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In Vivo and In Vitro Models of Demyelinating Disease: Endogenous Factors Influencing Demyelinating Disease Caused by Mouse Hepatitis Virus in Rats and Mice

O. SORENSEN, R. DUGRE, D. PERCY, AND S. DALES*

Cytobiology Group, Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1

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Intracerebral inoculation of JHM virus (JHMV), the neuropathic strain of mouse hepatitis virus, into Wistar Furth, Wistar Lewis, and Fischer 344 rats at various ages indicated that Wistar Furth rats are more susceptible to the virus than are the other strains. Fischer 344 and Wistar Lewis rats were more resistant to inoculation at 2 and 5 days of age and completely resistant by 10 days of age. In contrast, Wistar Furth rats which were very susceptible at both 2 and 5 days of age remained susceptible until 21 days of age. Intracerebral challenge of an F1 cross between Wistar Furth and Wistar Lewis rats at 10 days of age indicated that resistance to JHMV infection is dominant. Cyclophosphamide treatment 28 days after intracerebral inoculation exacerbated an inapparent infection, leading to paralysis in eight of nine and death in six of nine Wistar Furth test rats. In such immunosuppressed animals, grey- and white-matter lesions were noted throughout the central nervous system, in contrast to the purely demyelinating lesions noted previously. Since rats, unlike mice, were not susceptible to disease after intracerebral injection with the serorelated viscerotropic strain MHV-3, we wished to extend our understanding of the neurological disease process elicited by the two viruses in rodents. For this reason, various mouse strains, including some with recognized immunodeficiencies, were challenged by different routes of inoculation. Intraperitoneal infection of nude and beige mice with JHMV indicated that lack of natural killer cell functions does not markedly enhance the susceptibility to virus, whereas T-cell activity appears to be essential for resisting infection. JHMV and MHV-3 replication in peritoneal macrophages from highly resistant A/J mice was reduced in comparison with that noted in macrophages from susceptible C57BL6/J mice. An initial intraperitoneal inoculation of JHMV was able to protect C57BL6/J mice against fatal intracerebral challenge within 3 days, whereas A/J mice remained susceptible beyond day 3. The protective effect did not appear to result from increased levels of circulating interferon, preceded elevation in serum JHMV-neutralizing antibody titers, and persisted for at least several weeks after intraperitoneal inoculation. Based on the combined studies described here and on previous work by us and others, it appears that the factors influencing the outcome of coronavirus disease in rodents are age at inoculation, route of challenge, genetic constitution of the virus and host, and competence of the immune system, particularly cellular immunity involving T-cells.

Our previous experiments (27) demonstrated that intracerebral (i.c.) inoculation of suckling rats with JHM virus (JHMV), the neurotropic strain of mouse hepatitis virus (MHV), offers an attractive model for investigating virus-induced chronic demyelinating disease. Both inbred and outbred rats are susceptible to this virus (1, 7, 18, 27). Genetic and age-dependent susceptibility, apparent from tests conducted on outbred Wistar and inbred Fischer 344 (F344), Wistar Lewis (W/L), and Wistar Furth (W/F) rats (27), also occurs in selected strains of mice. For example, in SJL mice, the only known strain showing resistance to i.c. inoculation with JHMV (10, 28, 29), lack of susceptibility is age dependent (31) and apparently connected with the ability to replicate in neurons (10), which is determined by a single dominant locus (10) or perhaps by two loci of which one is dominant and the other is *H-2* linked (28). Similar genetically determined and age-related control of susceptibility to MHV-3 has been documented (13). In F1 crosses between susceptible C57BL/6 and resistant mice or inbred C3H and A2G mice,

there developes an age-related resistance to hepatic disease within the first 3 months of life (13). However, these strains may become paralyzed and manifest lesions in the central nervous system (CNS) several months after intraperitoneal (i.p.) inoculation of MHV-3.

Concerning the influence of the immune system, nude mice or other strains which have been thymectomized or immunosuppressed (4, 14, 24, 25, 45) invariably develop viscerotropic disease after i.p. inoculation with MHV-3. Dupuy et al. (4) and Levy-Leblond and Dupuy (14) have shown that in the absence of T-cell functions, an increased susceptibility to MHV-3 occurs in mice of the normally resistant A strain. These observations emphasize the interplay between hereditary and immunological factors influencing the disease processes caused by coronaviruses in rodents.

In this article we present additional comparative observations on the histopathology, serology, and influence of the immune state among rats and mice infected with either JHMV or MHV-3.

MATERIALS AND METHODS

Viruses and infection. The source of JHMV and MHV-3, the procedures used for propagating and assaying virus on L-2 murine fibroblasts, and the preparation of suspensions of these viruses for injection have been described previously (27). Control animals were inoculated with a suspension of uninfected L-2 cells processed in the same manner as the virus inoculum. In i.c. inoculation of rats and mice, animals received 5×10^4 PFU of JHMV or 4×10^4 PFU of MHV-3. Mice injected i.p. received 3×10^5 PFU of JHMV or MHV-3 or 5×10^4 PFU of JHMV by intranasal (i.n.) inoculation.

All animals were monitored for clinical indications of neurological disease for 1 year postinfection (p.i.). Mice reinoculated i.c. with JHMV 1 year after i.p. inoculation with JHMV were monitored for an additional 4 months.

Sources of animals. Female W/F rats were purchased from Microbiological Associates, Bethesda, Md., and male W/F rats were kindly provided by G. Strejan, University of Western Ontario, London, Ontario, Canada. For experimentation, W/F rats and the F1 cross between male W/F and female W/L animals, (W/L × W/F)F1, were bred in the Health Sciences Animal Quarters at the University of Western Ontario, F344 and W/L rats were purchased as pregnant animals from Canadian Breeding Laboratories, St. Constant, Quebec, Canada.

Mouse strains A/J, C57BL6/J, DBA/2, B6AF1 [(C57BL6/J × A/J)F1], and B6D2F1 [(C57BL6/J × DBA/2)F1] either 4 or 10 weeks of age were purchased from the Jackson Laboratory, Bar Harbor, Maine. Heterozygous C57 beige (*bg*/+) breeding pairs were purchased from Jackson Laboratory, and homozygous C57 beige mice (*bg*/*bg*) were bred at our facility. Likewise, male A/J and female DBA/2 mice were purchased from the Jackson Laboratory, and their F1 cross [(DBA/2 × A/J)F1] was bred at our facility. Adult RNC (*nu*/*nu*) nude mice were purchased from O. P. Miniats, Ontario Veterinary College, Guelph, Ontario, Canada.

Histology. All animals which either had succumbed to disease or had been killed were necropsied. Tissue samples were collected and processed for routine histopathological evaluation from all animals not showing evidence of autolysis, as described previously (27). To achieve good fixation of the rat CNS, some animals were perfused with glutaraldehyde via the left ventricle, and the appropriate tissues were collected, cut into 1-mm cubes, postfixed, dehydrated, and embedded in epoxy resin (27). Specimens were sectioned at 1-μm thickness for examination by light microscopy, and selected material was thin sectioned for examination in an electron microscope, according to our published procedure (27). Additional material, including CNS, spleen, thymus, and liver tissue, was removed from mice, fixed by immersion in a solution of 3% glutaraldehyde in a 320 mosM phosphate buffer (27), dehydrated, embedded in epoxy resin, and sectioned for light microscopic evaluation.

Immunosuppression studies. To test the effect of immunosuppression on survivors of the i.c. inoculation with JHMV, nine W/F rats which survived for 28 days beyond inoculation when 5 days of age were injected i.p. daily for 14 consecutive days with 10 mg cyclophosphamide (Procytox, Frank W. Horner Ltd., Montreal, Quebec, Canada) per kg of body weight (45). The effectiveness of immunosuppression with cyclophosphamide was determined by hemagglutination titration of the circulating anti-sheep erythrocyte (SRBC) antibody in rats injected i.p. with SRBCs.

Virus isolation from animals. Brains were removed aseptically and disrupted in a Dounce homogenizer with Eagle minimal essential medium supplemented with 10% fetal calf serum (CMEM) as the suspending medium. The final volume was adjusted with CMEM to yield a 20% (wt/vol) suspension. The suspensions of brain material were inoculated onto monolayers of L-2 cells and monitored for 8 weeks for evidence of cytopathic effects. The identity of virus isolates was established by electron microscopy of infected L-2 cells and by the development of characteristic lesions after i.c. or i.p. inoculation of 4-week-old C57BL6/J mice with virus present in the culture medium.

Macrophage infection. Since resistance of certain strains of mice to hepatic disease, produced by i.p. inoculation of MHV-3, has been correlated with the ability of the virus to replicate in macrophages from the particular strain (2), peritoneal macrophages (PM) from A/J and C57BL6/J mice were tested after either i.p. inoculation or isolation and challenge in vitro with JHMV or MHV-3. Unstimulated PM were harvested by the method described by Virelizier and Allison (38) from 2- to 3-month-old mice by means of peritoneal lavage with 3 to 4 ml of CMEM. Glass cover slips were seeded to obtain confluent monolayers. After 1 h of incubation at 37°C to allow PM to attach, the cultures were washed three times with CMEM to remove nonadherent cells. In vivo infection of PM was accomplished by the i.p. injection of 3×10^5 PFU of JHMV 24 h before harvest. In vitro inoculation was made 24 h after explanting the PM with either 5×10^5 PFU of JHMV or 1.2×10^5 PFU of MHV-3 per 10^6 cells. The concentration of virus in the supernatant was assayed

TABLE 1. Age- and strain-dependent susceptibility of inbred rats to paralysis and death after i.c. inoculation with JHMV

Strain	Age at inoculation (days)	No. paralyzed/no. dead at post-inoculation days[a]:				No. inoculated
		1–7	8–14	15–21	>21	
F344	10	0	0	0	0	6
	15	0	0	0	0	11
W/L	10	0	0	1/1	0	12
	15	0	0	0	0	17
	21	0	0	0	0	14
	30	0	0	0	0	15
W/F	10	0	6/8	9/9	5/5	33
	15	0	0	3/3	2/2	14
	21	0	0	1/1	0	8
	30	0	0	0	0	18
W/F (immunosuppressed)	5				6/6[b]	9
(W/L × W/F)F1	10	0	0/5	0	0/1	47

[a] Rats which died or were killed in extremis.
[b] Two rats recovered from paralysis and are not included in this column.

daily as PFU on L-2 cells until the PM monolayers were destroyed.

Antibody and interferon assays. Neutralizing antibody titers against JHMV were determined by plaque reduction assays. Sera were heated to 56°C for 1 h to inactivate complement and then were mixed in a ratio of 1:10 with a standard JHMV suspension containing about 250 PFU/ml. After incubation for 1 h at 32.5°C, this mixture was inoculated onto L-cell monolayers, and the resultant plaques were counted 48 h later. Antisera were scored as positive if a 30% reduction in PFU occurred at a dilution of 1:10. In the case of very active antisera (>90% plaque reduction), higher dilutions were used as required to achieve the appropriate endpoint (<90% plaque reduction). Rat sera were tested individually, but all mouse sera were pooled from three or more individuals involved in the same experiment and killed at the same time. Some of the pooled mouse sera diluted 1:10 with CMEM were also screened for interferon (IF) activity. Monolayers of 17CL-1 cells (obtained from L. S. Sturman, Division of Laboratories and Research, New York State Department of Health, Albany) were incubated for 24 h in medium containing the test serum and then were inoculated with approximately 100 PFU of vesicular stomatitis virus, Indiana strain, in CMEM containing 0.7% methylcellulose. Sera which failed to reduce vesicular stomatitis virus titers were scored as negative for IF activity. To monitor the sensitivity of the IF assay, control 17CL-1 cell monolayers were treated with a dilution series of a standard mouse IF preparation (provided by the National Institutes of Health, Bethesda, Md.) containing 12,000 U of IF per ml. This assay could detect a minimum of 5 U of IF per ml.

RESULTS

Genetic influence on disease products by JHMV in rats. In an effort to expand our examination of the relationship between age at inoculation and development of the neurological disease produced by JHMV, three inbred strains of rats were injected i.c. at 10, 15, 21, or 30 days of age. The data reveal that F344 and W/L rats were resistant to the development of fatal encephalitis earlier than were W/F rats (Table 1). F344 rats inoculated at 10 or 15 days of age and most W/L rats inoculated at 10 to 30 days of age survived inoculation of the virus. The exception was 1 of the 10 W/L rats inoculated at 10 days of age. This animal developed posterior paralysis and died 15 days p.i. Histological examination of this animal revealed demyelinated lesions of the pons and spinal cord. In contrast, W/F rats remained susceptible to disease at 10 and 15 days of age, and in one instance a rat of this strain inoculated at 21 days of age was affected. In the W/F rats inoculated at 10, 15, or 21 days of age in which disease symptoms became manifested gradually, paralysis occurred before sacrifice in extremis. Histopathological examination revealed that such paralyzed animals had developed demyelinating lesions identical to those previously described (27).

To determine whether the neurological disease seen in rats inoculated i.c. with JHMV was genetically controlled, an F1 cross was produced between male W/F and female W/L rats. Of the 47 pups inoculated at 10 days of age, only 6 developed disease (Table 1). The observed low incidence of disease in crossbred rats was about the same as that of W/L rats challenged at 10 days of age, implying that the gene(s) for resistance to JHMV is dominant.

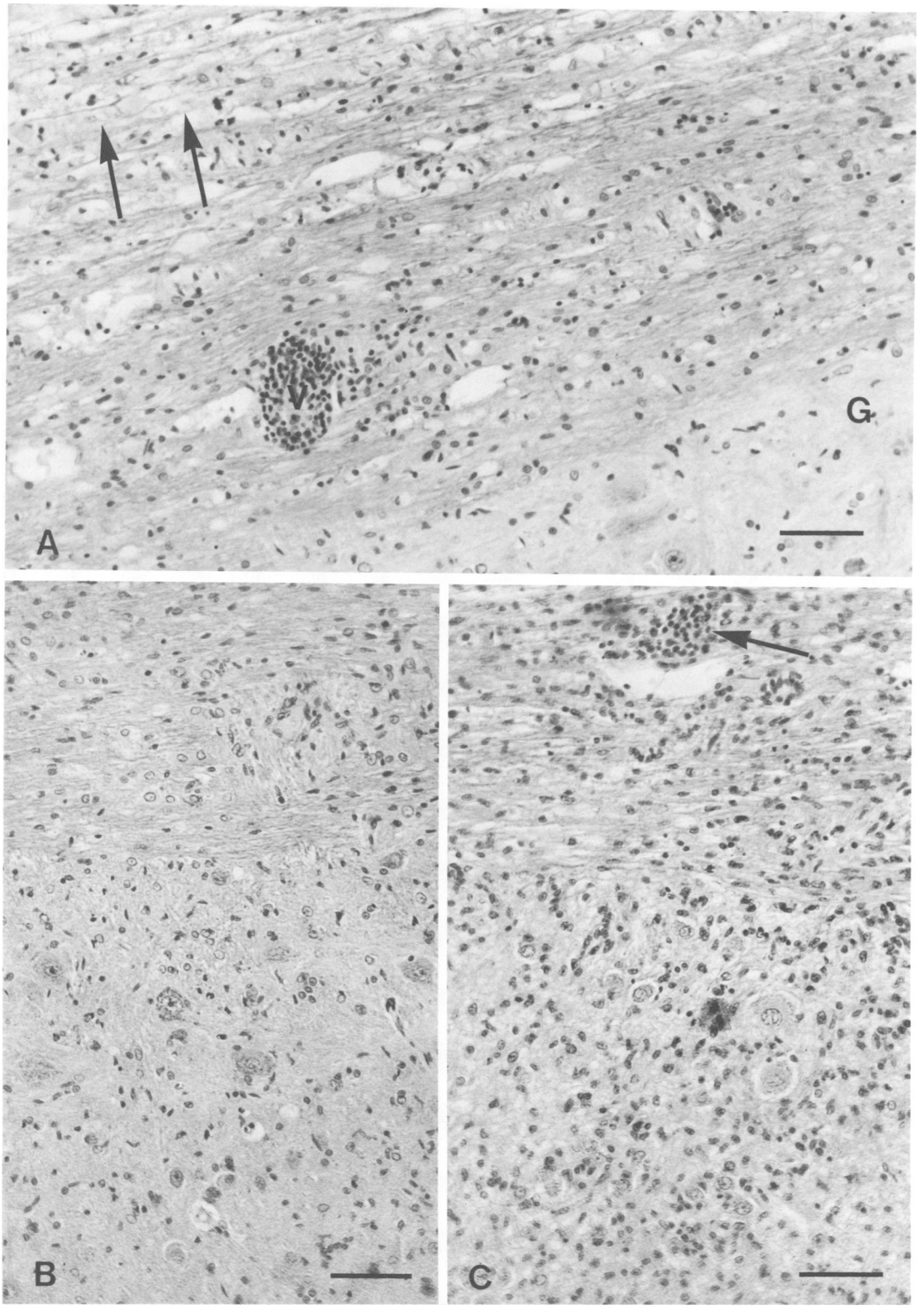

FIG. 1. Longitudinal sections of spinal cord from rats inoculated with JHMV at 5 days of age and sacrificed 110 days p.i. (A) or 47 days p.i. and 19 days after initiating cyclophosphamide treatment (B and C). In (A), note the white matter vacuolation (arrows), perivascular cuffing at the vessel (V), and lack of grey matter involvement (G). In (B), an apparently normal area illustrates organization of compact myelin in the upper half and unaffected grey matter in the lower half. In (C), a pathological focus from the same spinal cord as in (B) illustrates vacuolation and leukocytic infiltration within the white matter (arrow) and spongiform appearance of the grey matter. Bars = 0.35 mm.

Effects of immunosuppression on inoculated rats. The relatively high resistance of F344 and W/L rats to JHMV raised the possibility that the basis of increased resistance was due to the more efficient elimination of the inoculation virus. One approach to unmasking latent or persistent virus in the CNS of clinically normal rats is to induce immunosuppression. The high morbidity observed with W/F rats after i.c. challenge with JHMV made this the appropriate test strain. Thus, nine clinically normal W/F rats surviving the i.c. inoculation were treated for 14 days with cyclophosphamide beginning at 28 days p.i. In the period from 7 to 19 days after the initial cyclophosphamide injection, eight animals developed neurological signs, including incoordination, ataxia, tremors, and paresis or paralysis. Of the eight affected animals, two recovered completely by day 40 after the initial cyclophosphamide injection. Among the remaining six rats which survived for 40 to 55 days p.i., there were focal lesions in both the white and the grey matter of the brain and spinal cord. Lesions were characterized by mononuclear cell infiltration involving both the white and the grey matter (Fig. 1C). In previous experiments (27), rats developing fatal paralytic disease later than 21 days p.i. did not have grey matter involvement but had exclusively demyelinating lesions as in Fig. 1A.

Effectiveness of the immunosuppression regimen was confirmed by the absence of antibody responses to i.p. injection of SRBCs. Temporary abrogation of the immune response was demonstrated by the inability of sera from cyclophosphamide-treated animals to hemagglutinate SRBCs. However, rats immunized with SRBCs 5 weeks after the last cyclophosphamide infection did produce hemagglutinating antibodies with titers as high as those observed in untreated animals (data not shown).

Recovery of virus from infected rat CNS. Some rats inoculated i.c. with JHMV were without clinical symptoms of infection when killed up to 20 days p.i. However, JHMV was isolated from the brain tissue of all rats tested up to day 10 p.i. After longer incubation p.i., virus was isolated from the brains of rats only when the animals showed overt manifestations of neurological disease, in one case at 108 days p.i. Virus recovery was also made from the CNS of an affected rat after immunosuppression with cyclophosphamide. Tests showed that the isolated agent was indistinguishable from JHMV in 28-day-old C57BL6/J mice by morphology, in vitro cytopathology, or disease. These observations indicate that the virus can persist in the CNS for long periods after inoculation and in a small proportion of rats may cause demyelinating disease. In most rats made immunodeficient, CNS lesions were more extensive than previously observed and involved both grey matter lesions and demyelination.

Neutralizing antibodies in rats. Humoral immune responses were evaluated in sera from uninfected and JHMV-infected rats. All uninoculated animals tested possessed low levels of endogenous anti-JHMV antibodies, with the majority having endpoints (<90% plaque reduction) at dilutions of less than 1:10 (Table 2). This was interpreted to be due to the presence of antibodies to rat coronavirus, either sialodacryoadenitis virus or Parker's rat coronavirus. Indeed, clinical symptoms of sialodacryoadenitis virus infections were occasionally noted in both breeding and experimental animals. The cross-reactivity of this virus with viruses of the MHV group has been well documented (21). In general, serum antibody levels were not elevated after i.c. or i.p. inoculation of JHMV (Table 2). Only 2 of 101 rats tested displayed titers higher than those noted among uninoculated controls, and these had relatively low titers (1:50 or 1:100) (Table 2). The presence or absence of circulating JHMV-neutralizing antibodies thus did not appear to affect the course of the disease observed in rats since survival was not correlated with an antibody response to the virus.

In general, the outcome of MHV infection in rats appears to be determined by the genetic

TABLE 2. Serum antibody levels in JHMV-infected rats

Animals sampled for serum	No. of positive serum samples at endpoint dilution of[a]:				
	<1:10	1:10	1:25	1:50	1:100
Uninoculated controls, bled at various ages	26	2	1	0	0
Inoculated i.c., bled at various ages; no disease symptoms	71	10	1	1	1
Inoculated i.c., killed in extremis; disease evident at 1–21 days p.i.	3	1	0	0	0
Inoculated i.c., killed in extremis; disease evident at 21 days p.i.	1	0	0	0	0
Inoculated i.c., immunosuppressed, killed in extremis; disease evident	3	1	0	0	0
Inoculated i.c., killed after recovery from posterior paralysis	1	0	0	0	0
Inoculated i.p., bled at various ages; no disease evident	4	0	0	0	0

[a] Highest dilution of serum reducing JHMV plaque count by >90%. Sera unable to reduce JHMV plaque count by 30% or more at a 1:10 dilution were deemed to be negative.

constitution of the infectious agent (either MHV-3 or JHMV) and the host, the route of inoculation, and the state of the immune system. Diseases of the CNS were produced only when JHMV, the neurotropic strain of MHV, was inoculated at relatively high concentrations by the i.c. route into newborn or suckling rats. Infection of the serologically related viscerotropic MHV-3 strain fails to produce overt disease symptoms (27). Among inbred rats tested, only the W/F strain was susceptible to i.c. inoculation beyond 10 days of age. Resistance or susceptibility appears to be genetically related and may be influenced or controlled by a functional immune system.

Influence of the mouse or virus strain and the route of inoculation on the disease process. Genetic control of susceptibility and age-dependent development of resistance to JHMV and MHV-3 has been well documented in mice (12, 14, 28, 39). The previous findings and observations made with rats prompted us to initiate comparative studies with adults (>10 weeks of age) from selected strains of inbred mice. Animals more than 10 weeks of age were inoculated i.c. or i.p. with JHMV or MHV-3 and observed for at least 1 year.

JHMV inoculation i.c. into the strains tested invariably produced a rapidly fatal encephalomyelitis. Histological examination revealed widespread lesions in the CNS, with the most striking damage in the grey matter of the cerebrum and in both grey and white matter of the rhombencephalon. Lesions were minimal in the spinal cord. Focal hepatic necrosis was evident in most strains, although the lesions were minimal in comparison with those caused by i.p. inoculation with MHV-3. Nude mice, however, displayed extensive hepatic lesions as well as CNS lesions.

When the inoculation was i.p. with the standard dose of 3×10^5 PFU of JHMV, the mortality rate among all strains tested, with the exception of nude mice, was very low (Table 3). F1 crosses between A/J and DBA/2 or C57BL6/J mice failed to develop neurological disease during an observation period of more than 1 year (Table 3). Nude mice, however, developed an acute fatal encephalomyelitis with focal hepatic necrosis after a relatively long incubation period (Table 3).

Among all strains tested, only nude mice were susceptible to infection with JHMV by i.n. inoculation (Table 3). The average survival time after exposure to virus i.n. was 9 days, an interval intermediate between that observed after i.c. infection (average, 5 days) and i.p. infection (Table 3). After i.n. inoculation, the grey matter of the olfactory bulbs and cerebrum was most severely damaged (Fig. 2A and B), whereas hepatic lesions (Fig. 2C) and demyelinated lesions were less pronounced.

TABLE 3. Mortality resulting from i.p. and i.n. inoculation of JHMV into different mouse strains

Strain	Age at inoculation (wk)	No. inoculated/ no. dead[a]	Mean survival time of affected mice[a] (days)
Inoculated i.p.			
A/J	11–12	10/2	11
C57BL6/J	11–12	19/0	>300
DBA/2	15–17	6/1	11
C57 beige	10–14	7/0	>300
Nude	Adult[b,c]	15/15	21
B6D2F1	11–12	17/1	10
B6AF1	11–15	20/1	13
(DBA/2 × A/J)F1	11–17	29/0	>300
Inoculated i.n.			
A/J	11–12	4/0	>300
DBA/2	15–17	4/0	>300
C57 beige	10–14	12/0	>300
Nude	Adult[b]	9/9	9
(DBA/2 × A/J)F1	11/17	12/0	>300

[a] Mice which died or were killed in extremis.
[b] Various ages >24 weeks.
[c] Histological examination revealed lesions in the liver in two of four mice, in CNS grey matter in four of five mice, and in white matter in three of five mice.

Contrary to the findings with rats, i.c. inoculation of MHV-3 into all mouse strains tested caused an acute, rapidly fatal encephalomyelitis with concurrent hepatic lesions. The mortality rate in all strains was 100%.

After i.p. injection of MHV-3, mice, including a significant fraction of B6AF1 mice, developed a fulminating fatal hepatitis (Table 4). Our data on hybrid mice are at variance with those reporting a complete absence of fatal hepatitis among B6AF1 hybrids (13). The majority of B6AF1 mice which did not develop hepatitis after i.p. inoculation with MHV-3 manifested a chronic, protracted disease of the CNS and survived on the average for 199 days p.i. (Table 4). A similar disease pattern occurred in (DBA/2 × A/J)F1 animals (Table 4). In contrast, B6D2F1 hybrid mice were highly susceptible to i.p. injection with MHV-3, and all animals died rapidly with hepatic disease (Table 4).

Among B6AF1 and (DBA/2 × A/J)F1 mice which developed delayed neurological symptoms, the disease was initially manifested at 4 to 10 months p.i. as ataxia, incoordination, paresis, and occasional paraplegia. Despite the progression of neurological symptoms, affected mice usually survived for several months after the onset of disease. As the disease progressed, the mice suffered a marked weight loss and fre-

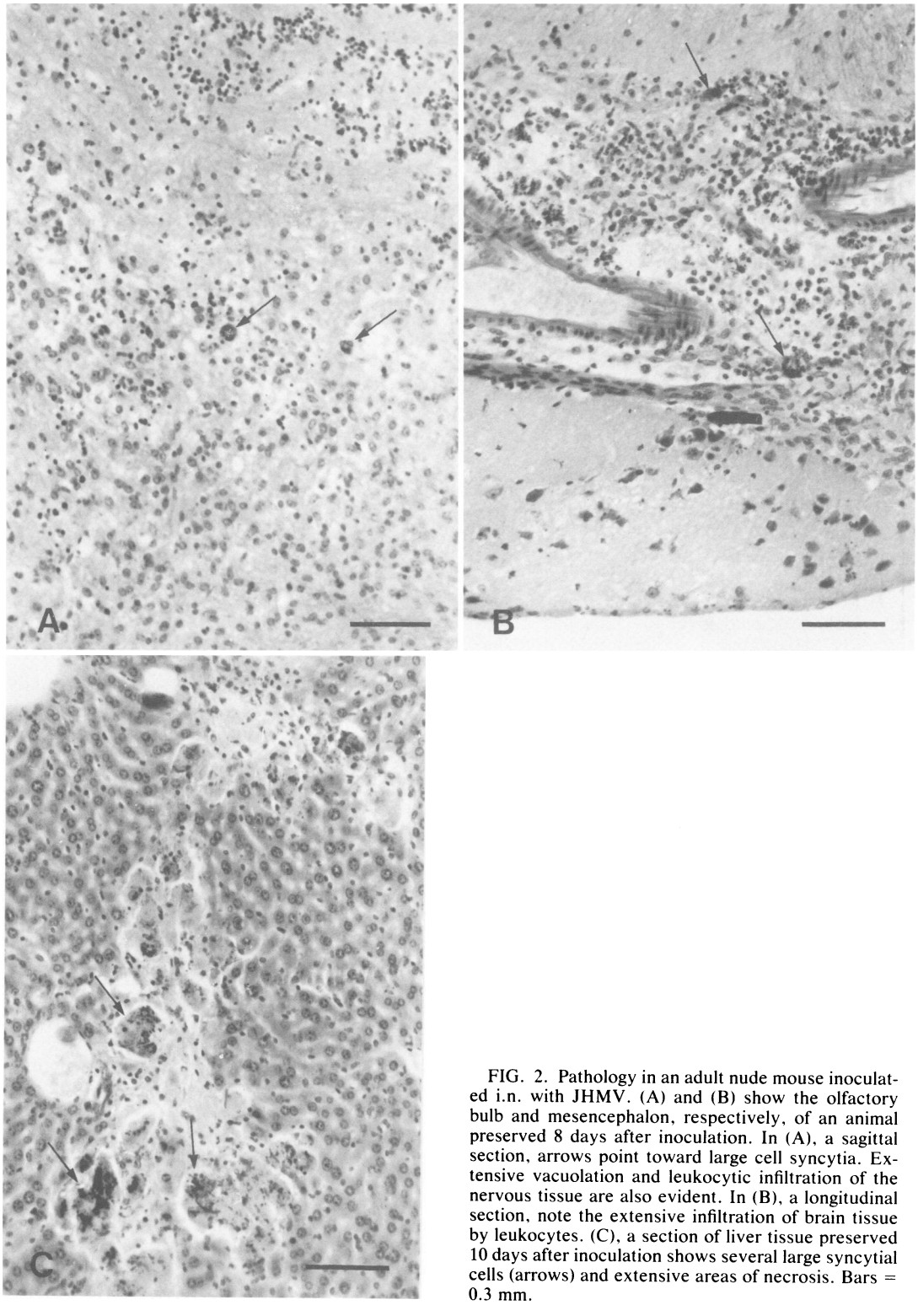

FIG. 2. Pathology in an adult nude mouse inoculated i.n. with JHMV. (A) and (B) show the olfactory bulb and mesencephalon, respectively, of an animal preserved 8 days after inoculation. In (A), a sagittal section, arrows point toward large cell syncytia. Extensive vacuolation and leukocytic infiltration of the nervous tissue are also evident. In (B), a longitudinal section, note the extensive infiltration of brain tissue by leukocytes. (C), a section of liver tissue preserved 10 days after inoculation shows several large syncytial cells (arrows) and extensive areas of necrosis. Bars = 0.3 mm.

TABLE 4. Pathology and mortality resulting from i.p. inoculation of MHV-3 into different mouse strains

Strain	Age at inoculation (wk)	No. inoculated/ no. dead[a]	Mean survival time of affected mice (days)	No. with lesions/total, based on histological evidence		
				Liver	CNS	
					Grey matter	White matter
A/J	12	10/1	11	ND[b]	ND	ND
C57BL6/J	11–12	19/19	4	4/4	0/4	0/4
DBA/2	15	5/4	5	2/2	1/2	0/2
C57 beige	10–14	6/6	6	2/2	1/2	0/2
Nude	Adult[c]	5/5	4	2/2	0/2	0/2
B6D2F1	11	9/9	5	3/3	0/3	0/3
B6AF1	12–15	19/6[d]	7	ND	ND	ND
		/6[e]	199	0/1	1/1	0/1
(DBA/2 × A/J)F1	11–17	25/1[d]	5	1/1	0/1	0/1
		/14[e]	268	0/3	3/3	3/3

[a] Mice which died or were killed in extremis.
[b] ND, Not determined.
[c] Nude mice of various ages >24 weeks.
[d] Death occurring within 14 days p.i.
[e] Death occurring later than 14 days p.i.

quently developed prominent domelike protrusions of the calvarium, indicative of hydrocephalus (Fig. 3A). Hydrocephalus was prominent at the time of death in 4 of 6 B6AF1 mice and 8 of 14 (DBA/2 × A/J)F1 mice displaying chronic neurological symptoms. Examination of brain sections revealed that, in general, the dilation involved the third, fourth, and lateral ventricles (Fig. 3A). Examination by light and electron microscopy showed that cells of the ependyma and choroid plexus retained their normal architecture, although obvious compression of the tissue had occurred. Perivascular cuffs and necrotic lesions, including foci of demyelination, were evident in the prosencephalon, rhombencephalon, and spinal cord. In some mice there was a concurrent necrotizing granulomatous vasculitis involving vessels in the neuropil and the leptomeninges of the brain and spinal cord (Fig 3B). Infectious MHV-3 was isolated from the CNS of each of the four chronically affected animals tested.

Virus replication in mouse macrophages. To confirm that the basis of resistance to infection i.p. with JHMV in mice was related to the capacity of the virus to replicate in PM, these cells were taken either from uninfected mice and infected in vitro or from mice 24 h after i.p. infection. Criteria used for characterizing the PM and methods for infecting them were similar to those previously described (38). Data on the yield of virus in culture fluids (Table 5) indicated that the neurotropic strain can initiate infection in PM in vivo and can continue to replicate upon explantation. After inoculation of PM in vitro, JHMV and MHV-3 were both more restricted in PM from the resistant A/J mice than in PM from the susceptible C57BL6/J strain (Table 5). The in vitro data, especially those on MHV-3, support the hypothesis that susceptibility or resistance to i.p. infection is controlled by the efficiency of coronavirus replication in PM.

Protection of mice from CNS disease conferred by JHMV inoculation. Different genetically determined responses to MHV inoculation of rats and mice, documented in this and previous studies, led us to examine whether any other endogenous factors controlled susceptibility to the virus. Our approach was to challenge mice surviving after an initial JHMV infection i.p. by a second inoculation i.c. with a lethal dose of virus. A/J and C57BL6/J mice were reinoculated i.c. at specified intervals after the i.p. injection, and survivors were observed for an extended period. Both strains tested could be protected within 7 days by preinjection. However, the sparing effect became evident about 4 days sooner in C57BL6/J than in A/J mice (Table 6). Protection appeared to prevail for intervals of several weeks or more.

Rapid fatalities caused by MHV-3 administered i.p. made parallel studies with this virus strain impractical.

One parameter examined in connection with the onset of resistance described above was the concentration of IF in the circulation. Assays of sera did not reveal an increase in circulating IF levels. Thus, a more profound IF response by C57BL6/J mice does not account for resistance developing more rapidly in C57BL6/J than A/J mice. In fact, only 2 of 10 samples taken 1 day p.i. and 1 of 6 samples collected 2 days after i.p. injection had detectable IF activity. None of the 20 samples taken at 3, 7, or 14 days p.i. con-

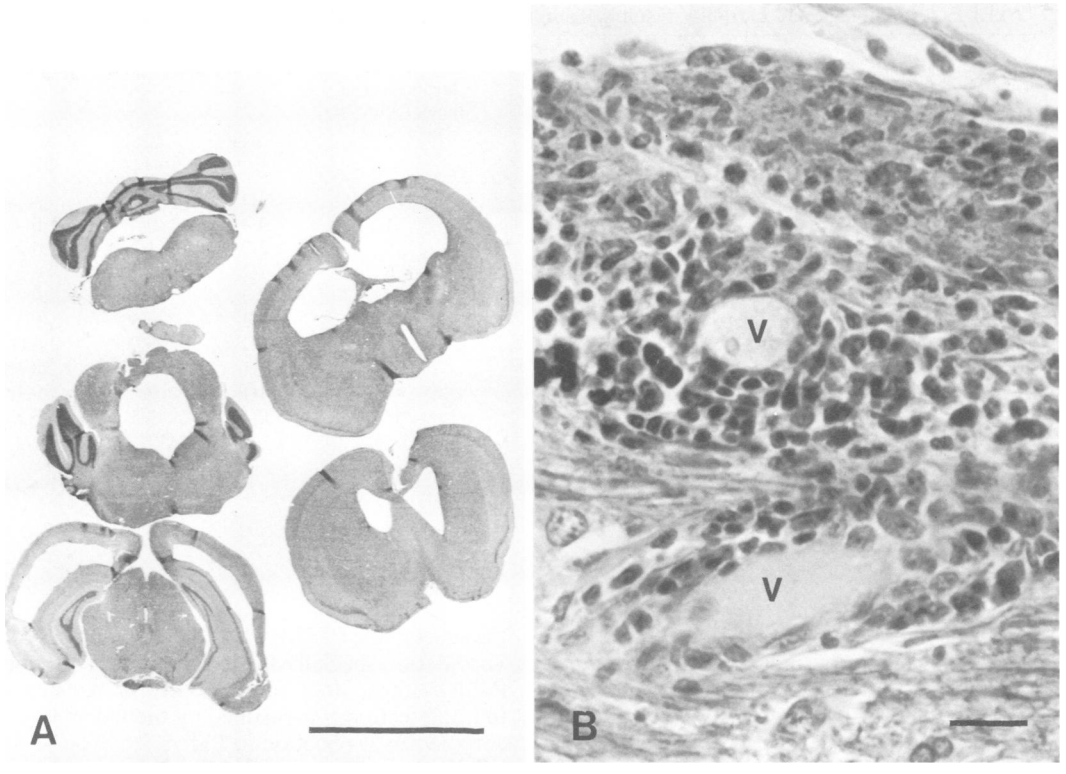

FIG. 3. Delayed hydrocephalus in (DBA/2 × A/J)F1 hybrid mice developing after i.p. inoculation with MHV-3. A coronal section through the brain of a mouse inoculated at 84 days of age and killed 285 days p.i. Note the extreme cavitation in the third, fourth, and lateral ventricles. B longitudinal section through the cervical spinal cord of another hydrocephalic mouse inoculated at 84 days of age, prepared 256 days p.i. Note the extensive granulomatous infiltration around vessels (V) in the meninges and spinal cord. Bars = 5 and 0.1 mm in (A) and (B), respectively.

tained detectable IF activity.

The neutralizing antibody data were somewhat ambiguous because, as in the case of rats (Table 2), both commercially acquired and laboratory-bred mice frequently had low levels of circulating anti-JHMV antibodies (Table 7). However, the seropositive uninoculated mice were infectable by either MHV-3 or JHMV (Tables 3 and 4), whereas those surviving a previous JHMV challenge were protected from a subsequent JHMV challenge (Table 6). The antigen inducing the preexisting antibodies remains unidentified. After i.p. inoculation, there was a trend toward increases in JHMV-neutralizing antibody titers (Table 7). Frequent sampling after the administration of JHMV i.p. showed that seroconversion was evident by day 7 p.i. in the strains tested (data not shown). However, effective protection to i.c. challenge occurred in C57BL6/J mice by day 3 (Table 6), implying that the development of resistance occurred by a mechanism other than the induction of antiviral antibodies.

TABLE 5. MHV replication in PM from A/J and C57BL6/J mice

Virus	Mouse strain	In vitro infection[a]	In vivo infection before isolation[b]
JHMV	C57BL6/J	1×10^5	2×10^4
	A/J	1.4×10^4	1.5×10^4
MHV-3	C57BL6/J	2×10^5	ND[c]
	A/J	3.5×10^3	ND

[a] PFU present in culture medium per milliliter 24 h after inoculation.

[b] PFU present in culture medium per milliliter 24 h after explanting the PM.

[c] ND, Not determined.

DISCUSSION

Heritable control of infection and disease. Our previous (27) and current data demonstrate a genetically conferred age-related susceptibility to neurological disease after i.c. inoculation of JHMV. If the neurological disease is manifested rapidly p.i., an acute fatal encephalomyelitis

TABLE 6. Mortality among different strains of mice inoculated i.p. with JHMV and challenged i.c. at intervals

Strain	Age at initial inoculation (wk)	Interval between i.p. and i.c. infection (days)	No. inoculated	No. dead
C57BL6/J	12	0	5	5
		1	5	5
		2	5	5
		3	5	1
		7	10	0
		14	5	0
		21	5	0
		42	5	2
		56	4	0
		>300	9	3
A/J	12	0	10	10
		1	5	5
		2	5	5
		3	5	5
		7	10	0
		14	5	0
		21	5	0
		42	5	0
	Adult[a]	0	5	5
		1	5	5
		2	5	5
		3	5	5
		7	5	0

[a] Various ages > 24 weeks.

develops, whereas progressive demyelination is associated with a chronic, progressive CNS disease (18, 27). Among the rats tested, most strains do not develop either type of disease if inoculated beyond 10 days of age (Table 1) (27). The more susceptible W/F strain is infectable in higher frequency and for a somewhat longer time after birth. To date, neither the number nor the chromosomal location of the gene(s) connected with control of sensitivity to JHMV has been established. It is known, however, that the major histocompatibility complex of the F344 and W/L strains differs from that of W/F, the former bearing H-1 haplotype 1 and Ag-B haplotype 1, whereas the latter has H-1 haplotype W and Ag-B haplotype 2 (5). It might be instructive to ascertain whether, in rats, H-1 and Ag-B haplotypes control susceptibility to JHMV. This could be tested by infecting Wistar Albino Glaxo, BN-BZ, Osborne-Mendel, or Louvain (5) rats since these strains bear the same H-1 and Ag-B haplotypes as the W/F strain.

Although resistance to JHMV infection i.c. is clearly dominant in rats, the number of genes influencing this trait is unknown because the appropriate breeding experiments involving backcrosses have not been performed. In contrast, age-related resistance of SJL mice to i.c. inoculation of JHMV has been subjected to more detailed crossbreeding and histopathological analysis (10, 28). These studies revealed that the efficiency of virus replication in the neurons of SJL mice is controlled by a single gene locus (10) or perhaps two loci, one of which may be *H-2* linked (28).

On the other hand, MHV-3 usually elicits a viscerotropic disease when inoculated i.p. into mice but evokes no disease in rats. Genetic control of susceptibility or resistance to coronaviruses is amply documented by previous (13, 15, 39) and current findings. Resistance in mice of the A strain, which develops at about 10 weeks of age (12), might be correlated with low efficiency of virus replication in PM. Among other strains, including C3H, A2G, and F1 crosses between resistant A and susceptible DBA/2 or C57BL6/J mice, partial age-related resistance also becomes evident by week 10 (14, 39). Levy-Leblond et al. (15) have suggested that both the severity and the type of disease produced by MHV-3 in mice are controlled by two host genes, of which the recessive confers susceptibility to hepatitis and the other, linked to *H-2*, influences the development of a chronic CNS syndrome.

Current data and those of others (14) indicate

TABLE 7. Relationship between route of inoculation and appearance of anti-JHMV neutralizing antibodies in mice[a]

Treatment of mice	No. of positive serum samples at endpoint dilution of[b]:				
	<1:100	1:100	1:500	1:1000	1:5000
Uninoculated	5	1	1	0[c]	0
Inoculated i.c.	2	0	0	0	0
Inoculated i.p.	10	5	2	4	1

[a] Each sample represents sera pooled from three or more mice of one of the strains (C57BL6/J, C57 beige, or A/J) without disease symptoms. Animals were sampled at various ages.

[b] Highest dilution of serum reducing JHMV plaque count by >90%. Antisera capable of reducing JHMV plaque count by >90% were deemed positive. Those unable to reduce JHMV plaque count by 30% or more at a 1:10 dilution were deemed to be negative.

[c] 0 indicates that none of the sera tested had this titer.

that B6AF1 and (DBA/2 × A/J)F1 hybrid mice succumb to the hepatic form of MHV-3 disease less frequently than do their susceptible parents. However, after a prolonged incubation period the hybrid mice frequently develop chronic neurological disease. Therefore, progeny from crossbred resistant and susceptible parents are, by 12 weeks of age, less susceptible to the viscerotropic effects of highly virulent MHV-3, but they tend to develop a chronic neurological disease. These findings emphasize the paramount role exercised by the genetic constitution of the host in controlling the type of disease caused by one subtype of MHV.

Observations on pathology. Since Bailey et al. (1) first described the neurological disease of rodents caused by JHMV, including demyelination, our own observations and those of others have revealed that certain similarities as well as important differences distinguish the diseases of rats from those of mice (14, 27, 30, 43). In rats, neurological disease follows one of two patterns regulated primarily by the genetic background and age of the animals rather than by the concentration of virus administered (27). The acute form of disease is associated with extensive destruction of grey matter and rapid killing. Among animals surviving beyond day 21 p.i., the lesions developing in the optic nerve, rhombencephalon, and spinal cord are confined primarily to the white matter (27). Foci of demyelination are probably caused by death of the oligodendrocytes, since JHMV has been demonstrated in these cells by electron microscopy and immunofluorescence (17, 27).

In mice, myelinoclasis also appears to result from virally caused damage to and death of the oligodendrocytes (11, 20). The nature of the disease may, in part, be related to the quantity of JHMV administered i.c. since high titers cause an acute, rapidly fatal encephalitis involving primarily the grey matter, whereas low titers provoke a more protracted encephalomyelitis characterized by damage to the white matter (21). In mice, MHV infections are not confined to the CNS. Viscerotropic strains such as MHV-3, when inoculated i.p., cause extensive lesions, particularly in the liver, but also in the spleen, thymus, and lymph nodes (40). In contrast, neither MHV-3 nor JHMV elicits any overt hepatic necrosis in rats (27). However, in chronic JHMV infections of rats, involution of the thymus and spleen may also occur (1; O. Sorensen, unpublished data).

Concerning age-related susceptiblity in rats, most strains are infectable i.c. with JHMV only during the first 2 weeks after birth, whereas mice remain susceptible for at least 1 year. Furthermore, i.p. infection of young mice with the neurotropic JHMV is possible (40), but rats appear to be completely resistant to inoculation by this route (27). Rats surviving more than 21 days after i.c. inoculation of JHMV frequently develop hind-limb paralysis but retain use of their forelimbs. In contrast, mice, after receiving JHMV i.c., seldom become chronically paralyzed but may exhibit progressive, irreversible neurological symptoms culminating in death. Of particular interest was the hydrocephalus which developed in F1 crosses between the C57BL6 or DBA/2 and the A strains which were chronically affected after the i.p. inoculation of MHV-3 (13). The pathogenesis of MHV-3-associated hydrocephalus in mice has not been determined. However, if the mechanism is due to impaired circulation of cerebrospinal fluid, the obstruction appears to occur distal to the fourth ventricle.

Immunological factors. Previous and current observations reveal the paramount importance of the immune system in controlling the outcome of MHV infections in rodents. In rats surviving JHMV infection beyond 30 days without overt symptoms, immunosuppression can exacerbate a latent infection and precipitate the associated fatal neurological disease. An analogous situation was shown here to occur with athymic nude mice in which clinical symptoms of CNS infection became evident, regardless of whether the JHMV inoculation was given i.p., i.c., or i.n. Likewise, thymectomy (25) and immunosuppression with cyclophosphamide (45), antilymphocyte serum (14), and X-irradiation (4) induce susceptibility to MHV among strains of mice naturally resistant to the virus. In the above experimental approaches, the T-cells appear to be a common factor controlling the outcome of the infectious process (14). A detailed future analysis of T-cell activities after infection of genetically defined rats and mice offers a useful approach for elucidating the role of cellular immunity in the control of CNS disease produced by MHV.

C57 beige (*bg/bg*) mice have a partial impairment of natural killer (NK) cell functions (2, 9, 16) without alteration in activities of their B- or T-cells involved in humoral or cellular immunity (21, 22, 23). The normal response to JHMV infection in these mice indicates that the lack of the killing activity of NK cells does not influence the outcome of CNS disease in mice. On the other hand, nude mice, possessing an elevated NK cell response (42), are highly susceptible to JHMV infection, as the present study demonstrates.

The onset of age-related resistance to MHV-3 is clearly evident in A/J mice, which remain infectable i.p. for about 10 weeks after birth. The capacity for conferring resistance to young A/J mice is associated with spleen cells of adults which, upon injection, provide protection

against MHV-3 (14, 35, 37). Such protection has been ascribed to the cotransfer of T-lymphocytes and an adherent spleen cell (14) and to an NK-like cell designated M (35). Data on C57 beige mice presented here suggest that if the M-cell is an NK cell, its killing function is not essential for its role in resistance.

Similar protection against i.c. infection with JHMV can be accomplished by transferring from adults to 6-week-old SJL mice the adherent spleen cells (29), again implying a protective function for macrophages. The role of macrophages in the control of coronavirus disease among resistant A/J and other mice may be related to inefficient replication and low virus yields demonstrated by in vitro and in vivo studies reported here and previously (2, 8, 26, 37, 38). Thus, production of limited quantities of virus by the initial target host cell might allow sufficient time for the induction of the appropriate immunological responses required for suppressing the infection. A correlation between the low rate of MHV production and the resistance of A/J mice to disease also holds in substrains of C3H mice infected i.p. with the PRI strain of MHV (26). The control of virus production in macrophages has been ascribed to soluble factors such as lymphokines (36) and could also involve hormonal regulation by the corticosteroids (44). It is not known whether the lymphokines are IF or IF-like substances.

As to the influence of humoral immunity, endemic infection of rats by rat coronavirus (3, 19), an agent serorelated to the MHV group (21), commonly occurs. Likewise, asymptomatic infections of laboratory rodents with MHV may occur (6, 31), making the interpretation of the role of circulating antibodies in the CNS disease pattern somewhat ambiguous. However, the current data provide no evidence to support the idea that neutralizing antibodies in the circulation in any way alter the disease process in the CNS of rats inoculated i.c. Also, mice first inoculated i.p. with JHMV are protected against an i.c. challenge before elevated humoral antibodies appear. These observations coincide with the findings on reinfection with another strain in this virus group, MHV-S, in mice as reported by Taguchi et al. (33), who ascribed the early protection to rapid sensitization of macrophages, the cell type shown to provide an initial defense in nude mice challenged i.p. with MHV (34).

One possible factor involved in early protection is IF. Evidence to this effect was provided by Verilizier and Gresser (40), who were able to suppress i.p. infection with MHV-3 by IF and to abrogate the inhibition with anti-IF antiserum. In contrast, our present analyses, albeit limited in scope, did not reveal elevation in circulating IF after i.p. inoculation of JHMV into mice, nor did they reveal any correlation between IF levels and the rapid onset of protection in C57BL6/J mice. Further analysis of this phenomenon would be appropriate to resolve the apparent discrepancy. In another study, Taguchi et al. (32) demonstrated a correlation in mice between age-dependent resistance to MHV-3 replication in PM and increased IF levels, a result consistent with the observed capacity of anti-IF antibodies to increase the severity of MHV-3 infection (40). However, since the natural resistance of A-strain mice to coronavirus infection could not be reversed by the injection of anti-IF antibody, the possibility remains that low levels of circulating IF during the chronic phase of infection by MHV-3 lead to the development of an immunosuppressed state (41), thereby increasing susceptibility to disease produced by the virus.

In summary, previous and current observations demonstrate that infection of rats and mice by MHV, producing a spectrum of CNS diseases, provides suitable models for continued investigation of the factors controlling latency, persistence, and demyelination. W/F rats appear to be particularly useful for investigating progressive demyelination, since with this strain a predictably high morbidity can be achieved. To achieve a better understanding of factors involved in the development of chronic progressive demyelination long after the initial infection has occurred, future studies might profitably focus on such parameters as the age-related and genetically controlled development of resistance to infection and the control of virus persistence in the CNS.

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