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Susceptibility of Inbred Mice to Chronic Central Nervous System Infection by Theiler's Murine Encephalomyelitis Virus

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The present study demonstrated that the clinicopathological expression of the late demyelinating disease due to chronic central nervous system infection by Theiler's mouse encephalomyelitis virus was dependent, at least in part, on the strain of mouse used as host. A range of involvement was observed, with late disease being most severe in the SJL strain, intermediate in the CBA and C3H/He strains, and least in C57BL/6 mice. The lack of clinical signs in seven other inbred strains of mice indicates that their response to chronic infection was similar to C57BL/6 mice. SJL, CBA, C3H/He, and C57BL/6 mice all generated similar levels of neutralizing antibody. A correlation between the severity of late disease and central nervous system virus content was not demonstrated, which indirectly suggests an immunopathological rather than a cytolytic mechanism of myelin injury during the late disease period. Finally, in addition to being more extensive, SJL demyelinating lesions contained a disproportionately large number of macrophages compared with those of similar lesions in CBA and C3H/He mice.

Theiler's murine encephalomyelitis virus (TMEV) is a natural enteric pathogen of mice which has been shown experimentally to produce chronic central nervous system (CNS) infection (5, 13). The first month after intracerebral (IC) inoculation of brain-derived DA virus, virus replication and poliomyelitis-like pathological changes take place in neuronal populations in CNS gray matter (early disease). After this time, surviving animals have persistent CNS infection, but the cell type(s) in the nervous system in which this virus replicates remains to be elucidated. From 1 to 6 months after inoculation, these chronically infected mice have mononuclear cell infiltrates in their leptomeninges and white matter and develop lesions of primary demyelination (late disease). Myelin breakdown in this infection appears to be immunopathological, since it can be prevented by immunosuppression (7).

Previous studies of the pathogenesis of this infection suggested that there are differences in the susceptibility between outbred and inbred mice to TMEV (5, 8). Since the genetic constitution of the host plays a critical role in the outcome of many acute virus infections, it would be important to determine whether the host influences the clinicopathological expression of TMEV infection. For this purpose, selected parameters of TMEV infection in outbred mice and in 11 strains of inbred mice were investigated. In particular, clinical neurological involvement, histopathological changes, virus replication, and the humoral immune response were examined in selected inbred strains of mice.

MATERIALS AND METHODS

Animals and animal inoculations. Outbred Swiss male mice (CD-1) were purchased from Charles River Breeding Laboratories, Portage, Mich., A.SW/SN mice of both sexes were obtained from R. Graff (Jewish Hospital of St. Louis, St. Louis, Mo.), and A.TL mice of both sexes were provided by P. Doherty (Wistar Institute, Philadelphia, Pa.). The other inbred strains of mice were males, and they were purchased from Jackson Laboratory, Bar Harbor, Maine. All of the mice were 4 to 8 weeks old, except the AKR and A.TL strains, which were 10 to 12 weeks old.

Mice were inoculated in the right hemisphere with 0.03 ml of virus. Titrations of brain-derived DA virus were performed, using five to eight mice per 10-fold dilution, and the 50% paralytic dose (PD_{50}) and 50% lethal dose (LD_{50}) end points per 0.03 g were calculated by the method of Reed and Muench (12).

Viruses. Brain-derived DA virus was prepared from the third suckling mouse passage as a 10% clarified suspension of brain in Hanks balanced salt solution. Cell culture-adapted DA virus was prepared as a thrice-plaque-purified stock in BHK21 cells (6).

Cells and media. BHK21 cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.1 mM L-glutamine, 100 μg of streptomycin, and 100 U of penicillin per ml and 10% heat-inactivated fetal calf serum.

The same medium containing 2% heat-inactivated fetal calf serum was used for cell maintenance.

Histology. Anesthetized mice were sacrificed by perfusion with either 10% buffered Formalin or 3% glutaraldehyde in phosphate buffer (pH 7.4). Spinal cords were fixed and embedded in paraffin or Epon, and sections were stained as previously described (3).

Virus assay. The virus content of clarified CNS homogenates was determined by standard plaque assay as previously described (10).

Neutralizing antibody. Twofold dilutions of heat-inactivated (56°C, 30 min) serum were incubated with approximately 100 plaque-forming units (PFU) of DA virus at 24°C for 60 min. Each mixture was assayed in duplicate on BHK cell monolayers, and neutralizing antibody was considered present if there was a 50% reduction in PFU compared with virus-diluent controls.

RESULTS

Clinical involvement. To determine whether the host influences the development of chronic CNS disease in TMEV infection, DA virus was titrated in 11 strains of inbred mice and in outbred Swiss mice. This investigation was initially undertaken by using brain-derived DA virus which produces a biphasic illness after IC inoculation. The PD_{50} during the early disease period was similar to the LD_{50} for all strains except AKR and SJL (Table 1). In the AKR and SJL strains, the amount of virus which produced 1 PD_{50} was 30 to 120 times less than the LD_{50}. All strains showed a similar susceptibility to early disease, with CBA, AKR, and SJL strains demonstrating somewhat greater resistance to lethal infection. The lack of more striking differences in LD_{50} and PD_{50} might be attributed to the fact that the DA virus used in these studies was of only moderate neurovirulence; the maximum LD_{50} was $10^{3.5}$/0.03 ml.

In prior studies where Swiss and SJL mice were observed for as long as a year, the clinical manifestations of late disease developed by 5 months (5, 8). For this reason, mice inoculated with tenfold dilutions (10^{-1} to 10^{-5}) of DA virus and surviving the early disease period were checked weekly up to 5 months for clinical signs of late disease. Affected mice with late disease become less active and have a spastic (waddling) gait, and when the cage is jarred they tend to lose their balance, developing prolonged extensor spasms of their limbs (8). Only outbred Swiss, C3H/He, CBA, and SJL mice showed clinical evidence of late disease (Table 1). Although only occasional surviving C3H/He mice developed these clinical signs, many of the surviving CBA and SJL mice developed late disease. In another titration, 6 of 9 CBA, 1 of 8 C3H/He, and 11 of 12 SJL mice surviving a dose of 0.1 to 1.0 LD_{50} of DA virus developed clinical late disease by 5 months postinfection. Late disease was always more severe in SJL mice.

Histopathology. Outbred Swiss mice and five strains of inbred mice were sacrificed for histology after the onset of late disease or at 5 months after inoculation. All strains of mice showed mononuclear cell infiltrates in their spinal cord leptomeninges and white matter, and frequently microcystic changes were seen in the white matter as well (Table 1). As expected, no gray matter involvement was observed at this

TABLE 1. *Susceptibility of adult mice to TMEV infection after IC inoculation of brain-derived DA virus*

Strain	*H-2*	Early disease[a]		Late disease[b]	
		LD_{50}	PD_{50}	Clinical expression	Pathological changes[c]
Swiss		1.0	1.0	+	+
A	*a*	3.4	4.2	0	+
C57BL/6	*b*	2.2	2.6	0	+
BALB/c	*d*	2.8		0	ND[d]
DBA/2	*d*	2.8	3.2	0	ND
C3H/He	*k*	3.5	3.5	+	+
CBA	*k*	<1.0	<1.0	+	+
AKR	*k*	1.4	3.5	0	ND
DBA/1	*q*	2.3	3.4	0	ND
SJL	*s*	1.8	3.2	+	+
A.SW/SN	*s*	3.0	>3.5	0	ND
A.TL	*tl*	>3.5	>3.5	0	+

[a] Reciprocal of $\log_{10}$ dilution giving LD_{50} and PD_{50} end points by day 30 per 0.03 ml inoculum. An animal was considered to be paralyzed if one or more limbs showed flaccid paralysis.

[b] Defined as the development of spastic paralysis between 30 and 150 days after IC inoculation.

[c] A plus in this column indicates the presence of leptomeningeal and white matter inflammation seen in paraffin-embedded material.

[d] ND, Not done.

time postinfection. Although the changes were clearly more severe in SJL mice, there was little difference in the pathological changes among the other strains of mice, judging from the paraffin-embedded material. The fact that not all of the animals were sacrificed at the same time precludes quantifying these pathological changes.

Therefore, 4- to 6-week-old inbred mice representing the differing clinical responses to late disease were inoculated IC with approximately one LD_{50} of brain-derived DA virus. At 3 months after inoculation, two to four mice of each strain were perfused with 3% glutaraldehyde, and spinal cord segments were embedded in Epon and stained with toluidine blue. This method is more sensitive for detecting demyelination than staining replicate sections of paraffin-embedded tissue with specific stains for myelin and axis cylinders. Obvious differences in the pathology were found in four inbred strains of mice (Table 2). The thoracic spinal cord segments were more severely involved than the cervical or lumbar regions; thus, these sections were used to quantify the lesions. SJL mice had the most marked involvement, with almost all thoracic sections showing prominent mononuclear cell infiltrates, macrophages, primary demyelination, and astrocytic gliosis in the anterior and lateral columns (Fig. 1). The majority of thoracic sections from C3H/He mice showed less extensive lesions in which there was only moderate inflammation and demyelination, and even less of a macrophage and glial response (Fig. 2). Only occasional thoracic cord sections from CBA mice showed involvement. These lesions were small, confined to a single column, and showed few macrophages and little gliosis (Fig. 3). In contrast to the other strains, C57BL/6 mouse spinal cord sections appeared to be normal (Fig. 4). The lack of changes in the Epon material from C57BL/6 mice while some mononuclear cells were seen in the paraffin sections is probably indicative of the fact that Epon sections are less sensitive in detecting pathological changes. Paraffin sections are thicker (6 versus 1 μm), and the spinal cord can be cut so that its entire length fits on one slide.

TABLE 2. *Light microscopy of Epon-embedded spinal cord material from inbred mice 3 months after IC inoculation of one LD_{50} of brain-derived DA virus*[a]

Strain	Inflammation		Demyelination	Gliosis
	Mononuclear cells[b]	Macrophages		
C57BL/6	0	0	0	0
C3H/He	++	+	++	+
CBA	+	±	+	±
SJL	+++	+++	+++	+++

[a] Histological changes were graded as follows: 0, none; ±, trace; +, mild; ++, moderate; +++, severe.
[b] Lymphocytes, plasma cells, and monocytes.

CNS virus content. To determine whether differences in clinicopathological expression of late disease can be correlated with virus replication, the CNS virus content of Swiss and four inbred strains of mice was determined. The mice were inoculated IC with approximately 10^4 PFU of tissue culture-adapted DA virus and sacrificed on day 21 for assay. Tissue culture-adapted DA virus was used, since brain-derived virus does not grow directly in cell culture. Day 21 is a time after an initial phase of logarithmic virus growth but at the beginning of the chronic stage of infection (5). There was considerable scatter in the virus titers in brains and spinal cords for the different strains of mice (Table 3). Except for CBA mice, mean titers were similar for all of these strains. Although CBA mice had a higher mean titer in the brain, the mean titer in the spinal cord was the same as for the other strains. Thus, there did not appear to be a correlation between the severity of late disease and the level of virus in the CNS.

Since these data represent only one point in time in a chronic infection, the temporal course of virus replication was studied. SJL and C57BL/6 mice which represent the extremes of late disease involvement were inoculated with approximately 10^4 PFU of tissue culture-adapted DA virus and sacrificed at the times indicated in Fig. 5. The growth of virus in both strains was similar until days 35 and 49. On these days, the SJL mean titers were one $\log_{10}$ greater than those of C57BL/6 mice; however, there was considerable scatter in individual titers, and the difference in mean virus titers on days 35 and 49 was not significant (Student's t test for unpaired samples; $P < 0.05$). Furthermore, on day 59 the virus titers were essentially the same in both strains of mice. Also (Fig. 5), 5 of 20 SJL mice and 6 of 15 C57BL/6 mice did not have detectable levels of virus during the chronic phase of infection (day 21 and later). This difference was not significant (chi-square test; $P < 0.05$). Therefore, although it would appear that somewhat higher amounts of virus are maintained in SJL CNS in the chronic phase of infection, this was not confirmed.

Serum neutralizing antibody titers to virus. Humoral immunity is considered to be more important than cellular immunity in defense against picornavirus infections (2, 11). Therefore, the same strains of inbred mice used for virus assay were examined to see whether they produced measurable levels of neutralizing antibody. All of these strains generated an antibody response to DA virus, and there was little

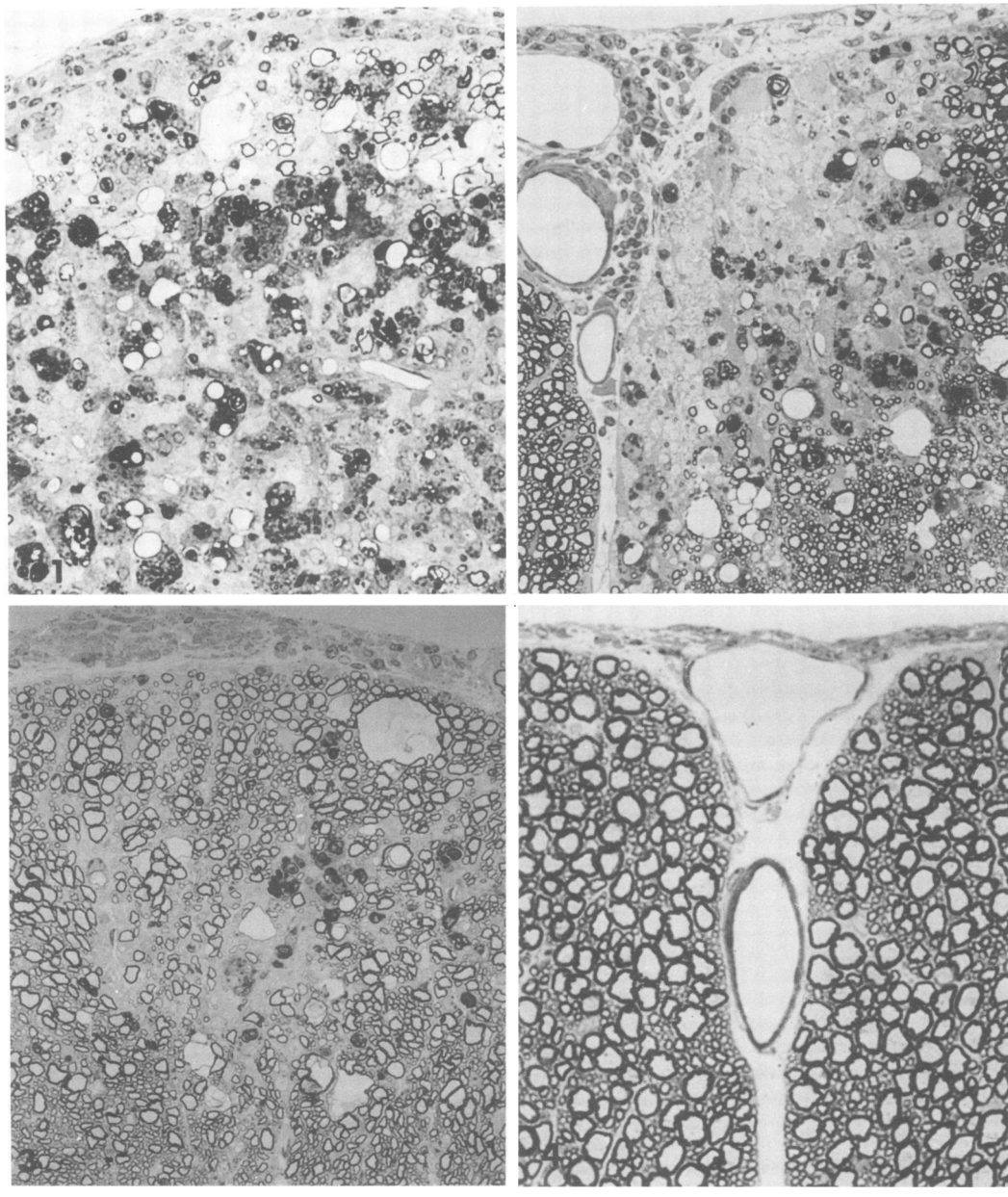

FIG. 1–4. *Epon-embedded thoracic spinal cord sections (1 μm thick) stained with toluidine blue from inbred mice sacrificed 3 months after IC inoculation with one LD_{50} of brain-derived DA virus.*

FIG. 1. *Anterior column from an SJL mouse, showing mononuclear cells in the leptomeninges and an extensive area of demyelination in the underlying white matter. Numerous naked axons and debris-laden macrophages are present. ×260.*

FIG. 2. *Anterior column from a C3H/He mouse, showing a demarcated plaque of demyelination in the white matter. Leptomeningeal inflammation, naked axons, and macrophages are present. ×300.*

FIG. 3. *Lateral column from a CBA mouse, showing some mononuclear cells in the leptomeninges and a small area of demyelination. ×255.*

FIG. 4. *Anterior columns of a C57BL/6 mouse, showing no abnormality. ×370.*

difference in the geometric mean titers (Table 4). The largest difference, between SJL and C57BL/6 mice, was not significant by the Student *t* test for unpaired samples. Therefore, the strain differences in the expression of late disease described above cannot be explained by a

TABLE 3. *CNS virus content on day 21 after IC inoculation of DA virus*

Strain	Virus titer[a]	
	Spinal cord	Brain
Swiss	2.8 (2.0–3.2)	2.1 (<2.0–2.7)
C57BL/6	2.3 (2.0–2.6)	2.8 (<2.0–3.3)
C3H/He	3.2 (2.3–3.7)	2.2 (2.0–2.3)
CBA	3.9 (2.3–4.4)	2.3 (<2.0–2.6)
SJL	2.7 (<2.0–3.1)	2.4 (<2.0–2.8)

[a] The mean virus titers, in $\log_{10}$ PFU per gram of tissue, for four animals per strain are given, with the range of values in parentheses. The level of sensitivity of the plaque assay was 100 PFU/g of tissue.

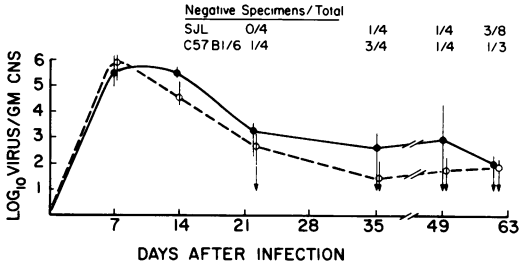

FIG. 5. *Growth of virus in the CNS (brainstem and spinal cord from each mouse combined) of 4- to 6-week-old SJL (●) and C57BL/6 (○) mice after IC inoculation of approximately 10^4 PFU of DA virus. Each vertical bar represents the range of titers for four specimens (except day 59), with the curve intersecting the mean values for each time. The level of sensitivity of the plaque assay was 100 PFU/g of CNS. Negative specimens are indicated by a downward pointing arrow on the vertical bars, and the number negative per total for each day is shown in the upper right.*

failure to generate an adequate humoral immune response.

DISCUSSION

The present study has demonstrated that the clinicopathological expression of late disease in mice due to TMEV infection is dependent at least in part on the strain of mouse used as host. A spectrum of involvement was observed, with late disease being most severe in the SJL strain, intermediate in C3H/He and CBA strains, and least in C57BL/6 mice (Tables 1 and 2). Although the CNS histopathology from the other inbred mice was either not studied or was only ascertained from paraffin-embedded material, their involvement may be similar to that of C57BL/6 mice, since they failed to develop clinical signs of late disease. It is of added interest that susceptibility to late disease could not be correlated with a particular *H-2* type (Table 1).

Initially it seemed possible that the more severe involvement and greater incidence of late disease in SJL mice was merely due to an increased rate of survival of these infected animals. It was shown that the amount of virus producing one PD_{50} was approximately 30 times less than the LD_{50} dose in SJL mice; therefore, in comparison with most of the other inbred strains, more SJL would survive and be subject to chronic infection. However, this does not appear to be the only factor responsible, since the PD_{50} was 100 times less than the LD_{50} in AKR mice, and none of the AKR mice developed clinical signs of late disease. Furthermore, when C57BL/6, C3H/He, CBA, and SJL mice were given the same amount of virus (one LD_{50}), pathological changes in the SJL strain were clearly more severe than in the other strains (Table 2). Therefore, SJL mice do appear to be more susceptible and to develop more severe clinical manifestations of late disease.

A correlation between the severity of late disease in inbred mice and CNS virus content was not demonstrated in the present study (Table 3, Fig. 5). This observation needs further confirmation and is currently under investigation as part of an immunogenetic analysis of the susceptibility of SJL and C57BL/6 mice to TMEV infection (unpublished data). The failure to document that SJL mice have higher levels of virus in their CNS than C57BL/6 mice during the chronic phase of this infection would favor an immunopathological rather than a cytolytic mechanism of myelin injury. Previously, we showed that myelin destruction was prevented by immunosuppression, which also suggested an immunopathological mechanism of demyelination (7). Because of the lack of viral antigen in white matter during late disease, as shown by fluorescent-antibody staining (5), it was proposed that myelin damage may be a nonspecific consequence of the interaction between (i) antibodies, or (ii) sensitized lymphoid cells and virus, or both i and ii. In this circumstance, the immune response directed to extracellular virus may inadvertently damage myelin membranes

TABLE 4. *Neutralizing antibodies in inbred mice inoculated IC with tissue culture-adapted DA virus*

Strain	Antibody titer[a]	
	Expt 1	Expt 2
C3H/He	512 (256–1024)	—
CBA	344 (16–1024)	—
SJL	460 (64–1024)	656 (64–1024)
C57BL/6	—	160 (64–256)

[a] Mean antibody titers (range in parentheses) for experiment 1 represent six mice of each strain sacrificed on day 42, and for experiment 2 represent four mice of each strain sacrificed on day 35. The titers are expressed as the reciprocal of the dilution inhibiting 50% of virus plaques. —, Not done.

that are in close proximity ("bystander effect").

It is interesting that the demyelinating lesions in infected SJL mice appeared to contain a disproportionately large number of macrophages in comparison with similar lesions in the other inbred mice that were studied (Table 2). This observation has been consistent but hard to accurately quantitate. Since some of the macrophages contain myelin debris (3), it might be assumed that they are merely performing a phagocytic function. However, this does not explain their apparent excess in SJL lesions, and it is worth considering whether macrophages may play an effector role in damaging myelin in this infection. In this connection, it is intriguing that SJL mice have been reported to possess endogenously hyperactive macrophages (R. Gallily and N. Haran-Ghera, Fed. Proc. **31**:1841, 1978). Furthermore, Cammer et al. (1) have shown that myelin basic protein and, to a lesser extent, other myelin proteins are degraded by at least two proteolytic activities secreted by stimulated macrophages. Plasminogen activator is one of several neutral proteases secreted by activated macrophages (4), and one of the proteolytic activities was found to be plasminogen dependent. Cammer and co-workers (1), therefore, postulated that the release of neutral proteases by activated macrophages with amplification by the plasminogen-plasmin system may play a significant role in myelin breakdown in inflammatory demyelinating diseases. This function of a macrophage is probably nonspecific; however, macrophage recruitment and activation no doubt would have immunological specificity as it does in the killing of certain microbial organisms and tumor cells (9).

The differences in susceptibility of inbred mice to late disease demonstrated in this study has provided initial information needed for investigating the immunogenetics of this chronic infection and an impetus for probing the role, if any, of macrophages in demyelination in TMEV infection.

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