that epitopes recognized by MAbs K29 and K71 are involved in binding of the H proteins to CNS receptors, because in vitro escape variants isolated with these two MAbs are not neurovirulent and treatment of infected rats with these MAbs does not suppress the neuropathogenic changes normally induced by the virus. Furthermore, brain reisolates do not have the characteristics of escape clones. Since the parent CAM contains multiple variants, both with and without neurovirulence properties, a possible explanation is that following in vivo transfer, MAbs K71 and K29 are unable to efficiently neutralize the neurovirulent variants present in the pool of CAM. If, however, escape variants are generated in vitro, MAb K71 or MAb K29 is present in excess and for prolonged time periods, allowing for neutralization of MV, including CAM/L77 or CAM/NC32 variants, even if the affinity and/or avidity of MAb K71 to these variants is low. The in vitro escape variant thus is nonneurovirulent. On the other hand, MAb NC32 neutralizes most MV variants and allows for the survival of only escape variant CAM/NC32. Since this variant is present only in low concentrations in CAM/RB, it takes time to grow to sufficient quantity after in vivo transfer of MAb NC32 and thus causes modulated disease.

The nucleotide changes at positions 603 and 619 which lead to the generation of a new potential glycosylation site have been found to be present in all MAb escape mutants. However, it is unlikely that the changes are important for neurovirulence; rather, they may represent changes that occur concomitantly

with the tissue culture adaptation which was required for isolation of the mutants. These changes have been observed in all investigated tissue culture-adapted MV strains, including EDM. Differences in glycosylation sites of H protein appear to be important for virulence in other viral systems, as shown in avian influenza virus (14). They are also found as characteristic for MV and other paramyxovirus lineages and strains (8, 24, 34) and in MAb escape, such as in mumps virus (16). It is possible that the presence of a carbohydrate side chain masks epitopes recognized by specific MAbs and changes the properties of a virus variant. In the case of the nonneurovirulent variant CAM/K29, the aforementioned sequence changes are the only ones found between the wild-type CAM strain and the escape mutant. In that sense, the mutations at positions 377, 378, and 388 observed in the CAM/L77 and CAM/NC32 escape variants appear to restore the neurovirulence characteristic of CAM. Double escape mutants will be used to study this question further and to rule out mutations outside the H gene as factors influencing the phenotype.

It is interesting that MAbs K71 and NC32 bind to partially overlapping epitopes, as observed in the competition binding assays. The escape mutations appear to be localized close, either C or N terminally, to cysteine 391, whereas the escape mutations for L77 MAb are further N terminal to this residue. This region of the H protein must represent a major antigenic site arranged on the surface so that different antibodies can bind to it in close proximity to each other without competition. Recently, mutations in this region have been found in escape mutants isolated with one of five H-reactive neutralizing MAbs, but nothing is known about their neurovirulence properties (12).

As is the case with other viruses, analysis of the H-gene and protein sequences of various MV strains has not been able to highlight the presence of hypervariable regions that may reflect immune selection pressure. Comparative sequence analysis has provided an insight into conserved regions of the protein (8), and when the H proteins of all paramyxoviruses and morbilliviruses are aligned and MAb escape mutations are localized on such an alignment, it becomes clear that the area between residues 368 and 396 in MV represents a part of the surface of the H molecule. Independently, it was proposed that a peptide sequence (residues 368 to 377) is localized on the surface because antipeptide sera have neutralizing activity (20). However, neither the functional significance of this sequence nor the tertiary structure of paramyxovirus attachment proteins is known.

In summary, the neurovirulence of various neutralizing MAb escape mutants of the rodent-adapted CAM strain of MV has been correlated with specific protein changes, localized to a major antigenic surface determinant of the H protein of MV. It is likely that the changes in the H molecule of escape mutants induced by the selecting MAb are responsible for the biological properties of MV, including neurovirulence.

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REFERENCES

1. **Alkhatib, G., and D. J. Briedis.** 1986. The predicted primary structure of the measles virus hemagglutinin. Virology **150**:479–490.
2. **Baczko, K., J. Lampe, U. G. Liebert, U. G. Brinckmann, V. ter Meulen, I. Pardowitz, H. Budka, S. L. Cosby, S. Isserte, and B. K. Rima.** 1993. Clonal expansion of hypermutated measles virus in a SSPE brain. Virology **197**:188–195.
3. **Baczko, K., U. G. Liebert, M. Billeter, R. Cattaneo, H. Budka, and V. ter Meulen.** 1986. Expression of defective measles virus genes in brain tissues of patients with subacute sclerosing panencephalitis. J. Virol. **59**:472–478.
4. **Baczko, K., I. Pardowitz, B. Rima, and V. ter Meulen.** 1992. Constant and variable regions of measles virus proteins encoded by the nucleocapsid and phosphoprotein genes derived from lytic and persistent viruses. Virology **190**:469–474.
5. **Barrett, P. N., K. Koschel, M. Carter, and V. ter Meulen.** 1985. Effect of measles virus antibodies on a measles SSPE virus persistently infected C6 rat glioma cell line. J. Gen. Virol. **66**:1411–1421.
6. **Cattaneo, R., and M. A. Billeter.** 1992. Genetic diversity of RNA viruses. Curr. Top. Microbiol. Immunol. **176**:65–74.
7. **Coelingh, K. V. W., C. C. Winter, E. T. Tierney, S. L. Hall, W. T. London, H. W. Kim, R. M. Chanock, and B. R. Murphy.** 1991. The antibody response of human and non-human primates to individual antigenic sites of the hemagglutinin-neuraminidase and fusion glycoproteins after primary infection or reinfection with parainfluenza type 3 virus. J. Virol. **64**:3833–3843.
8. **Curran, M. D., D. K. Clarke, and B. K. Rima.** 1991. The nucleotide sequence of the gene encoding the attachment protein H of canine distemper virus. J. Gen. Virol. **72**:443–447.
9. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **137**:266–267.
10. **Fujinami, R. S., and M. B. A. Oldstone.** 1979. Antiviral antibody reacting on the plasma membrane alters measles virus expression inside the cell. Nature (London) **279**:529–530.
11. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72**:3961–3965.
12. **Hu, A., H. Sheshberadaran, E. Norrby, and J. Kövamees.** 1993. Molecular characterization of epitopes on the measles virus hemagglutinin protein. Virology **192**:351–354.
13. **Kasel, J. A., A. L. Frank, W. A. Keitel, L. H. Taber, and W. P. Glezen.** 1984. Acquisition of serum antibodies to specific viral glycoproteins of parainfluenza virus 3 in children. J. Virol. **52**:828–832.
14. **Kawaoka, Y., and R. G. Webster.** 1989. Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. J. Virol. **63**:3296–3300.
15. **Kobune, K., F. Kobune, K. Yamanouchi, K. Nagashima, Y. Yoshikawa, and M. Hayami.** 1983. Neurovirulence of rat brain-adapted measles virus. Jpn. J. Exp. Med. **53**:177–180.
16. **Kovamees, J., R. Rydbeck, C. Orvell, and E. Norrby.** 1990. Hemagglutinin-neuraminidase (HN) amino acid alterations in neutralization escape mutants of Kilham mumps virus. Virus Res. **17**:119–129.
17. **Liebert, U. G., C. Linington, and V. ter Meulen.** 1988. Induction of autoimmune reactions to myelin basic protein in measles virus encephalitis in Lewis rats. J. Neuroimmunol. **17**:103–118.
18. **Liebert, U. G., S. Schneider-Schaulies, K. Baczko, and V. ter Meulen.** 1990. Antibody-induced restriction of viral gene expression in measles encephalitis in rats. J. Virol. **64**:706–713.
19. **Liebert, U. G., and V. ter Meulen.** 1987. Virological aspects of measles virus induced encephalomyelitis in Lewis and BN rats. J. Gen. Virol. **68**:1715–1722.
20. **Mäkela, M. J., G. A. Lund, and A. A. Salmi.** 1989. Antigenicity of measles virus haemagglutinin studied by using synthetic peptides. J. Gen. Virol. **70**:603–614.
21. **Matloubian, M., T. Somasundaram, S. R. Kolhekar, R. Selvakumar, and R. Ahmed.** 1990. Genetic basis of viral persistence: single amino acid change in the viral glycoprotein affects ability of lymphocytic choriomeningitis virus to persist in adult mice. J. Exp. Med. **172**:1043–1048.
22. **Mims, C. A.** 1989. The pathogenetic basis of viral tropism. Am. J. Pathol. **135**:447–455.
23. **Reich, A., O. Erlwein, S. Niewiesk, V. ter Meulen, and U. G. Liebert.** 1992. Control of measles encephalitis in the rat by virus-specific CD4+ T cells. Immunology **76**:185–191.

24. **Rota, J. S., K. B. Hummel, P. A. Rota, and W. J. Bellini.** 1992. Genetic variability of the glycoprotein genes of current wild-type measles isolates. Virology **188:**135–142.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Schmid, A., R. Cattaneo, and M. A. Billeter.** 1987. A procedure for selective full length cDNA-cloning of specific RNA species. Nucleic Acids Res. **26:**3987–3996.
27. **Schneider-Schaulies, S., and U. G. Liebert.** 1991. Pathogenetic aspects of persistent measles virus infections in brain tissue. Semin. Neurosci. **3:**139–147.
28. **Schneider-Schaulies, S., U. G. Liebert, Y. Segev, B. Rager-Zisman, M. Wolfson, and V. ter Meulen.** 1992. Antibody-dependent transcriptional regulation of measles virus in persistently infected neural cells. J. Virol. **66:**5534–5541.
29. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53:**926–934.
30. **Summers, B. A., H. A. Greisen, and M. J. G. Appel.** 1978. Possible initiation of viral encephalomyelitis in dogs by migrating lymphocytes infected with distemper virus. Lancet **i:**187–189.
31. **Taylor, M. J., E. Godfrey, K. Baczko K., V. ter Meulen, T. F. Wild, and B. K. Rima.** 1991. Identification of several different lineages of measles virus. J. Gen. Virol. **72:**83–88.
32. **ter Meulen, V., S. Löffler, M. J. Carter, and J. R. Stephenson.** 1981. Antigenic characterization of measles and SSPE virus haemagglutinin by monoclonal antibodies. J. Gen. Virol. **57:**357–364.
33. **ter Meulen, V., J. R. Stephenson, and H. W. Kreth.** 1983. Subacute sclerosing panencephalitis, p. 105–159. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 18. Plenum, New York.
34. **Tsukiyama, K., M. Sugiyama, Y. Yoshikawa, and K. Yamanouchi.** 1987. Molecular cloning and sequence analysis of the rinderpest virus mRNA encoding the haemagglutinin protein. Virology **164:** 523–530.
35. **Weinmann-Dorsch C., and K. Koschel.** 1989. Coupling of viral membrane proteins to phosphatidylinositide signalling system. FEBS Lett. **247:**185–188.
36. **Zinnheimer-Dreikorn, J., and K. Koschel.** 1990. Antigenic modulation of measles subacute sclerosing panencephalitis virus in a persistently infected rat glioma cell line by monoclonal anti-haemagglutinin antibodies. J. Gen. Virol. **71:**1391–1394.