

Pathogenic Murine Coronaviruses

III. Biological and Biochemical Characterization of Temperature-Sensitive Mutants of JHMV

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JHMV is a neurotropic member of the heptoencephalitis group of murine coronaviridae. The characteristics of the biology and intracellular viral RNA synthesis and the intracellular viral protein synthesis of JHMV are discussed in the two previous papers, respectively. This paper describes the neuropathogenesis of JHMV and the isolation and characterization of 34 temperature-sensitive mutants of JHMV. These mutants were selected for their inability to induce syncytia formation after low multiplicity infection (m.o.i. = 0.1 iU) in BALB/c 17CL-1 cells at 38.5° as compared to the induction of syncytia at 33°. *N*-Methyl-*N'*-nitrosoguanidine (14 mutants) and 5-fluorouridine (20 mutants) were used as mutagens at a concentration that reduced infectivity by 90-95%. Characterization of these mutants included: induction of syncytia; synthesis of JHMV-specific intracellular RNA; progeny yields at 33, 37, and 38.5°; synthesis of JHMV-specific antigens as determined by indirect immunofluorescence and sodium dodecyl sulfate-polyacrylamide gel electrophoresis; virion thermostability; neuropathogenesis including isolation of virus from infected brain, immunofluorescence of infected brain, and histopathology of brain and spinal cord by light and transmission electron microscopy; ability to protect mice from a lethal JHMV infection; and complementation. RNA-minus (17/34), RNA-intermediate (14/34), and RNA-plus (3/34) groups were defined. One mutant, N3, produces chronic meningitis and demyelination without typical JHMV encephalitis in spite of the fact that neurons are infected as detected by immunofluorescence. This altered neuropathogenesis cannot be explained by "leakiness" or reversion. In addition, non-temperature-sensitive variants of JHMV have been selected for altered neuropathogenesis and are described.

INTRODUCTION

Coronaviruses are common viruses found in many animal species from birds to man [See Robb and Bond (1979a) for review]. They produce significant organ and systemic disease in their natural hosts, diseases that often persist and become chronic (e.g., encephalitis, hepatitis, pneumonitis, lymphadenitis). These viruses are transmitted by droplet inhalation and/or ingestion.

In the previous two papers, we have characterized the *in vitro* biology and the

intracellular virus-specific RNA and protein synthesis of two closely related murine coronaviruses: JHMV, a neurotropic virus, and A59V, a less neurotropic virus. This paper describes a murine model of coronavirus-caused encephalitis and primary demyelination (acute encephalomyelitis) and the preliminary characterization of our collection of 34 temperature-sensitive (*ts*) mutants of JHMV.

MATERIALS AND METHODS

Cell culture and virus titration. The growth and titration of methods of JHMV and A59V in 17CL-1 cells are presented in the first of the two previous papers (Robb and Bond, 1979b).

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Viral mutagenesis. The doubly cloned JHMV wild-type (wt) "parent" (Robb and Bond, 1979b) was subjected to *N*-methyl-*N'*-nitrosoguanidine (NTG) or 5-fluorouridine (5FU) mutagenesis. The concentration of each drug was chosen so that infectivity was reduced by 90–95% after mutagenesis. Cells were infected in suspension with a m.o.i. = 0.10. After a 30-min adsorption at 33° with agitation every 5 min, the cells were either treated with NTG or 5FU in the following manner. NTG: Infected cells were plated into MultiWell plates (Falcon) using 6×10^5 cells per well in 1.0 ml DEB2. After a 4-hr incubation at 33°, 4 μ g of NTG in 8 μ l of sterile deionized water was added to each well. After an additional 4-hr incubation at 33°, the NTG-containing medium was aspirated, the wells rinsed once with 1.0 ml DEB2, and the cells incubated with 1.0 ml DEB2 per well for an additional 16 hr at 33°. 5FU: Infected cells were plated into MultiWell plates at 6×10^5 cells per well in 1.0 ml DEB2 containing 500 μ g/ml 5FU. After a 6-hr incubation at 33°, the medium was aspirated, the infected cells were rinsed once with 1.0 ml DEB2 per well, and the cells incubated with 1.0 ml DEB2 per well for an additional 18 hr at 33°.

Isolation of independent temperature-sensitive mutants. A modified version of the previously described (Robb, 1973) replica-plating microtechnique for the isolation of *ts* animal virus mutants was developed. The modification was necessary because the titer of any given clone of JHMV was not high enough for reproducible replica plating. Three types of plastic culture vessels (Falcon) were used: the mutagenesis procedure used MultiWell plates with 24 wells/plate and 1.0 ml/well; the procedure for cloning mutagenized virus used Microtest plates with 60 wells/plate and 10 μ l/well ("master plates"); and the replica plating procedure used Microtest II plates with 96 wells/plate and 0.10 ml/well ("replica plates"). The mutagenized viruses from a single MultiWell were plated into Microtest master plates at a dilution that would infect about half of the 60 wells in each plate. This initial plating of mutagenized virus used a sterile Hamilton syringe with repeating dispenser to inocu-

late each "master well" with the virus and 1000 17CL-1 cells in 10 μ l of DEB2 (Robb, 1973). These master wells were incubated at 33° for 48 hr and all infected wells were replica plated into two Microtest II replica plates in the following manner. Each "replica well" was first filled with 0.10 ml of DEB2 containing 4×10^4 17CL-1 cells. Five microliters from an infected master well was then added to each of two corresponding replica wells of the two replica plates. When all the master wells derived from a single MultiWell were replica plated into replica wells, one set of replica wells was placed at 38.5° and the other set at 33°. After a subsequent 48-hr incubation at the respective temperatures, the replica wells were examined with phase contrast microscopy for the presence of JHMV-specific syncytia formation. The replica wells that had syncytia at 33°, but not at 38.5°, contained potential temperature-sensitive JHMV mutants. Only one potential mutant was picked from each original MultiWell of mutagenized virus, a process that insured the independence of each mutant isolate.

Each potential mutant was tested to confirm its *ts* nature. The cells in the 33° replica well containing the presumed mutant were scraped with a sterile rubber policeman, sonically disrupted, and cloned by the endpoint dilution microtechnique (Robb and Bond, 1979b). Two clones of a potential mutant were then picked from each of the independent isolates, incubated with 5×10^5 17CL-1 cells in 0.5 ml DEB2 at 33°, plated into individual wells of a MultiWell plate with an additional 0.5 ml DEB2, and incubated at 33°. Each of the two clones thus grown was harvested and titered at 33 and 38.5°. The clone with the greatest inhibition of syncytia formation at 38.5° (reduction in the 38.5° titer) was grown into a stock of mutant virus and the other clone was destroyed. Only mutants having a 1000-fold or greater reduction in their ability to form syncytia at 38.5° were studied further. The "restrictive" temperature of 38.5° was chosen to provide the largest temperature differential (33° vs 38.5°) while staying within the body temperature of a mouse (37–39°). Perma-

nent designations will be assigned to each mutant according to the nomenclature for animal viruses developed for mutants (Robb, *et al.*, 1972) after the affected protein or complementation group has been identified.

Analysis of virus-specific intracellular RNA. One million 17CL-1 cells were infected with wt JHMV or *ts* mutants of JHMV at an m.o.i. = 0.1. Virus adsorption was at 33° for 30 min. Following virus adsorption, the cells were divided into four aliquots and duplicate samples were incubated in 1 ml of DEB2 at 33 and 38.5°. One microgram of actinomycin D (Merck) was added at 6 hr postinfection (hpi) (38.5°) or 12 hpi (33°), and 15 min after the addition of the actinomycin D 25 μ Ci of [³H]uridine (New England Nuclear) (specific activity 40–50 Ci/mmol) was added. After a further 2-hr incubation at either 33 or 38.5°, the dishes were placed on ice, the medium was removed, the cells were extracted with 1.0 ml 1% Nonidet-P40 (NP40) in phosphate-buffered saline (PBS) pH 7.4, and the cell extract was pooled with the previously removed medium. These 2.0-ml samples were brought to 1% sodium dodecyl sulfate (SDS) to inhibit RNase activity and were stored at –70°. After thawing, duplicate 50- μ l aliquots from each sample were assayed for acid-precipitable radioactivity.

Radioimmune precipitation. The radioimmune precipitation technique using poly-specific mouse anti-JHMV serum to precipitate [³⁵S]methionine-labeled protein has been previously described (Robb, 1977). The properties of this anti-serum is described in the previous paper (Bond *et al.*, 1979).

SDS-polyacrylamide slab gel electrophoresis and fluorography. Total viral-specific intracellular proteins and radioimmune precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described in the previous paper (Bond *et al.*, 1979).

Immunofluorescent microscopy. The fixation techniques using absolute methanol or formaldehyde-Triton X-100 (cell culture) and 95% ethanol (brain) are presented in the first of the previous two papers along with the preparation of the

mouse anti-JHMV serum (Robb and Bond, 1979b). The antiserum reacts with all JHMV-specific proteins observed by SDS-polyacrylamide electrophoresis as determined by radioimmune precipitation (Bond *et al.*, 1979). Mouse brains were prepared for immunofluorescent microscopy using the ethanol fixation-paraffin embedding procedure of Sainte-Marie (1962). Serial sections of each specimen were examined, one section by light microscopy using a hematoxylin/eosin stain and the other sections by immunofluorescent microscopy using sera from seronegative-uninfected, JHMV-infected, or A59V-infected adult BALB/c mice.

Electron microscopy. All procedures used in the preparation of mouse brain for transmission electron microscopy have been previously described (Lampert, *et al.*, 1973).

Inoculation of animals. There were three types of inoculation. (i) Intracerebral (ic) inoculation: Animals were inoculated with 10 μ l of fluid in the right fronto-temporal region just posterior and superior to the eye. (ii) Intranasal (in) inoculation: Animals received 10 μ l of fluid on one nostril and were watched until they inhaled the droplet. (iii) Intraperitoneal (ip) inoculation: Ten microliters of fluid was injected within the peritoneal cavity of each animal. All inoculations were carried out with a Hamilton syringe in a repeating dispenser. Sterile, disposable, 27-gauge, 0.5-in. needles were used for all inoculations. Infected animals were anesthetized with phenobarbital (ip) and soaked in 70% ethanol before dissection.

Infectivity assay of JHMV in mouse brain. One aseptically removed mouse brain, approximately 1.0 ml in volume, was mixed with 4.0 ml PBS, pH 7.4, containing 25 mg/titer tetracycline. The mixture was homogenized in a sterile Ten-Broeck homogenizer using 10–15 strokes. The homogenized fluid was clarified at 733 g in a Sorvall GLC-1 centrifuge at 25° and stored at –70° or immediately titered. The infectivity assay of the clarified brain homogenate fluid was performed in the following manner. Fluid, 0.5 ml, was added to 1×10^6 17CL-1 cells and incubated for

TABLE 1
CHARACTERISTICS OF 34 TEMPERATURE-SENSITIVE MUTANTS OF JHMV

Mutant	Ability to form syncytia ^a	Virus-specific RNA synthesis ^b	Progeny yield (iU/ml) ^c			ic LD ₅₀ ^d (iU)	Neurotropism ^e		
			33°	37°/33°	38.5°/33°		Brain FA	Brain infection	Disease H & E
JHMV	1.0	1.00	4.3 × 10 ⁵	0.62	0.31	4	wt	0/5 <i>ts</i> ^f	AEM
A59V	1.0	1.00	9.7 × 10 ⁵	0.81	0.08	490	wt	0/5 <i>ts</i>	AEM
RNA-minus group (17 mutants)									
N1	10 ⁻⁵	0	4.6 × 10 ⁵	ND ^g	ND	>10,000	0	ND	Neg
N2	10 ⁻⁵	0	2.6 × 10 ⁵	<10 ⁻⁴	<10 ⁻⁴	650	wt	1/3 <i>ts</i>	CM/D
N3	10 ⁻³	0.01	4.8 × 10 ⁵	<10 ⁻³	<10 ⁻⁴	85	wt	6/6 <i>ts</i>	CM/D
N32	10 ⁻⁵	0	9.7 × 10 ⁵	0.01	<10 ⁻⁴	3,500	wt	0/1 <i>ts</i>	AEM
N48	10 ⁻⁴	0	6.8 × 10 ⁵	0.31	<10 ⁻⁴	600	wt	2/2 <i>ts</i>	AEM
N60	10 ⁻⁵	0	4.0 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
N64	10 ⁻⁵	0.01	3.1 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
2FU	10 ⁻⁴	0	4.2 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
3FU	10 ⁻⁴	0.05	3.8 × 10 ⁵	ND	10 ⁻³	>10,000	0	ND	Neg
5FU	10 ⁻⁴	0	5.4 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
6FU	10 ⁻⁴	0	4.6 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
16FU	10 ⁻⁵	0	2.8 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
17FU	10 ⁻⁵	0	4.0 × 10 ⁵	ND	<10 ⁻⁴	>10,000	0	ND	Neg
23FU	10 ⁻³	0	5.6 × 10 ⁵	0.09	10 ⁻³	>10,000	0	0	Neg
24FU	10 ⁻⁵	0	1.5 × 10 ⁴	0.23	0.007	>10,000	0	ND	Neg
29FU	10 ⁻⁵	0.04	3.2 × 10 ⁵	<10 ⁻⁴	<10 ⁻⁴	>10,000	0	0	Neg
38FU	10 ⁻⁵	0	3.3 × 10 ⁵	<10 ⁻⁴	<10 ⁻⁴	>10,000	0	0	Neg
RNA-intermediate group (14 mutants)									
N4	10 ⁻⁶	0.53	2.4 × 10 ⁵	<10 ⁻⁴	<10 ⁻⁴	>10,000	0	ND	Neg
N7	10 ⁻⁴	0.12	1.6 × 10 ⁵	<10 ⁻³	<10 ⁻³	>10,000	0	ND	Neg
N16	10 ⁻⁵	0.11	2.3 × 10 ⁵	0.04	<10 ⁻⁴	>10,000	0	ND	Neg
N18	10 ⁻⁴	0.42	2.2 × 10 ⁵	0.19	<10 ⁻⁴	>10,000	0	ND	Neg
N19	10 ⁻³	0.22	1.3 × 10 ⁴	0.08	0.05	>10,000	0	ND	Neg
N70	10 ⁻⁴	0.11	1.8 × 10 ⁵	0.19	<10 ⁻⁴	>10,000	0	ND	Neg
N71	10 ⁻⁵	0.11	2.0 × 10 ⁵	0.18	<10 ⁻⁴	>10,000	0	ND	Neg
8FU	10 ⁻⁴	0.36	1.4 × 10 ⁵	0.14	<10 ⁻³	>10,000	0	ND	Neg
9FU	10 ⁻³	0.22	1.0 × 10 ³	<0.01	<0.01	>10,000	0	ND	Neg
20FU	10 ⁻³	0.22	2.2 × 10 ⁵	0.55	0.30	4,200	wt	ND	AEM
31FU	10 ⁻⁴	0.25	8.7 × 10 ⁴	<10 ⁻³	<10 ⁻³	3,800	0	0	Neg
33FU	10 ⁻⁵	0.20	2.8 × 10 ⁴	0.36	<10 ⁻³	>10,000	0	0	Neg
37FU	10 ⁻⁴	0.10	1.3 × 10 ⁵	0.32	<10 ⁻⁴	>10,000	0	0	Neg
42FU	10 ⁻⁴	0.50	4.2 × 10 ⁴	0.01	<10 ⁻³	>10,000	0	0	Neg
RNA-plus group (3 mutants)									
4FU	10 ⁻⁴	1.4	1.1 × 10 ⁵	<10 ⁻³	<10 ⁻⁴	>10,000	0	0	Neg
10FU	10 ⁻⁴	3.4	1.6 × 10 ⁵	<10 ⁻³	<10 ⁻⁴	>10,000	0	0	Neg
15FU	10 ⁻⁴	1.4	6.7 × 10 ⁴	<10 ⁻³	<10 ⁻³	3,100	0	0	Neg

^a Ability to form syncytia at 38.5°: ratio of the titer in Syncytial Forming Units (SFU)/ml at 38.5° to the titer at 33°. Virus was grown at 33° from an m.o.i. = 0.0001.

^b Total virus-specific RNA synthesis: [³H]uridine was added in the presence of 1 μg/ml actinomycin D to 17CL-1 cells infected at a m.o.i. = 0.1 (see Robb *et al.*, 1979 for details). The ratio of mutant/wt RNA synthesis at 38.5° was divided by the ratio of mutant/wt RNA synthesis at 33° to provide the data shown. RNA-minus is defined as a value of less than 0.10. RNA-intermediate is defined as a value between 0.10 and 0.75. RNA-plus is defined as a value greater than 0.75.

^c 17CL-1 cells were infected with an m.o.i. = 0.0001, adsorbed at 33° for 30 min, and plated into replicate 32-mm petri dishes. Duplicate dishes were incubated at 33, 37, and 38.5° for 48 hr. The total yield of virus in each dish was then titered in duplicate.

30 min at 33 or 37° with agitation every 5 min. Eight milliliters of DEB2 was then added, and 0.2 ml of the cell-brain fluid suspension was plated into each of four 32-mm petri dishes (Falcon). The dishes were incubated at 33° for 72 hr and scored for infectivity by observing the presence of syncytia formation along two perpendicular diameters. If infectivity was not detected, the remaining 4.5 ml of the brain homogenate fluid was assayed for infectivity. If infectivity was observed, the brain homogenate fluid was quantitatively titered by the endpoint dilution microtitration method described above. Reconstruction experiments using noninfected brain homogenate with a known amount of virus that was added immediately after brain homogenization or brain homogenate that was made immediately after the intracerebral injection of a known amount of virus, have provided a lower limit of detection of 1–3 infectious units (iU) per brain.

Assays for serum neutralization of virus infectivity. Two microculture techniques were used to assay specific JHMV neutralization activity in sera. One technique measured the loss of infectivity by immunofluorescence and the other technique measured the loss of cytopathic effect. Both assays gave similar quantitative results. Virus infection and addition of serum was similar for both assays, but JHMV was used at a m.o.i. of 0.1 for the immunofluorescent assay and at a m.o.i. of 0.01 for the cytopathic assay. JHMV, at 5×10^5 iU/ml DEB0 (no FBS) for the immunofluorescent assay and at 5×10^4 iU/ml DEB0 for the cytopathic assay, was mixed with the test serum. Additional DEB0 was added to provide a constant amount of virus

in 0.10 ml with 20, 10, 5, 2.5, or 0% serum. These mixtures were incubated at 37° for 30 min. After the incubation, 10 μ l of the virus-serum mixture was added to 0.10 ml of 1×10^6 17CL-1 cells in DEB2 for immunofluorescence or to 5×10^5 cells/ml for cytopathic effect. The mixtures were plated into Microtest plates using 2 μ l/well. This plating procedure provided 100 iU/well for the immunofluorescent assay and 10 iU/well for the cytopathic effect assay. The plates were incubated at 33° for 7 (immunofluorescent) or 24 hr (cytopathic effect). The reduction in the number of immunofluorescent foci or in the number of infected wells (cytopathic effect) was determined. The reciprocal of the serum dilution producing a 50% reduction from control values (0% serum) was used as the neutralization titer. Fetal bovine serum (20%) and mouse anti-JHMV serum (3%) were used as negative and positive controls, respectively.

RESULTS

Ability to Form Syncytia at 38.5°

The basis of mutant selection was the inability of the mutants to induce syncytia at 38.5°. The ratio of the ability to induce syncytia at 38.5° versus 33° was determined by titering the cloned mutant stock at each temperature, as the microtiter assay is based upon syncytia induction. All mutants had a 38.5°/33° titer ratio of 10^{-3} or less (Table 1).

Production of Total Virus-Specific Intracellular RNA at 38.5°

Three groups of mutants were defined by the relative ability of mutants to syn-

^d Number of infectious units (SFU) required to kill 50% of 4-week-old BALB/c mice after ic inoculation as calculated by the procedure of Reed and Nuench, 1938.

^e Detection of brain infection after ic inoculation: Brain tissue was examined for JHMV-specific immunofluorescence, for infectious virus, and for neuropathology by light microscopy (H & E) as described under Materials and Methods. The "wt" designation for the mutants indicates that, in the animals that became ill after infection, the immunofluorescent staining pattern was similar to that after JHMV infection. AEM, acute encephalomyelitis; CM/D, chronic meningitis with demyelination in spinal cord, but without evidence of an acute encephalitis; Neg, no evidence of neuropathology in brain or spinal cord by light microscopy.

^f The type of virus recovered from infected brains (e.g., 1/3 *ts* = the recovered virus in one of three brains from which infectious virus could be recovered was a temperature-sensitive mutant that could not be distinguished from the inoculated mutant).

^g Not done.

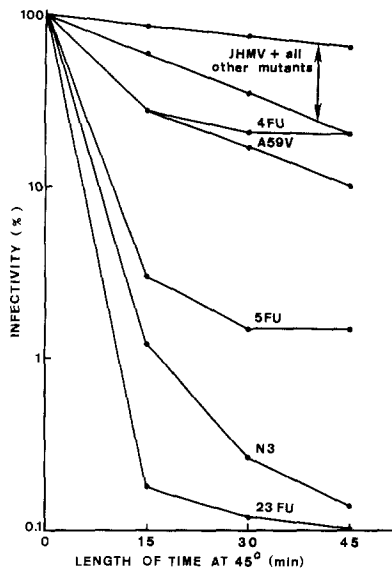


FIG. 1. Thermostability of virions. Four 0.1-ml aliquots of each virus were incubated in a $45^{\circ} (\pm 0.2^{\circ})$ constant temperature water bath for 0, 15, 30, or 45 min. Duplicate microtitrations were made on each sample. The range of variability for JHMV is shown by the upper two lines (arrow) using six independent determinations and three pools of virus (two determinations for each pool). All mutants deviating from JHMV were retested independently at least twice. Residual infectivity is plotted as a function of length of incubation.

thesize virus-specific RNA at 38.5 and 33° relative to wild-type JHMV RNA synthesis at both temperatures. The three groups are shown in Table 1 and represent "RNA-minus" mutants, "RNA-intermediate" mutants, and "RNA-plus" mutants. Mutants derived by both mutagens are represented in the minus and intermediate groups, but only mutants derived by 5FU mutagenesis are represented in the plus group.

Progeny Virus Yields at 33, 37, and 38.5°

The body temperature of a mouse is quite variable and ranges between 37 and 39° . It was therefore necessary to determine the ability of the mutants to multiply at both 37 and 38.5° as compared to 33° to allow suitable correlations between altered neuropathogenesis and mutant genotype (i.e., is the altered neuropathogenesis due to the mutation or simply to "leakiness" of

the mutant at temperatures less than 38.5°). The data are presented in Table 1. In spite of the fact that syncytia induction was inhibited to less than 10^{-3} at 38.5° , progeny virus production was significant at 38.5° for N19 and 20FU, both RNA-intermediate mutants. In fact, 20FU was distinguished from JHMV only because it did not induce syncytia at 38.5° . In spite of their "leakiness" at 37° , several mutants had LD_{50} 's of greater than 10,000 iU: RNA-minus; 23FU, 24FU; RNA-intermediate, N16, N18, N19, N70, N71, 8FU, 33FU, and 37FU.

Virus-Specific Antigen and Protein Synthesis at 38.5°

The JHMV-specific immunofluorescence was similar for JHMV and all 34 mutants at 33° using living, methanol-fixed, and formaldehyde-Triton fixed infected cells. At 38.5° , however, no viral specific antigens were detected by immunofluorescence in infected cells with the following exceptions: N19, 4FU, and 15FU all induced viral antigens which gave wt immunofluorescence although the amount of syncytia induction was markedly reduced. Cells infected with 8FU or 10FU and were incubated at 38.5° were indistinguishable from those infected with wt JHMV when tested by immunofluorescence. These two mutants were capable of inducing syncytia in infected cells. The fraction of infected cells incubated at 38.5° which contained viral-specific antigens detectable by immunofluorescence was approximately the same as the ratio of virus yields at $38.5^{\circ}/33^{\circ}$ for 20FU, 23FU, and 42FU. Radioimmune precipitation of JHMV proteins was not as sensitive as immunofluorescence for detecting viral antigens. When infected cells were incubated at 38.5° and radioimmune precipitation was used as an assay for viral-specific protein synthesis, only 4FU, 8FU, and 42FU induced detectable JHMV-specific proteins, all at a greatly reduced level when compared with wt JHMV. The analysis of total intracellular proteins for JHMV-specific proteins synthesized by the mutants at 38.5° by one-dimensional gel electrophoresis gave results comparable to that found by radioimmune precipitation.

Thermostability of Virions

The data in Fig. 1 show that wild-type JHMV loses 15–40%, 25–65%, and 25–80% of its original infectivity after 15-, 30-, and 45-min incubations at 45°, respectively. A59V is more thermolabile than JHMV. The mutants fall into three groups: 30 mutants similar to JHMV; 4FU with a modest initial loss; and N3, 5FU, and 23FU with marked thermoinstability.

Demonstration of Neurovirulence and Selection of Mouse Strain

Weiner (1973) and Lampert *et al.* (1973) demonstrated that intracerebral and intraperitoneal inoculation of JHMV produced panencephalitis with primary demyelination in the central nervous systems of weanling outbred Swiss-Webster mice. Four-week-old outbred Swiss-Webster (Strong Research Foundation), inbred SJL

(Jackson Laboratories), and inbred BALB/c (Strong Research Foundation and Jackson Laboratories) mice were examined for encephalitis and/or demyelination after intracerebral inoculation with the doubly cloned wild-type JHMV. Figure 2 demonstrates that BALB/c mice were very sensitive to the intracerebral infection. The Swiss-Webster mice were less sensitive than the BALB/c mice and exhibited an all-or-none response independent of the dose of JHMV. The SJL mice were also less sensitive than the BALB/c mice, but the infection was dependent upon the dose of JHMV unlike the infection in the Swiss-Webster mice. The BALB/c strain was chosen for further investigation because of the sensitivity and reproducibility of JHMV infection in this strain. Another advantage of the BALB/c strain is that the 17CL-1 cells were derived from the BALB/c strain and are syngeneic. The third, fourth, and

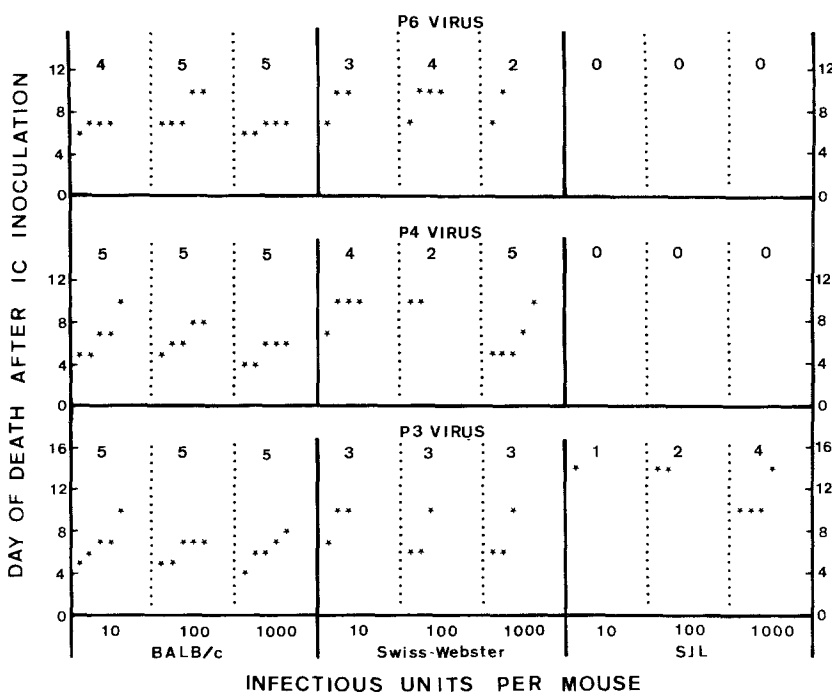


FIG. 2. Production of lethal encephalomyelitis in three strains of 4-week-old mice after intracerebral inoculation with various doses of JHMV. Three strains of mice were tested: BALB/c, Swiss-Webster, and SJL. JHMV, 10, 100, or 1000 infectious units, was inoculated into five mice. The animals were observed for 16 days. The day of death is given for each mouse. The total number of animals that died is given at the top of each column for each dose of virus for each strain. JHMV was tested after its third (P3), fourth (P4), and sixth (P6) passages in 17CL-1 cells at 33°.

sixth passages of wild-type JHMV were equally neuropathogenic in BALB/c and Swiss-Webster mice showing that the neurovirulence is not reduced by six passages of the wild-type JHMV *in vitro*. The fourth and sixth passages of JHMV, however, were increasingly less neurovirulent in the SJL mice. This finding indicates that some change must have taken place in virus structure and/or multiplication during *in vitro* cultivation. The maximum titer of JHMV was about 10^5 iU per infected brain, and was independent of the amount of inoculated virus between 1 and 1000 iU per inoculation.

Three routes of infection were examined in 4-week-old BALB/c mice to determine the ability of the doubly cloned JHMV to produce lethal encephalomyelitis. The data are given in Table 2. Intraperitoneal infection using up to 10,000 iU/mouse produced no clinical disease. Intracerebral infection was very effective in producing a lethal encephalitis with an LD₅₀ of about 2-4 iU depending on the source of the mice. Intranasal infection was also effective, but 100-fold more virus is required by this route than by the direct intracerebral route.

Figure 3A demonstrates by immunofluorescence JHMV-specific antigens in the

hippocampus. Astrocytes, oligodendrocytes, and neurons are infected during the first week of infection. Figures 3B and C demonstrate by transmission electron microscopy the presence of JHMV ribonucleoprotein and budding JHMV in a glial cell. The distribution of lesions and the neuropathology observed in BALB/c mice are similar to that described by Bailey *et al.* (1949) for this virus in outbred Swiss mice. An electron micrograph of a demyelinating lesion is shown in Fig. 4. Demyelinating lesions were found in the spinal cords of all animals. These lesions were similar to those described by Lampert *et al.* (1973).

Selection of JHMV and A59V Variants with Altered Neuropathogenesis

Biochemical analysis of variants of JHMV and A59V might reveal differences in virus structure or multiplication that could be correlated with the selected alteration in neuropathogenesis. To this end, we selected the JHMV-R strain which is less neuropathogenic than JHMV by repeated passages of JHMV in 17CL-1 cells using a m.o.i. = 0.0001. An A59V-R strain which is more neuropathogenic than A59V was selected by five intracerebral passages of A59V in 4-week-old BALB/c mice selecting the

TABLE 2

LETHAL ACUTE ENCEPHALOMYELITIS PRODUCED IN 4-WEEK-OLD BALB/c MICE BY INTRACEREBRAL, INTRANASAL, AND INTRAPERITONEAL INFECTION WITH JHMV AND A59V AS A FUNCTION OF VIRUS DOSE

Route of infection	Virus	Infectious units inoculated per animal (No. dead/No. inoculated) ^a						LD ₅₀ ^b
		10,000	1000	100	10	1	0.1	
IC	JHMV	ND ^c	15/15	19/20	28/35	2/10	0/10	3.7
	A59V	ND	4/10	3/10	1/10	1/10	0/10	490
IN	JHMV	5/5	4/5	2/5	0/5	0/5	ND	210
	A59V	0/5	0/5	0/5	0/5	0/5	ND	>10,000
IP	JHMV	0/5	0/5	0/5	0/5	0/5	ND	>10,000
	A59V	0/5	0/5	0/5	0/5	0/5	ND	>10,000

^a Animals were observed for 3 weeks after inoculation. Focal spinal cord demyelination occurred in all animals surviving the ic inoculation of JHMV and A59V at the 100 and 10 iU/animal doses. None of the survivors at the 1 or 0.1 iU/animal doses developed demyelination, probably as a result of not being infected as calculated by the Poisson distribution. Hepatitis was not detected by light or immunofluorescent microscopy.

^b LD₅₀ calculated by the procedure of Reed and Muench, 1938.

^c Not done.

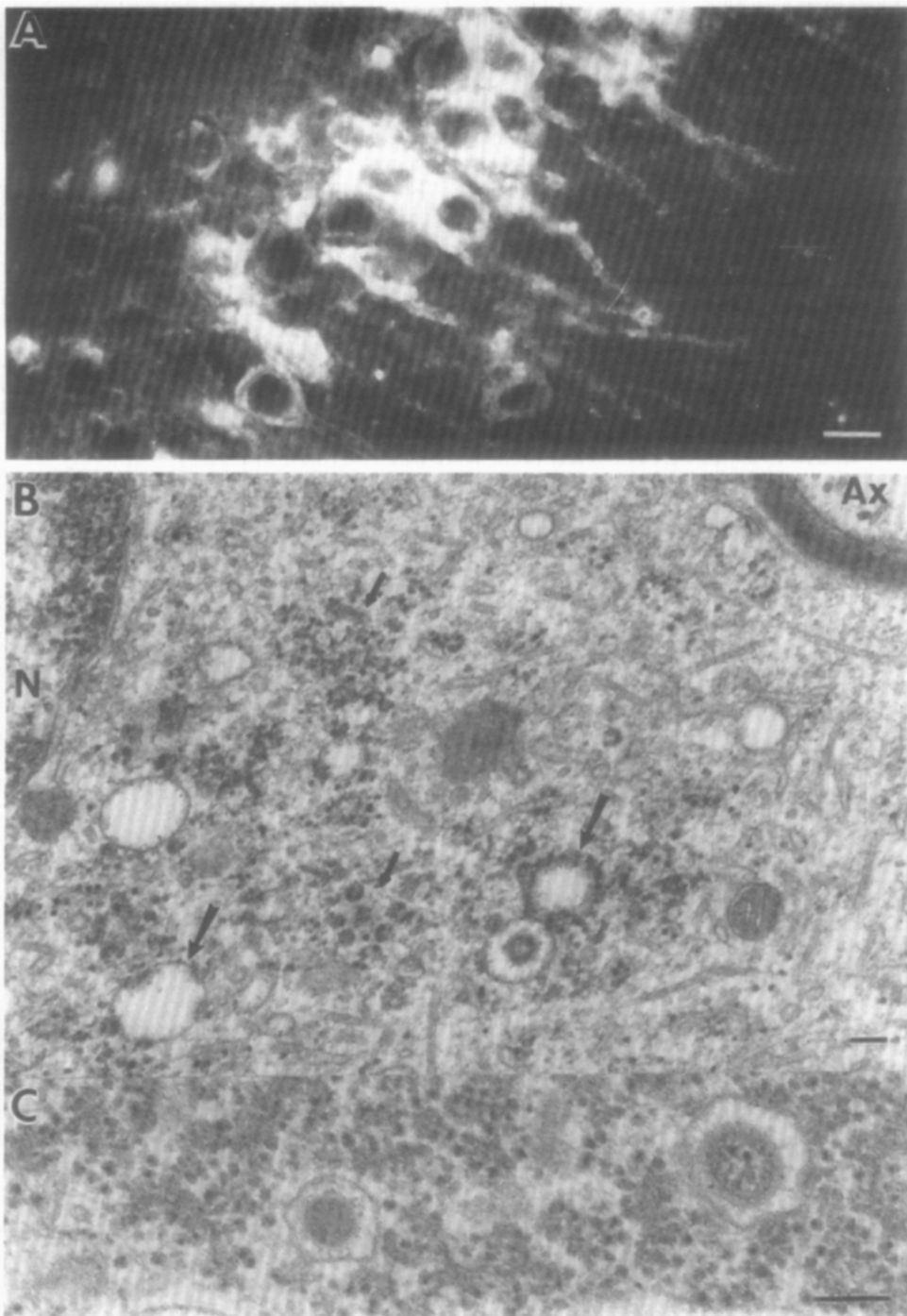


FIG. 3. Demonstration of JHMV by immunofluorescence and electron microscopy in brains from 4-week-old BALB/c mice. (A) demonstrates the infection of neurons and glial cells in the hippocampus by JHMV as assayed by immunofluorescence with mouse anti-JHMV serum. (B) demonstrates the presence of viral ribonucleoprotein (small arrows) and the budding of this viral ribonucleoprotein into cisternae of the endoplasmic reticulum (large arrows) of a glial cell, most likely an oligodendrocyte. Ax is a myelinated axon and N is the nucleus. (C) demonstrates mature virus particles within cisternae of the endoplasmic reticulum. Scale bar in (A) is 10 μm and in (B) and (C) is 100 nm.

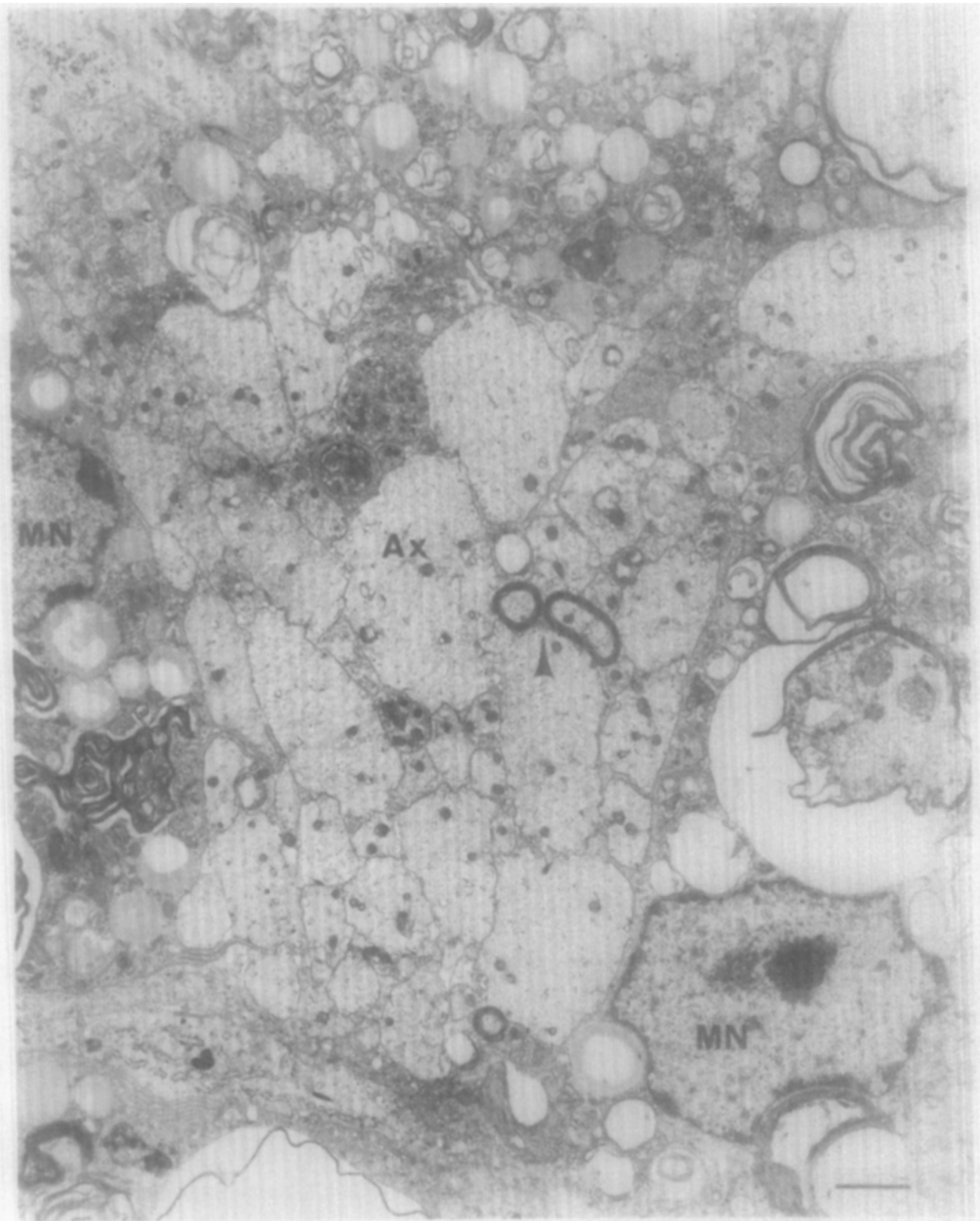


FIG. 4. Demyelination in the brain of a BALB/c mouse. A 4-week-old BALB/c mouse was inoculated with 10 iU of JHMV by the intracerebral route 21 days before examination by transmission electron microscopy. Two normally myelinated small axons (arrow) are present within a group of demyelinated axons (Ax). MN indicates the nuclei within two macrophages that have removed the damaged myelin from the demyelinated axons. Scale bar is 1 μ m.

sickest mouse each time for the donor brain homogenate (Table 3). The virus-specific proteins found in cells infected with the parent A59V or JHMV or the variants JHMV-R or A59V-R are all pre-

cipitated by anti-JHMV or anti-A59V sera as determined by radioimmune precipitation and slab gel electrophoresis. These variants provide a model for acute viral-induced demyelination in which almost

100% of inoculated animals survive low dose IC infection and have extensive spinal cord demyelination, usually without clinical symptoms. Almost all of the animals recover completely by 2–3 months. A second phase of demyelination does not occur except in a few animals that also seem to have foci of remyelination at 2–3 months after infection (Table 3).

The Neuropathogenesis of 34 Temperature-Sensitive Mutants of JHMV

JHMV has an LD₅₀ of 2–4 iU by the intracerebral route in 4-week-old BALB/c mice. Each mutant was tested using five mice for each dose: 10¹, 10², 10³, and 10⁴ iU/animal. The LD₅₀ was calculated, and the brains and spinal cords of the infected animals were examined for virus infection as shown in Table 1. Of the mutants, 13/17 RNA-minus, 12/14 RNA-intermediate, and 2/3 RNA-plus mutants had an LD₅₀ of greater than 10⁴ iU and produced no detectable neuropathology; 1/17 RNA-minus (N32) and 1/14 RNA-intermediate (20FU) mutants had an LD₅₀ of greater than 10³ iU and had a neuropathology similar to that in JHMV-infected animals in only the ani-

mals that became sick. The virus recovered from one sick N32-infected animal was not temperature sensitive. The healthy animals infected with these mutants had no detectable neuropathology. This neuropathology can be accounted for by the mutants' "leakiness" at 37°. Reversion to wt-JHMV may also account for part of these findings. An LD₅₀ greater than 10³ iU was found in 1/14 RNA-intermediate (31FU) and 1/3 RNA-plus (15FU) mutants, but no neuropathology was detectable even in the sick and dying animals. N48 (RNA-minus) has an LD₅₀ of 600 and produced a neuropathology similar to that in JHMV-infected animals. Temperature-sensitive virus was recovered from 2/2 sick animals. "Leakiness" of N48 at 37° can account for this neuropathology.

N2 and N3 are very interesting mutants because they produce only a chronic meningitis and demyelination of the brain and spinal cord without an acute encephalitis (i.e., killing of neurons as detected by light microscopy). Neurons, however, do contain JHMV-specific antigens when examined by immunofluorescence. An alteration in neuronal function without neuronal death may account for the lethality of the

TABLE 3

ISOLATION OF LESS NEURTROPIC JHMV (JHMV-R) AND MORE NEURTROPIC A59V (A59V-R) VARIANTS

Virus	LD ₅₀ (iU)	ic dose (iU)	Percentage of animals (intracerebral inoculation)					
			<14 Days postinfection			60–100 Days postinfection		
			Sick ^a	Dead	Spinal cord demyelination ^b	Sick ^a	Dead ^c	Demyelinated
JHMV	4	100	100(20/20)	95(19/20)	100(19/19)	0	0	100(1/1)
JHMV-R	150	50	100(30/30)	17(5/30)	100(15/15)	0	0	7(1/15)
		500	100(30/30)	10(3/30)	100(15/15)	0	0	0(0/15)
A59V	490	1000	50(5/10)	40(4/10)	100(5/5)	0	0	0(0/5)
A59V-R	370	100	100(15/15)	7(1/15)	100(5/5)	10(1/10)	0	20(2/10)
		1000	100(15/15)	47(7/15)	100(10/10)	20(1/5)	0	40(2/5)

^a All animals that became sick within 14 days after infection had clinical encephalitis. The animals that survived became clinically well and stayed well except for those few that received JHMV-R and developed hindlimb paralysis between 60 and 100 days after infection.

^b Longitudinal sections of at least 50% of each animal's spinal cord were examined by light microscopy. Transmission electron microscopy demonstrated focal remyelination with a macrophage infiltrate in the A59V-R-infected animals that relapsed at 60–100 days postinfection.

^c Number of additional animals that died after 14 days.

TABLE 4

PROTECTION OF 4-WEEK-OLD BALB/c MICE BY INFECTION WITH A59 VIRUS AGAINST REINFECTION BY WILD-TYPE JHMV AS A FUNCTION OF ROUTE OF INOCULATION

Primary inoculation ^a	Primary infection (iU/animal)	Animals surviving/ animals inoculated ^b		Protection
		Primary infection with A59V	Reinfection (ic) with JHMV	
ic	0 ^c	5/5	2/5	0
	10	5/5	5/5	+
	100	5/5	5/5	+
	1000	5/5	5/5	+
in	0	5/5	2/5	0
	10	5/5	2/5	0
	100	5/5	3/5	0
	1000	5/5	3/5	0
ip	0	5/5	2/5	0
	10	5/5	3/5	0
	100	5/5	3/5	0
	1000	5/5	4/5	±

^a ic means intracerebral inoculation using 10 μ l/animal. in means intranasal inoculation using 10 μ l/animal. ip means intraperitoneal using 10 μ l/animal.

^b The animals surviving the primary infection were reinfected 2 weeks after their primary inoculation using 100 iU/animal in 10 μ l ic in the contralateral side of the head from the primary inoculation when the ic route was used for the primary inoculation. The animals were observed for 3 weeks after their reinfection.

^c A dose of 0 iU/animal means the animals were inoculated with a lysate of noninfected 17C1-1 cells (mock infection) using a dilution similar to that of the highest concentration of JHMV.

infection [LD₅₀ of 85 (N3) and 650 (N2)] because neither the "leakiness" of these mutants at 37° (Table 1) nor the reversion of these mutants can account for the animal's death. The viruses recovered from the brains of six sick animals infected with N3 were all temperature sensitive. The viruses recovered from the brains of sick N2-infected animals were only temperature sensitive in one of three isolates suggesting a rather high reversion frequency for N2.

Protection of Mice from JHMV-Induced Death by Previous Infection with Temperature-Sensitive Mutants

Pilot experiments were performed to determine which routes of primary infection would protect mice from a subsequent lethal ic infection with JHMV. A59V was inoculated ic, in, and ip using doses of 0, 10, 100, and 1000 iU into each of five 4-week-

old BALB/c mice for every point. Two weeks after the primary infection, all animals were inoculated with 100 iU of JHMV in the contralateral cerebrum. The animals were observed for three weeks after the JHMV inoculation. Only the intracerebral route of the primary infection was protective (Table 4).

Fifteen mutants were subsequently tested in a similar manner for their ability to protect mice from a lethal JHMV ic infection. The data in Table 5 show that all the mutants tested were protective and that some mutants were more efficient than others in terms of the infectious units per animal required for this protection.

DISCUSSION

The murine and human coronaviruses are serologically related and extrapolations from the murine to the human disease are likely to be rewarding. Second, the diseases

produced by the viruses are naturally occurring diseases produced in their natural host using doses (1–100 iU/animal) of virus and either a natural (intranasal or intraoral) or experimental (intracerebral or intraperitoneal) route of infection. The use of our collection of temperature-sensitive mutants and selected variant non-temperature-sensitive viruses should enable us to elucidate the disease process caused by this group of viruses during natural infections in both mice and men.

Our mutants were selected by their inability to induce syncytia at low multiplicities of infection (“fusion from within”) rather than the inability to produce infectious progeny virus at restrictive temperature (38.5°). Of concern was the possibility that we would select a skewed collection of one or a few specific classes of mutants rather than a collection containing mutants having defects in many, if not all, the virus-coded proteins. Our collection, however,

has RNA-minus, RNA-intermediate, and RNA-plus mutants and at four tentative complementation groups. The identification of the affected protein by two-dimensional gel electrophoresis coupled to continuing attempts to define complementation groups should eventually allow the proper grouping of each mutant.

Our attempts to analyze the *in vivo* neuropathogenesis and immunology/immunopathology were curtailed by the serious problem that essentially all commercially available BALB/c mice had been previously infected with a murine coronavirus as detected by our microimmunofluorescence and microneutralization assays (data not shown). All of these animals were reported as “negative for mouse hepatitis virus” by the suppliers using complement fixation assays. Fortunately, the experiments using intracerebral inoculations are not altered by this finding because a previous infection by the nasal or oral route (the natural routes)

TABLE 5
PROTECTION BY INOCULATION WITH *ts* MUTANTS AGAINST IC CHALLENGE WITH WT JHMV

Virus used in primary infection ^a	Primary infection (iU/animal)	Animals surviving/animals inoculated		Protection
		Primary infection	Contralateral ic infection with JHMV ^b	
JHMV	0 ^c	5/5	2/5	0
	10	1/5	1/1	+
	100	1/5	1/1	+
<i>ts</i> N3	100	4/5	4/4	+
	1000	3/5	3/3	+
<i>ts</i> N60,15FU	100	5/5	2/5	0
	1000	4/5	4/4	+
<i>ts</i> N64,N70,N71, 10FU	100	5/5	2-3/5	±
	1000	5/5	5/5	+
<i>ts</i> N2,N4,16FU, 23FU,29FU, 37FU,38FU, 42FU	100	5/5	5/5	+
	1000	5/5	5/5	+

^a Primary infection used 10 μ l/animal.

^b Reinfection was accomplished 14 days after the primary infection using 100 iU JHMV/10 μ l/animal in the contralateral cerebral hemisphere from the primary inoculation. The animals were observed for 3 weeks after their reinfection. At the time of the reinfection with JHMC, the animals were 6 weeks old and more resistant as shown by the death rate of 40% (2/5) for JHMV using 100 iU compared to a death rate of 95% in 4-week-old animals (Table 2).

^c A dose of 0 iU/animal means the animals were inoculated with a lysate of noninfected 17CL-1 cells (mock infection) using a dilution similar to that of the 10 iU dose of JHMV.

does not interfere with a subsequent intracerebral infection (Table 5). Therefore, the data in Tables 1, 3, and 5 are valid. The data in the other tables, where intraperitoneal or intranasal inoculation appear to be valid, but must be confirmed by further experiments using germ-free mice or mice that have been shown to be seronegative by immunofluorescence. Further work is necessary to define the mechanisms of protection afforded by intracerebral inoculation of A59V (Table 4) and some mutants (Table 5).

The altered neuropathogenesis produced by mutant N3 is of interest. Intracerebral infection by this mutant produces chronic meningitis and demyelination with an LD₅₀ of 85 that cannot be explained by "leakiness" or reversion. Histologically, neurons are not killed as they are by JHMV infection (encephalitis), but are infected as detected by JHMV-specific immunofluorescence. The anti-JHMV serum used for this immunofluorescence is polyspecific and recognizes intracellular JHMV-specific proteins (Bond *et al.*, 1979). Monospecific antisera will have to be used to define further the protein(s) synthesized in the N3-infected neurons. One intriguing possibility is that the N3-infected neurons are simply "dysfunctional" and not lethally altered or made susceptible to immune attack. This type of dysfunctional attack is probably only clinically important in an organ such as the brain, where the neurons cannot be replaced by newly regenerated parenchymal cells as occurs in the liver, lung, or kidney. Such a hypothesis may gain some support from the work of Oldstone *et al.*, (1977) who showed that the differentiated protein synthesis (e.g., choline acetyl transferase, acetylcholine esterase) in murine neuroblastoma cells persistently infected with the neurotropic lymphocytic choriomeningitis virus was selectively inhibited compared to uninfected cells and to general protein synthesis in the persistently infected cells. The role of defective interfering JHMV particles in the N3-altered neuropathogenesis is remote because 40 serial undiluted passages of JHMV *in vitro* do not decrease the production of infectious progeny JHMV (Robb, unpublished observation).

We had hoped that by selecting wild-type

variants with altered neuropathogenesis, we could develop a "two-phase" (acute and persistent) model for virus-caused demyelination, possibly analogous to human multiple sclerosis. Although we can produce primary demyelination in almost 100% of infected animals without death the animals recover and very few have persistent demyelination beyond 2-3 months (Table 3). One temperature-sensitive mutant of JHMV has been isolated by Haspel *et al.* (1978) that produces both acute and persistent demyelination after intracerebral inoculation.

Present work is focused on identifying the biochemical differences between the temperature-sensitive mutants, the wild-type non-temperature-sensitive variants JHMV and A59V. We are also biologically and biochemically characterizing 17CL-1 and murine neuroblastoma cells that are persistently infected with JHMV and A59V, and the viruses recovered from these cells, to identify some of the mechanisms used by these viruses in establishing persistent disease.

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REFERENCES

- BAILEY, O. T., PAPPENHEIMER, A. M., CHEEVER, F. S., and DANIELS, J. B. (1949). A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin: II, Pathology *J. Exp. Med.* **90**, 195-221.
- BOND, C. W., LEIBOWITZ, J. L., and ROBB, J. A. (1979). Pathogenic murine coronaviruses: II, Characterization of virus-specific proteins of murine

- coronaviruses JHMV and A59V. *Virology* **94**, 371-384.
- HASPEL, M. V., LAMPERT, P. W., and OLDSTONE, M. B. A. (1978). Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination. *Proc. Natl. Acad. Sci. USA* **75**, 4033-4036.
- LAI, M. M. C., and STOHLMAN, S. S. (1978). RNA of mouse hepatitis virus. *J. Virol.* **26**, 236-248.
- LAMPERT, P. W., SIMS, J. K., and KNIAZEFF, A. J. (1973). Mechanism of demyelination in JHM virus encephalomyelitis: Electron microscopic studies. *Acta Neuropathol.* **24**, 76-85.
- OLDSTONE, M. B. A., HOLMSTOEN, J., and WELSH, R. M. (1977). Alterations of acetylcholine enzymes in neuroblastoma cells persistently infected with lymphocytic choriomeningitis virus. *J. Cell. Physiol.* **91**, 459-472.
- REED, L. J., and MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27**, 493-497.
- ROBB, J. A., TEGTMEYER, P., MARTIN, R. G., and KIT, S. (1972). Proposal for a uniform nomenclature for simian virus 40 mutants. *J. Virol.* **9**, 562-563.
- ROBB, J. A. (1973) Replica plating. In "Tissue Culture-Methods and Applications" (P. F. Kruse and M. K. Patterson, eds), pp. 270-274. Academic Press, New York.
- ROBB, J. A., and BOND, C. W. (1979a). Coronaviridae. In "Comprehensive Virology" (H. Fraenkel-Conrat and R. Wagner, eds.), Vol. 14. Plenum, New York, in press.
- ROBB, J. A., and BOND, C. W. (1979). Pathogenic murine coronaviruses: I, Characterization of biological behaviour *in vitro* and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59V viruses. *Virology* **94**, 352-370.
- SAINTE-MARIE, G. (1962). A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**, 250-256.
- WEINER, L. P. (1973). Pathogenesis of demyelination induced by a mouse hepatitis virus (JHM virus). *Arch. Neurol.* **28**, 298-303.