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Genetic Variation of Neurotropic and Non-neurotropic Murine Coronaviruses

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SUMMARY

The murine coronavirus strains MHV JHM, MHV 1, MHV 2, MHV 3 and MHV A59 were tested for their neurovirulence in weanling rats. The strain JHM was found to be highly neurovirulent for weanling rats, whereas the other strains were not, or only slightly, neurovirulent. MHV 1 caused no lesions in weanling rats. The other strains (MHV 2, MHV 3 and MHV A59) induced predominantly subclinical infections in weanling rats as demonstrated by an increase of antibodies and inflammatory lesions in the liver. Analysis of these strains by cross-neutralization revealed variable degrees of antigenic relationship between these viruses which were not related to their neurovirulence. However, when these strains were compared by analysing the T_1-RNase-resistant oligonucleotides of virion RNA, the highly neurovirulent strain JHM was found to differ significantly in its nucleotide sequence from the other less-neurovirulent strains.

INTRODUCTION

Murine coronaviruses are widespread in nature and cause a variety of diseases ranging from inapparent infections to acute diseases such as hepatitis, enteritis and encephalomyelitis (Gledhill & Andrewes, 1951; Nelson, 1952; Dick *et al.*, 1956; Manaker *et al.*, 1961; Bailey *et al.*, 1949; Cheever *et al.*, 1949). The original isolates of murine coronaviruses were recovered from different mouse strains and, so far, animal experiments to test the organ tropism of these viruses and to study the pathogenic mechanisms by which these strains induce diseases have mainly been carried out in mice (Lampert *et al.*, 1973; Weiner, 1973). Recently, however, it has been shown in weanling rats that the murine coronavirus JHM induces different types of central nervous system (CNS) disease which can be accompanied by marked demyelination (Nagashima *et al.*, 1978*a*, *b*, 1979). This finding led us to investigate the organ tropism of a number of other murine coronaviruses in rats. The antigenicity and oligonucleotide pattern of the virion RNA of these viruses were also analysed to further characterize these agents. The results obtained indicate that, of the strains analysed, JHM is the only murine coronavirus highly neurotropic for rats. This strain can also be readily distinguished from the others serologically and by the T_1 oligonucleotide pattern of its virion RNA.

METHODS

Source and maintenance of virus strains. The mouse hepatitis virus strains MHV JHM and MHV 2 were obtained from L. Weiner, Johns Hopkins University, Baltimore, Md., U.S.A. The strains MHV A59, MHV 1 and MHV 3 were a gift from P. Carthew, MRC Laboratory Animal Centre, Surrey, U.K. and originated from the American Type Culture Collection. All stock virus strains were propagated on monolayers of Sac(–) cells (Weiland *et*

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al., 1978) grown in minimal essential medium containing 5% foetal calf serum at an m.o.i. of 0·1 $TCID_{50}$/cell. The infected cell cultures were incubated at 37 °C and harvested 20 to 24 h post-infection (p.i.), when more than 75% of the cells were fused. The original virus stocks were cloned by three consecutive plaque passages.

Production of antisera. Specific pathogen-free mice (strain NMRI) were immunized by five intraperitoneal injections of virus (0·4 ml clarified medium from infected cell cultures, corresponding to 1×10^6 $TCID_{50}$ of virus) at weekly intervals. The surviving animals were bled 10 days after the last injection.

Neutralization test. The virus was diluted to contain 100 $TCID_{50}$ and incubated for 1 h at 37 °C with twofold dilutions of inactivated antiserum in a final vol. of 0·1 ml. The incubation mixture was then transferred to monolayers of Sac(−) cells in microtitre plates (96 × 0·2 ml) using four replicas per virus serum dilution. The test was scored for cytopathology after 48 h at 37 °C.

Animal experiments. Specific pathogen-free rats, strain CHBB/Thom were obtained from Thomae, Biberach, F.R.G. Virus was inoculated with a dispenser syringe in vol. of 40 μl into the left brain hemisphere. Virus isolation and histology were performed as described previously (Nagashima *et al.*, 1978*a*, *b*, 1979). Weanling rats were infected when 20 to 24 days old.

Production of virus labelled with ^{32}P. Suspension cultures of Sac(−) cells were starved overnight with phosphate-free Joklik's minimal essential medium containing 5% dialysed foetal calf serum. Prestarved cells (6×10^8) were infected at an m.o.i. of 0·2 $TCID_{50}$/cell as described previously (Siddell *et al.*, 1980) and incubated in 300 ml phosphate-free medium for 18 to 26 h at 37 °C, depending on the growth kinetics of the individual virus strains used. Six h after infection, 30 mCi ^{32}P-orthophosphate (The Radiochemical Centre, Amersham; PBS 11) were added for labelling of virion RNA. The labelled virus was purified from the medium of the infected culture as described previously (Wege *et al.*, 1978) and the pellet of purified virus was immediately used for extraction of RNA.

Extraction and purification of virion RNA. The purified virus pellet was resuspended in 10 ml extraction buffer (0·05 M-sodium acetate, 0·1 M-NaCl, 0·0025 M-EDTA, 1% SDS pH 4·6) and 100 μg cytoplasmic RNA from Vero cells were added. After thorough mixing, the solution was decanted and the viral RNA was extracted with 10 ml water-saturated phenol as previously described (Stephenson *et al.*, 1977). The RNA pellet from the final ethanol precipitation was resuspended in 0·4 ml gradient buffer (0·1 M-LiCl, 50 mM-tris pH 7·5, 1 mM-EDTA, 0·1% SDS) and purified by centrifugation for 4·5 h at 18 °C and 200000 *g* on 15 to 30% sucrose gradients. The virion RNA of murine coronaviruses sediments between 52 and 56S in aqueous gradients (Lai & Stohlman, 1978; Wege *et al.*, 1978). The pooled RNA of this size was precipitated overnight at −20 °C with 3 vol. absolute ethanol and 50 μg cytoplasmic RNA from Vero cells. Virion RNA prepared by this method showed no detectable contamination by cellular species (<1% as determined by scanning the gradient at A_{260} and <0·1% as measured by determining the radioactivity in various fractions of the sucrose gradient). In addition, the oligonucleotide maps from the virion RNA showed no evidence of containing spots which were specific for rRNA. As the profile of radioactive RNA on the sucrose gradient gave a single symmetrical peak of between 52 and 56S, the RNA was assumed to be homogeneous.

Preparation and analysis of T_1 *oligonucleotides.* The ethanol precipitate of purified RNA was centrifuged at 5000 *g* for 30 min and dried in a desiccator. The dry pellet was digested with T_1 RNase (Sigma) and the products analysed by two-dimensional electrophoresis as described by de Wachter & Fiers (1972), but with the following modifications. As these large RNAs contain a large number of specific oligonucleotides, the resolution of the gel was improved by increasing the thickness of the gel twofold and thus reducing the size of spots.

Table 1. *Neurovirulence of murine coronaviruses in weanling rats*

Virus	Dose $TCID_{50}$/rat i.c.	Neurological disease within 24 days p.i.	Virus isolation* 4 to 8 days p.i.	Liver inflammatory lesions 24 days p.i.	Neutralizing antibody titre 24 days p.i.
MHV JHM	$4{\cdot}0 \times 10^4$	25/30	8/8†	0/5	1:56
	$2{\cdot}0 \times 10^6$	10/10			
MHV 1	$4{\cdot}0 \times 10^4$	0/13	0/8	0/5	<1:8
MHV 2	$4{\cdot}0 \times 10^4$	0/15	0/8	2/5	1:70
	$4{\cdot}8 \times 10^6$	1/10			
MHV 3	$4{\cdot}0 \times 10^4$	0/15	0/8	1/5	1:52
	$3{\cdot}4 \times 10^6$	0/10			
MHV A59	$4{\cdot}0 \times 10^4$	0/15	0/8	3/5	1:76
	$6{\cdot}4 \times 10^6$	0/10			

* Brain, spinal cord and liver.
† Liver samples negative

Table 2. *Antigenic relationship of murine coronaviruses by serum neutralization test**

	Antiserum				
Antigen	MHV JHM	MHV 1	MHV 2	MHV 3	MHV A59
MHV JHM	**283**	8	1624	262	39
MHV 1	9	**2130**	38	384	967
MHV 2	230	28	**618**	191	19
MHV 3	35	13	136	**2725**	72
MHV A59	35	155	49	272	**1112**

* Neutralizing antibody titres are expressed as the reciprocal factor of the serum inhibiting 50% of the input virus infectivity (100 $TCID_{50}$) within 2 days. Data of the homologous reaction are in bold type. Strong cross-reaction: heterologous titre within 1/4 × of homologous titre; weak cross-reaction: heterologous titre within 1/16 × of homologous titre.

The improvement in resolution by reduction in spot size was further enhanced by using 0·13% bisacrylamide in the second dimension, which enabled the gels to be dried before exposure. The use of dried gels substantially reduced diffusion of the spots when exposures longer than 24 h were needed.

RESULTS

Neurovirulence of different murine coronaviruses for rats

For comparison of neurovirulence, different cloned murine coronavirus strains were intracerebrally (i.c.) inoculated into weanling rats. Each animal received 4×10^4 $TCID_{50}$ of virus (Table 1). Eight animals of each group were killed 4 to 8 days p.i. and samples of brain, spinal cord and liver were removed for virus isolation. After 24 days, five clinically healthy animals of each group were sacrificed for determination of antibodies and histological examination. In addition, groups of 10 animals were injected i.c. with the highest virus dose available. The doses injected/rat ($TCID_{50}$) were 2×10^6 for MHV JHM, $4{\cdot}8 \times 10^6$ for MHV 2, $3{\cdot}4 \times 10^6$ for MHV 3 and $6{\cdot}4 \times 10^6$ for MHV A59. For MHV 1, no doses higher than 4×10^4 $TCID_{50}$ could be obtained.

As summarized in Table 1, JHM is the only coronavirus strain which gave a high incidence of neurological disease in weanling rats. The infected animals developed an acute

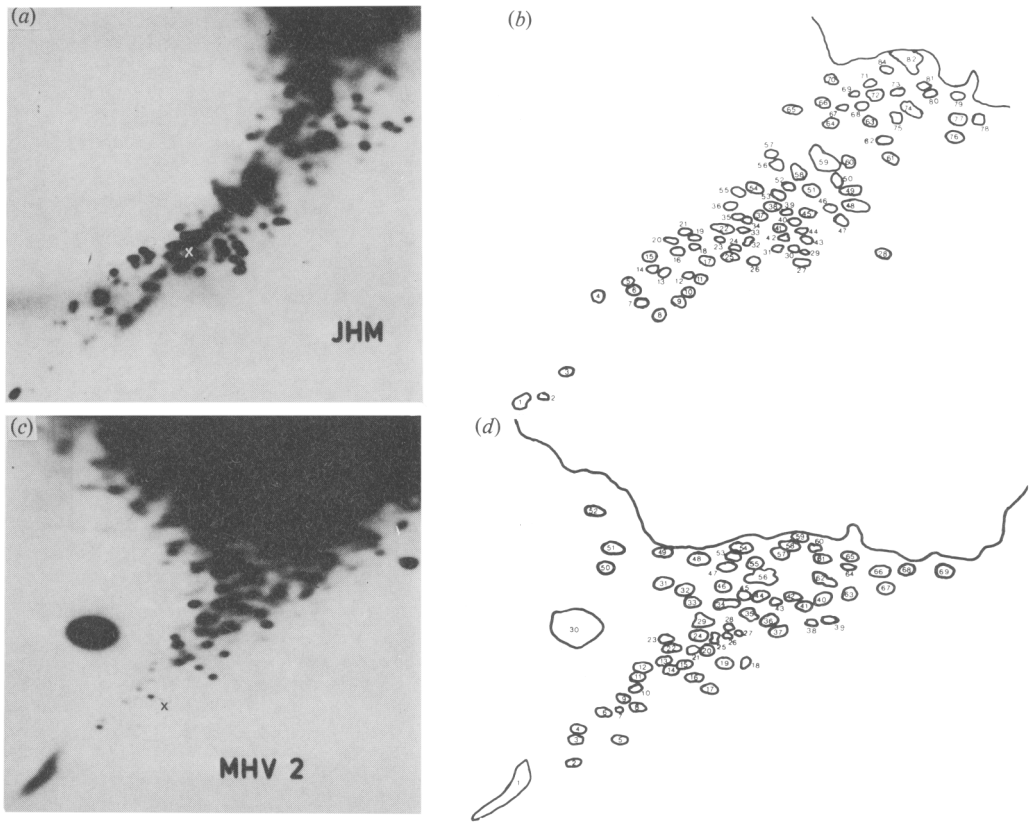
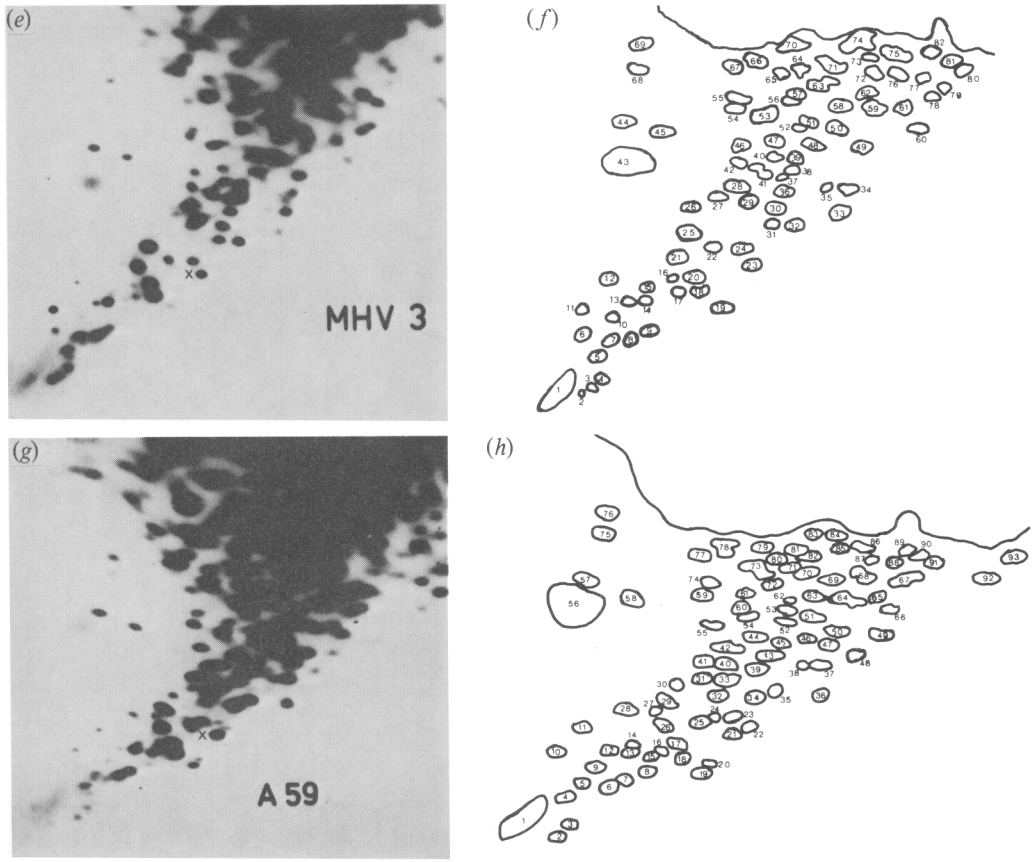


Fig. 1. Two-dimensional electrophoresis of T_1-resistant oligonucleotides of virion RNA. Electrophoresis in the first dimension was from left to right and in the second dimension from bottom to top. (*a,c,e,g*) Autoradiograms of the two-dimensional gels; (*b,d,f,h*) corresponding diagrams made from the original autoradiograms. X marks the position of xylene cyanol FF dye.

encephalomyelitis 7 to 12 days p.i. Neuropathological lesions were distributed in most parts of the CNS. Both neurones and degenerating oligodendroglia cells contained virus particles. Diseased animals developed paralysis and died within a few days. JHM virus was easily recovered from clinically healthy rats within the first 8 days after infection and from all diseased animals. In the clinically healthy rats, the titre of infectious virus in brain was $2{\cdot}8 \times 10^3 \pm 1{\cdot}8$ $TCID_{50}$/g and $5{\cdot}4 \times 10^2 \pm 1{\cdot}7$ $TCID_{50}$/g in spinal cord homogenates. In diseased animals, the titre of infectious virus was usually between 1×10^3 and 1×10^4 $TCID_{50}$/g and depended upon the time between appearance of clinical symptoms and the final dissection of the animal. Inflammatory lesions were not found in the liver or other organs. The other murine coronavirus strains induced no neurological disease in weanling rats, even when the highest available dose of infectious virus was applied, with the exception of one case of an acute encephalomyelitis, accompanied by necroses of the liver, after infection with MHV 2. This observation was supported by the unsuccessful attempts to re-isolate infectious virus from CNS tissue within 4 to 8 days after infection. However, all animals, except those inoculated with MHV 1, showed inflammatory lesions of the liver. Neutralizing antibodies, which were detected within 24 days after inoculation in animals inoculated with MHV 2, MHV 3 or A59, indicated that the animals had been infected. No antibodies to MHV 1 were found, suggesting that the rat strain used in these experiments is resistant to this virus. Suckling rats were also not susceptible to MHV 1.



Cross-neutralizing antibodies induced by cloned MHV viruses

The strains of murine coronaviruses were differentiated by the cross-neutralization test (Table 2). The strains JHM and MHV 2 are very closely related. A strong cross-reaction was also noted between MHV 1 virus and A59 serum, whereas MHV A59 was only weakly neutralized by MHV 1 serum. The other strains showed weak cross-reactions. Bilateral minor cross-reactions were observed between the virus strains JHM and MHV 3, MHV 2 and MHV 3, and finally A59 and MHV 3. Unilateral cross-neutralizations were seen between A59 virus and JHM serum, and also MHV 1 virus and MHV 3 serum.

Comparison of viral genomes by oligonucleotide analysis

Fig. 1 (*a*, *c*, *e*, *g*) shows autoradiograms of the two-dimensional electrophoretic analysis of T_1 oligonucleotides from four strains of murine coronaviruses. MHV 1 virus was not included in this analysis as it did not grow to sufficiently high titres. To facilitate comparative analysis, diagrams of each map were made using the original autoradiogram and not the photographs shown (Fig. 1*b*, *d*, *f*, *h*), since these were overexposed to reveal also less-dense spots. The irregular shaped spot no. 1 from MHV 2, MHV 3 and A59 probably represents the poly(A) tract of the virion RNA. A similar tract has been reported for JHM virus (Lai & Stohlman, 1978; Wege *et al.*, 1978) and was seen occasionally in the T_1 digests as a faint irregular spot.

As the digestion of such a large RNA species necessarily involves the analysis of a large number of oligonucleotides, the possibility that some spots contain multiple species must be

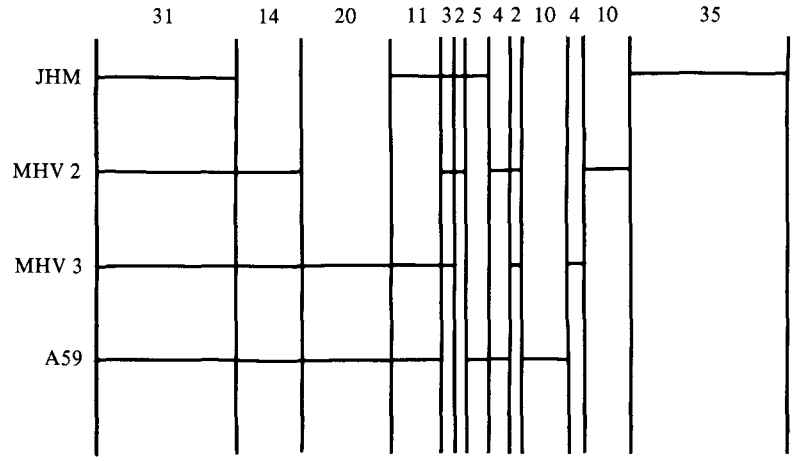


Fig. 2. Diagrammatic representation of the common and unique oligonucleotides of various murine coronaviruses. The numbers above each vertical block represent the number of oligonucleotides represented by it. The width of each block is approximately equal to the number of oligonucleotides.

considered. In addition, it is obvious from Fig. 1 that there is a variability in the intensity of spots that is not in a direct relationship to their electrophoretic mobility. However, such variabilities did remain constant over several analyses of the same isolate and also when identical spots from different isolates were analysed. Therefore, only those spots which were identical in shape, relative intensity and mobility were scored as being identical. Similarly spots whose shape clearly indicated that they contained doublets or triplets were scored as two or three spots respectively. Only the spots numbered in the diagrams were included in the analysis.

The diagram of each individual fingerprint was compared and a summary of this comparison is shown in Fig. 2. This summary reveals the number of oligonucleotides which each virus has in common with the others. Thus, there are 31 oligonucleotides common to all four viruses and a further 14 common to three of them but missing in JHM etc. Similarly, all strains have a number of unique spots. The strain JHM shows the highest degree of variation from the other viruses.

DISCUSSION

The results presented in this study clearly indicate that murine coronavirus strains are very different in their neurovirulence for weanling rats. After i.c. injection of comparable doses of each virus strain only the JHM virus was highly neurovirulent for weanling rats. The strain MHV 1, by contrast, did not seem to be infectious for either weanling or suckling animals. This observation is in accordance with the resistance of cotton rats injected with the original isolate of MHV 1 (Gledhill & Andrewes, 1951). However, the other strains tested (MHV 2, MHV 3 and A59) infected weanling rats but induced no detectable diseases of the CNS after injection of comparable virus doses. The antigenic relationship revealed signs of cross-neutralization not completely in agreement with results obtained by Hierholzer *et al.* (1979). In that study, a unilateral cross-reaction was found between JHM virus and MHV 1, which was not observed by us. In addition, we observed neutralization of MHV 1 virus by A59 serum, which was not found in their analysis. These different observations may be because serum specimens were used which were not prepared against cloned virus preparations in that study.

In general, the serological relationships between the viruses are reflected by differences in their genome sequence as revealed by their T_1 oligonucleotides. However, the serological relationships expressed by cross-neutralization reflect only the expression of a very small part of the genomic information responsible for the sequence of the structural proteins. Therefore, this relationship does not need to correlate exactly with the differences in the oligonucleotide patterns as is the case for some strains of togaviruses (Trent & Grant, 1980). Thus, although JHM virus and MHV 2 are closely related by cross-neutralization, these strains show several differences in their oligonucleotide patterns.

The virion RNA of JHM virus contained 35 oligonucleotide spots which are not represented in the other strains, whereas the other viruses contained only 4 to 10 unique oligonucleotides. It is possible that such differences could arise from non-specific degradation of virus RNA or from the presence of a contaminating defective RNA of similar size to the virion RNA. However, no evidence of such breakdown or contamination was obtained during the extraction procedure (see Methods). In addition, T_1 maps of other single-stranded RNAs from similar viruses such as vesicular stomatitis virus and spring viraemia of carp virus (Clewley *et al.*, 1977; Roy & Clewley, 1978) show similar patterns and the authors analysed a similar number of oligonucleotides. If the JHM RNA samples contained a defective RNA species of similar mol. wt. to that of the genomic RNA then the map of JHM should contain at least twice as many spots as those of the other virus isolates. As this is obviously not the case, we consider our results to represent a genuine variation in the nucleotide sequence of JHM. Therefore, assuming that these four strains have been originally derived from one parent and that the mutational frequency detected by this method is representative of that undergone by all the genome, these results suggest that the strains MHV 2, MHV 3 and A59 have all evolved at approximately equal rates. The strain JHM, however, shows many more mutations than the other viruses and therefore might have developed faster during evolution. It has been shown recently for vesicular stomatitis virus that during several years of persistent infection in tissue culture the number of mutations has increased substantially over those undergone by the same virus during continuous lytic passages (Holland *et al.*, 1979). A similar situation might also occur in the animal during the evolution of other viruses with a strong tendency to induce inapparent and persistent infections. Therefore, the fact that the strain JHM shows significant differences in its RNA sequence to other murine coronaviruses is consistent with the hypothesis that a virus arising from a persistent infection can cause subacute or chronic diseases of the CNS. The detailed characterization of mRNA and proteins of individual neurotropic and non-neurotropic strains and mutants may help to define biochemically the neurovirulence of murine coronaviruses.

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