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Demyelination induced by murine hepatitis virus JHM strain (MHV-4) is immunologically mediated

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Summary

The neurotropic mouse hepatitis viruses (MHV), in particular strain JHM (JHMV or MHV-4), cause experimental central nervous system demyelination that pathologically resembles multiple sclerosis, an important human demyelinating disease. The mechanism of JHMV-induced demyelination remains unclear, though its tropism for oligodendrocytes had led to the belief that JHMV causes demyelination by direct lysis of these myelin-producing cells. However, several studies have also implicated the involvement of immune responses in the demyelinating process. In this communication, we present evidence that generalized immunosuppression with gamma irradiation prevents JHMV-induced demyelination, a finding that was not limited to a particular strain of JHMV or to one strain of mouse. In addition, significant paralytic-demyelinating disease was restored to infected, irradiated mice after the adoptive transfer of nylon wool nonadherent splenic cells and appeared to be restricted by the major histocompatibility complex (MHC). These observations indicate that the principal mechanisms of JHMV-induced demyelination are most likely immunopathological.

Introduction

The murine coronavirus JHMV, also designated MHV-4, was originally isolated from mice which spontaneously developed hindlimb paralysis and demyelination (Bailey et al., 1949; Cheever et al., 1949). Subsequently, JHMV infection of rodents has been one of several model diseases which has been used to study demyelination experimentally. These models include experimental allergic encephalomyelitis (EAE) and infection by Theiler's virus, canine distemper virus, Semliki forest virus, A59 coronavirus, visna virus, and others (reviewed by Raine, 1984; Martin and Nathanson, 1979; Dal Canto and Rabinowitz, 1982; Stohlman and Kuywa, 1990).

For the viral models of experimental demyelination, Rodriguez (1988) has outlined a useful classification of the mechanisms by which viral infection may lead to myelin destruction. Within this classification, JHMV-induced demyelination has usually been considered to fall into the cate-
gory of direct viral cytopathic effect on oligo-
dendrocytes, the cells which produce and maintain
myelin; in this mechanism, the immune system
plays no role or merely has a scavenging function.
This hypothesis is supported by findings that
JHMV replicates and causes acute cytopathology
in oligodendrocytes (Lampert et al., 1973; Weiner,
1973; Powell and Lampert, 1975; Fleury et al.,
1980), as well as by several reports that animals
which are either immunosuppressed or immu-
nodeficient nonetheless develop JHMV-in-
duced demyelination to a variable degree (Sorensen et al., 1982, 1987). On the other hand, studies
have shown that MHV may elicit a variety of
potentially immunopathological responses, includ-
ing alteration in major histocompatibility complex
(MHC) antigen expression (Massa et al., 1986;
Suzumura et al., 1986), reactivity to myelin basic
protein (Watanabe et al., 1983), anti-viral antibod-
ies (Fleming et al., 1983) and anti-viral T cells
(Sussman et al., 1989). Despite the evidence show-
ing that JHMV is capable of causing (1) direct
cytopathology and (2) potentially immunopatho-
logical responses, controversy remains about the
extent to which either of these processes actually predominates in vivo.

Two approaches were taken to directly study
the role of the immune system in the JHMV-in-
duced demyelination. First, infected animals were
immunosuppressed by gamma irradiation early in
the disease course. Second, immunosuppressed mice were reconstituted by the adoptive transfer
of spleen cells from immune donors. These experi-
ments revealed that JHMV-induced demyelination
is prevented by gamma irradiation and partially
restored by the transfer of immune splenocytes,
providing direct evidence for a central role of the
immune system during JHMV-induced demyelina-
tion.

Materials and methods

Animals and viruses

Six-week-old male C57BL/6J and BALB/cJ
mice were obtained from Jackson Laboratories
(Bar Harbor, ME, U.S.A.). Mice were held for
48–72 h before intracerebral (i.c.) infection or
intraperitoneal (i.p.) immunization with virus. All
representative mice bled and tested by enzyme-
linked immunosorbent assay (ELISA; Fleming and
Pen, 1988) for antibodies to murine coronaviruses
were seronegative.

The isolation and characterization of JHMV
antigenic variant 2.2-V-1 (Fleming et al., 1986)
and a small plaque variant JHMV-DS (Stohlman
et al., 1982) have been described previously.
Viruses were propagated under serum-free condi-
tions and quantitated by plaque assay on DBT
cells (Stohlman and Weiner, 1978). Prior to inoc-
ulation, viruses were diluted in Dulbecco's minimal
essential medium. Mice were injected either i.c.
with 30 μl containing 10³ plaque-forming units
(PFU) of virus or i.p. with 0.5 ml containing 10⁶
PFU of virus. Infectious virus titers in brain ho-
logenates were measured on L2 cells and by
infectious center assays as previously described
(Stohlman and Weiner, 1978).

Gamma irradiation

Mice were irradiated with a ¹³⁷Cs gamma verti-
ical beam source at 150 rad/min (Gamma Cell 40,
Atomic Energy of Canada) under the experimen-
tal conditions noted below. In experiments con-
ducted to compare the effects of irradiation of the
central nervous system (CNS) with those of sys-
temic compartments, mice were anesthetized using
pentobarbital (75 mg/kg; i.p.), placed in a plastic
restrainer, and protected by 25 mm thick lead
shields introduced above and below the mice
longitudinally to either cover the CNS (systemic
exposure; 4 mm dorsal shield) or the non-CNS,
 systemic areas (CNS exposure; ventral shield to
within 4 mm of the back). Mock experiments and
dissections confirmed that differential irradiation
of the CNS (brain and spinal cord) or systemic
compartments (including spleen, lymph nodes, and
bone marrow) was achieved under these shielding
conditions.

Adoptive transfers

Donor mice were either not immunized (naive
donors) or immunized i.p. with approximately 10⁶
PFU of virus (immune donors) at 6 days prior to
adoptive transfer. Single-cell suspensions were
prepared from spleens of donor mice, and 5 × 10⁷
cells were injected intravenously (i.v.) into recipi-
ent mice which had received 850 rad of irradiation
Statistical analyses

Both clinical and histological scores were compared for statistical significance using the Mann-Whitney test for nonparametric samples (Statsoft statistical programs, Tulsa, OK, U.S.A.). A probability (p) ≤ 0.05 by this test was considered significant. In instances where observers disagreed, the histological score assigned was either the lower grade (one-step disagreement, n = 31/90 observations) or intermediate grade (two-step disagreement, n = 2/90 observations).

Results

Whole body irradiation

To study the immunosuppressive effects of irradiation, different amounts of whole body gamma irradiation were administered daily throughout the disease course in C57BL/6J mice after i.c. infection with JHMV variant 2.2-V-1. Irradiation dramatically reduced paralytic-demyelinating disease if 850 rad were administered at day 6 p.i. or earlier. At day 7 p.i. or later, however, disease was unaffected by as much as 1250 rad. Based on these data, we chose to administer 850 rad on day 6 p.i. in subsequent experiments in which clinical and histological diseases were monitored quantitatively.

Severe paralysis and demyelination were evident by day 12 p.i. in infected, untreated mice (Table 1, group 1) as reported previously (Fleming et al., 1986). Fig. 1 shows several histological features of the demyelinating lesions observed in group 1, including inflammatory hypercellularity (A, B, C, E), scanty viral antigen and the presence of naked axons (D). In contrast, infected mice irradiated at day 6 p.i. had a marked reduction in both paralysis and demyelination (Table 1, group 2; Fig. 2B). Irradiation given to uninfected, naive mice had no demonstrable clinical or histological effect (data not shown). To determine if the diminution in disease could be attributed to the inhibition of virus replication by irradiation, the virus titer in brains of irradiated (850 rad) and control mice were compared. Fig. 3 shows that the virus titer in the irradiated mice exceeded that of the unirradiated controls. These data indicate that the absence of disease was not due to an inhibi-
TABLE 1
IRRADIATION OF MICE PREVENTS JHMV-INDUCED PARALYTIC-Demyelinating Disease

| Group | Conditions | Clinical score c | Histological score c |
|-------|------------|-----------------|---------------------|
|       | Virus a    | Irradiation b   | Mean n              | Mean n              |
| 1     | +          | -               | 3.16 ± 0.69         | 0.69 ± 0.67         |
| 2     | +          | +               | 0.96 ± 1.06         | 0.64 ± 0.67         |

a Six-week-old male C57BL/6J mice were given $10^3$ PFU of JHMV 2.2-V-1 i.c. on day 0, as indicated by '+' sign.
b 850 rad of whole body irradiation were given at day 6 p.i., as indicated by '+' sign.
c Clinical observations and blinded histological evaluations were performed as indicated in Materials and Methods (0–4 scales, with grade 4 being maximal disease). The mean, standard deviation, and number (n) of mice tested are shown. Underlined values indicate a statistically significant difference ($p < 0.05$) between groups 1 and 2 as determined by the Mann-Whitney test for nonparametric samples.

Differential irradiation. In addition, immunohistochemical studies showed that following 2.2-V-1 challenge, viral antigen was scanty or absent in unirradiated mice (Fig. 1D) but was very abundant in irradiated mice (Fig. 2A). Many of the antigen-positive cells in irradiated, infected mice appeared to be oligodendrocytes (Fig. 2A); surprisingly, these cells showed few or no morphological abnormalities.

In control experiments paralleling those shown in Table 1, mice were infected with a second JHMV strain, JHMV-DS (Stohlman et al., 1982), and were either irradiated or not irradiated. Unirradiated mice demonstrated intense disease at day 9 p.i. By contrast, mice given 850 rad at day 6 p.i. had few histological changes at day 9 p.i. In addition, a second mouse strain, BALB/cJ mice infected with $10^3$ PFU of 2.2-V-1 also developed severe paralytic-demyelinating disease by day 12 p.i. Disease was also prevented in these mice by 850 rad of whole body irradiation at day 6 p.i. (data not shown). These findings are essentially identical to those obtained in C57BL/6J mice infected with 2.2-V-1, suggesting that abrogation of demyelination by irradiation is not dependent on an unusual characteristic of a particular JHMV strain or mouse strain.

Differential irradiation

In view of the finding that whole body irradiation at day 6 p.i. prevents JHMV-induced paralytic-demyelinating disease, differential irradiation studies were conducted to determine whether critical radiosensitive targets reside in the systemic or

TABLE 2
ADOPTIVE TRANSFER OF SPLENOCYTES PARTIALLY RESTORES PARALYTIC-DMYELINATING DISEASE TO JHMV-INFECTED, IRRADIATED MICE

| Group | Conditions | Clinical score c | Histological score c |
|-------|------------|-----------------|---------------------|
|       | Donor cells a | Recipient mice b | Mean n              | Mean n              |
| 3     | Immune     | Virus           | 2.17 ± 0.99         | 1.67 ± 1.41         |
| 4     | Naive      | Virus           | 2.10 ± 0.97         | 1.13 ± 1.41         |
| 5     | Immune     | Naive           | 0.12 ± 0.35         | 0.00 ± 0.00         |

a Immune donor mice were 6-week-old C57BL/6J males given $10^6$ PFU of JHMV 2.2-V-1 i.p. 6 days prior to transfer. Naive donors were identical mice not given virus. In each group, $5 \times 10^7$ spleen cells were transferred i.v. into recipient mice on day 6 p.i.
b Recipient mice were 6-week-old C57BL/6J male inoculated (Virus) or not (Naive) with $10^3$ PFU of JHMV 2.2-V-1 i.c. on day 0. All recipient mice were given 850 rad prior to adoptive transfer on day 6 p.i.
c Scoring was performed as indicated in Table 1. Underlined values are clinical or histological scores which are significantly greater than respective scores of JHMV-infected, irradiated mice not given splenocytes (Table 1, group 2) ($p < 0.05$ by the Mann-Whitney test). Italicized value is of borderline significance ($p = 0.0587$).
CNS compartments. When 850 rad were delivered to the CNS only (brain and spinal cord) at day 6 after 2.2-V-1 infection, marked white matter pathology was observed (Fig. 4A, B). In the converse experiment in which 850 rad were delivered at day 6 p.i. to systemic regions (including spleen, lymph nodes, and bone marrow) but not to the CNS, white matter appeared normal (Fig. 4C, D). These findings are essentially identical to those found in EAE (Hickey and Kimura, 1988) and

Fig. 1. Spinal cord from a C57BL/6J mouse inoculated i.c. with JHMV 2.2-V-1 and examined at day 12 p.i. (A) LFB-stained section showing marked hypercellularity in a demyelinated area due to infiltration of mononuclear inflammatory cells (×500). (B) Different field of the same section showing normal myelination (lower brackets) and an area of demyelination (upper brackets) in which inflammatory hypercellularity is also evident (×250). The gray matter is unaffected. (C) Higher magnification (×1000) of the area indicated in (B) by arrowhead. (D) Immunoperoxidase staining of an adjacent section showing little or no viral antigen in the demyelinated area. Note the presence of naked axons (arrowheads; ×1000). (E) More advanced demyelinating focus (asterisk) in the same section as (A) is characterized by severe white matter disruption in which inflammatory cellular infiltration is less apparent (×250).
suggest that radiosensitive cells residing in the systemic compartment at day 6 p.i. are essential for subsequent lesion development.

Adoptive transfers

To identify systemic cells which may be linked to JHMV-induced disease, spleen cells from donor mice were transferred into irradiated recipient mice (Table 2). When 2.2-V-1-infected, irradiated mice received immune spleen cells (group 3, Table 2), significant disease was restored (clinical score, \( p = 0.0023 \); histological score, \( p = 0.0587 \)). While this effect was often quite dramatic in individual mice (Fig. 5A), the mean clinical and histological scores in these animals were not as high as those of virally infected, unirradiated mice (group 1, Table 1). Surprisingly, adoptive transfer of naive spleen cells into infected, irradiated recipients (group 4, Table 2; Fig. 5B) also produced moderate disease. On the other hand, following transfer of immune splenocytes to naive, uninfected mice (group 5, Table 2) recipients were completely normal (Fig. 5C), indicating that the presence of virus itself or target cells altered by virus are required for disease production.

The ability of spleen cells from naive mice to induce disease in infected, irradiated mice (group 4, Table 2) raised the possibility that a non-MHC-restricted cell, such as a macrophage, might be responsible for mediating this effect. To test this hypothesis, allogeneic transfers were performed, using BALB/cJ (H-2^d\) donor and C57BL/6J (H-2^b\) recipient mice. As shown in Table 3 (groups 6 and 7), allogeneic transfers did not restore disease, suggesting that MHC restriction is, in fact, required.

To further define the characteristics of cells which are active in the adoptive transfer of disease, syngeneic immune spleen cells were separated by nylon wool to yield T cell-enriched (nylon wool nonadherent) and T cell-depleted (nylon wool adherent) fractions. As shown in Ta-

![Fig. 3. Viral replication in the brains of C57BL/6J mice inoculated with JHMV 2.2-V-1 on day 0 and sacrificed at the indicated time points, each of which represents the mean titer from a group of 4–6 mice. Mice were either not irradiated (○), or subjected to 850 rad of whole body irradiation at either day −1 (⊙) or at day +6 (●) relative to the day of viral inoculation.](image-url)
Fig. 4. Differential irradiation of C57BL/6J mice infected with JHMV 2.2-V-1. (A) H&E-stained section of spinal cord from a mouse given 850 rad to the CNS (brain and spinal cord) at day 6 p.i. and examined at day 12 p.i. Note the tissue disruption and inflammatory cellular infiltration in the white matter (×500). (B) LFB staining of an adjacent section showing that the lesion noted in (A) is actually demyelinated (asterisk; ×250). Spinal cord sections stained with H&E (C; ×600) or LFB (D; ×500) from a mouse given 850 rad to the non-CNS, systemic compartments at day 6 p.i. and examined at day 12 p.i. Note the normal white matter.

TABLE 3
CHARACTERIZATION OF SPLEEN CELLS MEDIATING ADOPTIVE TRANSFER OF PARALYTIC-DEMYELINATING DISEASE INDUCED BY JHMV

| Group | Conditions | Clinical score c | Histological score c |
|-------|------------|------------------|---------------------|
|       | Donor cells a | Recipient mice b | Mean   | n  | Mean   | n  |
| 6     | Allogeneic immune | Virus | 1.14 ± 1.06 | 7  | 0.71 ± 1.11 | 7  |
| 7     | Allogeneic naive | Virus | 1.00 ± 0.81 | 7  | 1.29 ± 1.60 | 7  |
| 8     | Syngeneic immune, NWNA | Virus | 2.00 ± 0.76 | 8  | 1.38 ± 1.51 | 8  |
| 9     | Syngeneic immune, NWA | Virus | 0.63 ± 0.74 | 8  | 0.50 ± 1.07 | 8  |

a Allogeneic (BALB/cJ, H-2d) or syngeneic (C57BL/6J, H-2b) donor mice were given 10⁶ PFU of JHMV 2.2-V-1 i.p. 6 days prior to transfer (Immune) or not (Naive), as noted in Table 2. In groups 6 and 7, 5 x 10⁷ spleen cells were transferred i.v. into each recipient at day 6 p.i. NWNA indicates nylon wool nonadherent cells, and NWA indicates nylon wool adherent cells (selected from a total of 5 x 10⁷ spleen cells) transferred i.v. to recipients at day 6 p.i.

b In all groups, recipient mice were 6-week-old C57BL/6J males given 10³ PFU of JHMV 2.2-V-1 i.c. on day 0 and irradiated on day 6 p.i. prior to transfer.

c Scoring was also performed as in Table 1. Underlined values are those which are significantly greater than respective scores of JHMV-infected, irradiated mice not given splenocytes (Table 1, group 2) (p ≤ 0.05 by the Mann-Whitney test).
Fig. 5. LFB-stained sections of spinal cord from C57BL/6J mice following irradiation and subsequent intravenous adoptive transfer of splenocytes at day 6 p.i. and examined at day 12 p.i. (A) Transfer of immune donor splenocytes is able to induce demyelination (asterisk) in JHMV 2.2-V-1-infected recipient (×250). (B) Demyelination (asterisk) is also induced by transfer of naive donor splenocytes into JHMV 2.2-V-1-infected recipient (×250). Note the lesions of (A) and (B) are similar to, though more pronounced than advanced demyelination shown in Fig. 1E. (C) Transfer of immune donor splenocytes into uninfected recipient does not result in demyelination (×500).

Table 3, the ability to transfer clinical disease was contained in the T cell-enriched population (group 8). Although histological scores were elevated in these mice relative to irradiated, infected mice not given spleen cells (group 2, Table 1), this value did not achieve statistical significance, possibly due to the relatively small number of animals in the experiment. Taken together, the characteristics of MHC restriction and nylon wool nonadherent suggest that spleen cells which are most active in adoptive transfers are likely to be T lymphocytes.

Discussion

The major finding of this study is that immunosuppression of JHMV-infected mice by means of gamma irradiation abrogates viral-induced demyelination. This result strongly argues that JHMV causes demyelination through immunopathological mechanisms. However, our investigation cannot exclude some contribution of direct viral cytolysis of oligodendrocytes (Lampert et al., 1973) to the JHMV pathogenesis. Clearly, however, the role of this mechanism, if present, must be minor, since it should be unaffected or even enhanced by an immunosuppressive dose of irradiation. In fact, infected irradiated mice show little or no evidence of demyelination or cell destruction (Fig. 2), despite marked increases in both viral antigen-positive oligodendrocytes (Fig. 2A) and brain viral titers (Fig. 3). Prior studies in athymic (Sorensen et al., 1982, 1987), immunosuppressed (Sorensen et al., 1982; Zimmer and Dales, 1989), or lethally challenged (Stohlman et al., 1986; Sussman et al., 1989) rodents have established a protective role for cellular immunity early in the JHMV pathogenesis by limiting virus infection in susceptible cells, such as oligodendrocytes and neurons. Again, these studies have limited relevance to the study of JHMV-induced demyelination, since early challenge of animals with severe
immunodeficiency or with large amounts of virulent virus primarily results in an acute, fulminating encephalitis, with little or no demyelination.

Further support for an immunopathological mechanism of JHMV-induced demyelination comes from the adoptive transfer studies. These experiments indicate that populations of murine donor spleen cells, which are enriched for T lymphocytes and appear to be MHC-restricted, restore demyelination to infected, irradiated recipient mice (Tables 2 and 3). Two features of the adoptive transfers were unexpected, however, and indicate that these experiments must be interpreted cautiously. First, cells from naive donors (Table 2, group 4) were nearly as effective as cells from immune donors (Table 2, group 3) in transferring paralytic-demyelinating disease at day 6 p.i. This result may reflect the fact that in an established cellular immune response within the CNS, the majority of lymphocytes present locally may not be antigen-specific (Ceredig et al., 1987; Fallis et al., 1987). Second, although the degree to which adoptive transfers reconstituted disease was clearly significant in the aggregate (Table 2) and often dramatic in individual animals (Fig. 5A, B), the mean clinical and histological scores of reconstituted mice were considerably lower than those of the infected unirradiated mice (Table 1, group 1). Thus, the disease was, on average, only partially restored by adoptive transfer of spleen cells. This result may reflect technical factors, such as the quantity or quality of transferred cells or the ability of transferred cells to reach specific targets. Alternatively, it is possible that irradiation at day 6 p.i. irreversibly damages critical elements in the cascade leading to demyelination. In order to address these questions, further experiments in which adoptive transfers performed during the early, inductive phase of disease are in progress.

An immunopathological mechanism has not been established previously for JHMV-induced demyelination, possibly due to the use of a relatively virulent virus strain coupled with cyclophosphamide-mediated immunosuppression applied simultaneously with virus infection (Lampert et al., 1973; Weiner, 1973). In this setting, the majority of mice died of fulminant acute encephalitis and did not manifest typical JHMV-induced subacute or chronic paralysis and demyelination.

The two JHMV strains, 2.2-V-1 and JHMV-DS, used in the present study are essentially identical to most JHMV strains in all respects except neurovirulence (Weiner, 1973; Stohlman et al., 1982; Fleming et al., 1986). In terms of neurovirulence, they resemble the original JHMV isolates, which in early passages primarily caused a nonfatal paralytic disease (Bailey et al., 1949; Cheever et al., 1949); only after many i.c. passages did the virus acquire marked neurovirulence. Most importantly, the minimal neurovirulence of JHMV 2.2-V-1, JHMV-DS, and similar JHMV strains (Hirano et al., 1981; Knobler et al., 1982; Dalziel et al., 1986) allows virus-induced demyelination to be studied directly, by minimizing the confounding fatal encephalitis caused by other JHMV strains.

The only previous study of irradiation during JHMV infection was that of Love et al. (1987), who applied regional irradiation to the spinal cords of mice which had been infected with JHMV, strain ts8 (Knobler et al., 1982) 2 months previously. Under these circumstances, irradiation had no effect on the clinical or histological course of JHMV pathogenesis. We have confirmed the result of Love et al. (1987), that irradiation applied to the CNS alone or applied after disease has become firmly established has no effect on JHMV-induced demyelination. The suggestion of Love et al. (1987), that irradiation administered at an earlier time in the course of disease might have an effect on demyelination was shown to be correct in the present study (Table 1).

In conclusion, we have shown that JHMV-induced paralytic-demyelinating disease may be prevented by immunosuppressive dose of gamma irradiation and partially restored by the adoptive transfer of spleen cells, which are most likely T lymphocytes. Taken together, these findings indicate that the primary mechanism of JHMV-induced demyelination is immunopathological, rather than being due to direct viral lysis of oligodendrocytes.

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