

## Rabies Virus Neuritic Paralysis: Immunopathogenesis of Nonfatal Paralytic Rabies

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**Two pathogenetically distinct disease manifestations are distinguished in a murine model of primary rabies virus infection with the Evelyn-Rokitnicky-Abelseth strain, rabies virus neuritic paralysis (RVNP) and fatal encephalopathogenic rabies. RVNP develops with high incidence in immunocompetent mice after intraplantar infection as a flaccid paralysis restricted to the infected limb. The histopathologic correlate of this monoplegia is a degeneration of the myelinated motor neurons of the peripheral nerve involved. While, in this model, fatal encephalopathogenic rabies develops only after depletion of the CD4 subset of T lymphocytes and without contribution of the CD8 subset, RVNP is identified as an immunopathological process in which both the CD4 and CD8 subsets of T lymphocytes are critically implicated.**

Rabies is the neurological syndrome caused by the neurotropic and neuropathogenic rabies and rabies-related viruses (22) that are taxonomically classified as the genus *Lyssavirus* of the family *Rhabdoviridae*. The negative-stranded genomic RNA of about 12 kb, encoding the five viral proteins N, NS, M, G, and L, has recently been cloned and sequenced for the highly pathogenic strain PV (24) and the vaccine strain SAD B19 (4), which differs only in cell culture history from the Evelyn-Rokitnicky-Abelseth (ERA) strain (1). The neurotropism of the virus and its pathological consequences have been extensively studied and reviewed (2, 16). Rabies virus replicates in the neurons of the central nervous system, forming cytoplasmic inclusions, the Negri bodies, which are pathognomonic of rabies. The mode of neuroinvasion from a peripheral site of inoculation has been of long-standing interest (20) and was brought to a conclusion by recent elegant studies (5, 21) showing that rabies virus can enter sensory and motor nerve endings without prior replication in extraneuronal cells and reaches the cell bodies in the respective ganglia by retrograde axonal transport. After a primary wave of replication within the ganglia, virus invades the central nervous system and also moves back to the peripheral nerve endings by anterograde axonal transport.

It has been known since the earliest days of rabies research that rabies virus infection can result in two forms of disease, encephalitic disease and paralytic disease (9). On occasion, flaccid paralysis of the afflicted extremity was seen as the outstanding clinical feature of human rabies (9), and in murine models of nonfatal rabies, paralysis persisted in so-called survivors cum sequelae (2). Interestingly, survivors with paralysis proved to be more resistant against a subsequent challenge infection than healthy survivors. In retrospective interpretation, this early finding has already suggested a pathogenetic linkage between the efficacy of the immune response and the manifestation of paralytic disease. Evidence was provided later by demonstrating the absence

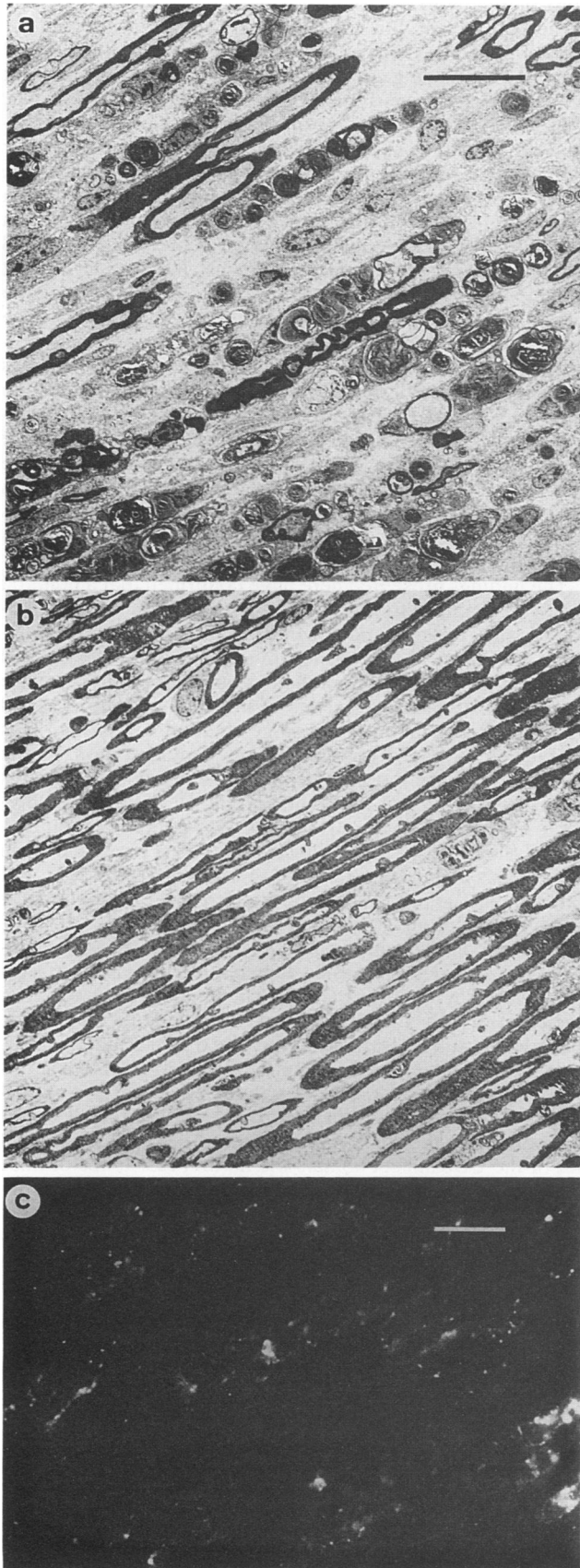
of paralytic disease but rapid progression to fatal encephalitic disease in immunocompromised mice infected with rabies virus strains CVS (11) as well as ERA (23). Yet the effector cell population responsible for the immunopathogenesis of paralytic rabies remained unidentified, and we see also a reason to bring the site of the damage back into discussion. Iwasaki et al. (11) correctly noted a coincidence between paralytic disease and mononuclear cell infiltration associated with parenchymatous tissue destruction in the brains of immunocompetent mice, in contrast to the absence of brain infiltrates during encephalitic disease in immunocompromised mice. However, the conclusion that paralysis results from the histopathology in the brain is contradicted by the early observation that, in fatal experimental rabies with a street isolate, limb paralysis was the first clinical sign of ascending rabies (20). Here we show that limb paralysis results from a peripheral immunopathological process, in which both subsets of T lymphocytes are implicated and which causes degeneration of the motor neurons of the local sciatic nerve. On the basis of these findings, we propose rabies virus neuritic paralysis (RVNP) as the name for this manifestation of rabies.

Intraplantar infection of 8-week-old immunocompetent BALB/c (MHC<sup>d</sup>) and C57BL/6 (MHC<sup>b</sup>) mice with 10<sup>6</sup> tissue culture infectious doses of the ERA strain of rabies virus (6) resulted in an incidence of ca. 60% of a monoplegia that became clinically manifest by days 12 to 14 after infection and was characterized as a flaccid paralysis restricted to the afflicted, ipsilateral limb. In none of these animals did disease proceed to fatal encephalopathogenic rabies, and during an observation period of 6 months, neither ascendance nor regression of paralysis was ever observed. RVNP is thus irreversible and local. Mice in which paralysis did not develop during the critical time period remained healthy. This clinical picture is essentially in accordance with that reported previously for ERA (23), whereas after intraplantar infection with the more virulent CVS ts2 strain, paralysis has been ascending and infection has proceeded to fatal rabies (11).

The histopathologic correlate of RVNP is a pronounced disintegration of myelin sheaths and degeneration of axons,

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as is shown in Fig. 1a for the great sciatic nerve, the nervus ischiadicus. Loss of the laminated structure of the myelin and hydropic swelling as well as shrinkage of the axoplasm are evident. Proliferation of Schwann cells, resulting in the formation of characteristic bands that signify areas of degeneration without regeneration, known as bands of Büngner, was seen occasionally. Infiltration of lymphocytes, macrophages, and in particular eosinophilic granulocytes was frequent, albeit not massive. Notably, at no stage of the development of RVNP was rabies virus ribonucleoprotein detectable in the axons by immunofluorescence (not depicted). In contrast, the myelinated axons in the nervus ischiadicus of a host immunocompromised by a sublethal dose of gamma irradiation (5 Gy) appear to be ultrastructurally intact (Fig. 1b) and yet harbor large amounts of virus (Fig. 1c). The latter finding is particularly instructive. First, as fluorescence is reportedly never seen in the axon before viral replication in the ganglion (5, 21), such replication must have occurred in the immunocompromised host; second, as virus travels back by anterograde axoplasmic flow (5, 21), the fluorescent axons not only appear ultrastructurally intact but must have remained functionally intact. These results have thus demonstrated that immunocompetence is associated with low virus production in the peripheral neurons and with a high incidence of RVNP, whereas immunoblative treatment results in high virus production and nevertheless prevents neuronal degeneration and paralysis. In conclusion, RVNP is not caused by neurolytic viral replication in the ganglia.

The data obtained so far pointed to an immunopathogenesis of RVNP. This conclusion was confirmed by specific *in vivo* depletion of the CD4 and CD8 T-lymphocyte subsets, accomplished by repeated intravenous infusion of milligram doses of purified rat monoclonal antibodies YTS 191.1 and YTS 169.4, respectively (3). The depletion regimen and control of depletion efficacy were performed as recommended by Cobbold et al. (3). Two-color cytofluorometric analyses of spleen lymphocytes revealed a depletion of CD4 T lymphocytes from the initial 21 to 32% to 0 to 4% and of CD8 T lymphocytes from an initial 11 to 17% to 1 to 3%. The incidences of RVNP and of encephalopathogenic rabies were monitored at 3 weeks after infection, when mice with encephalitic disease (groups B and D) were in a moribund stage just before the onset of mortality, which was complete by the fourth week (Fig. 2). On the basis of 20 individual mice per treatment group, the clinical status was correlated with the titer of neutralizing serum antibody, determined by the established rapid fluorescent-focus inhibition test, and by spread of the virus in the brain, detected by direct immunofluorescence histology in frozen tissue sections with fluorescein isothiocyanate-conjugated rabbit antiserum against rabies virus (HEP strain) ribonucleoprotein.

In immunocompetent mice (Fig. 2A), the incidence of

FIG. 1. Histopathology of RVNP. (a) Disintegration of myelin sheaths and degeneration of axons shown by electron microscopy at low magnification. Longitudinal section of nervus ischiadicus from an immunocompetent BALB/c mouse with clinically apparent monoplegia at day 14 after intraplantar infection. (b) Ultrastructurally intact myelinated axons. Nervus ischiadicus from an immunosuppressed (5 Gy of gamma irradiation) animal that showed symptoms of classical rabies but did not develop monoplegia. (c) Neuronal spread of rabies virus. Cryostat section of nervus ischiadicus corresponding to that shown in panel b. Detection of viral ribonucleoprotein by direct immunofluorescence histology. Bars, 2  $\mu$ m.

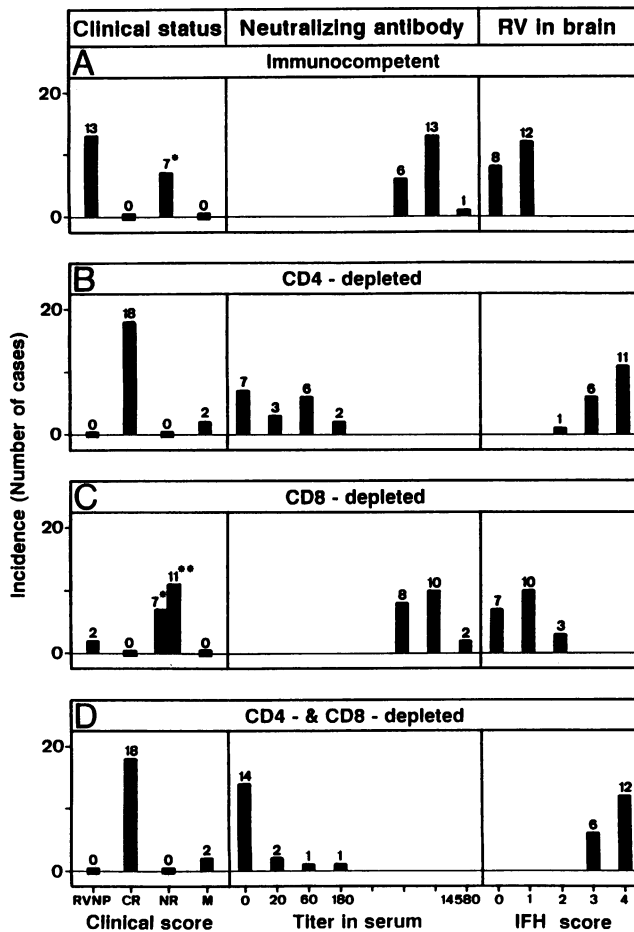


FIG. 2. Involvement of T-lymphocyte subsets in rabies virus pathogenesis. (A) Immunocompetent BALB/c mice infected in the left hind footpad with  $10^6$  tissue culture infectious doses of ERA. (B) Infusions of anti-CD4 at day  $-1$  and day  $+1$  relative to the day of infection (day 0). (C) Infusions of anti-CD8 at days  $-1$  and  $+1$ . (D) Double depletion at days  $-1$ ,  $+1$ , and  $+8$ . Left panel: Symptoms 3 weeks after infection. CR, classical encephalopathogenic rabies; NR, nonrabies cases, including apparently healthy cases (\*) and cases of idiopathic morbidity (\*\*); M, deaths. Center panel: Reciprocal of the serum dilution yielding 50% reduction in challenge virus standard CVS-11 as determined by the rapid fluorescent-focus inhibition test. Right panel: Detection of rabies virus (RV) by antiribonucleoprotein immunofluorescence histology (IFH) in rostral and nuchal brain tissue sections. Scores: 0, no immunofluorescence; 1, single immunofluorescent foci; 2 to 4, disseminated immunofluorescence with increasing density. For each panel, numbers above each bar indicate the number of animals from each group of 20.

RVNP was high (13 of 20 individuals), whereas no individual had developed encephalopathogenic rabies by the time of the screening, and accordingly, all survived later on. This situation was associated with high antibody titers in the serum and with low or absent virus spread in the brain. Conversely, mice depleted of the CD4 subset did not develop RVNP (Fig. 2B) but died in the fourth week from the typical neurological symptoms of fatal encephalopathogenic rabies (concluded from a separate experiment; not shown), which were associated with high virus replication in the brain. As a consequence of the abrogation of CD4 T-helper function, antibody

titers were low to negative. It is worth noting that depletion of the CD4 subset, but not of the CD8 subset, prevented the formation of perivascular infiltrates in the brain (concluded from the screening of many brain tissue sections; not shown). This observation allows two conclusions. First, CD4 T lymphocytes are not only obligatory for the production of neutralizing antibodies, but are also critical for perivascular brain infiltration. Second, even though the infiltrates are considered a prominent histopathological sign in rabies (11, 16), their presence correlates with protection rather than with pathogenesis. Mice depleted of the CD8 subset (Fig. 2C) were protected against RVNP and did not develop fatal encephalopathogenic rabies either. It should be mentioned that a proportion of the CD8-depleted mice appeared to be moribund but without the pronounced neurological signs of rabies. The pathogenetic basis for this idiopathic morbidity was not investigated further, and all the mice thus affected recovered and survived. CD4 T lymphocytes were clearly operative, as is indicated by high titers of neutralizing antibody, and accordingly, virus spread in the brain was controlled. Finally, after simultaneous depletion of both T-lymphocyte subsets (Fig. 2D), rabies pathogenesis was indistinguishable from that after depletion of the CD4 subset alone.

The findings presented here have confirmed that a nonfatal paralytic manifestation of rabies results from an immunopathological process and not from a direct neuropathogenicity of the virus. While earlier investigators have attributed paralysis to an immunopathology in the brain (11), we conclude that flaccid paralysis of the afflicted limb is based on a peripheral immunopathology, that is, a neuritis. This conclusion is based on histological demonstration of the destruction of the motor neurons in the sciatic nerve and is strengthened by our observation that CD8 depletion prevents limb paralysis even though it does not prevent infiltration of the brain by mononuclear cells. This particular form of paralysis is therefore referred to as RVNP. However, our data should not be mistaken as the only explanation for paralytic symptoms in rabies.

Encephalopathogenic rabies, the classical disease manifestation, also occurs in mice depleted of either both subsets of T lymphocytes or of the CD4 subset alone, but not after depletion of only the CD8 subset. This demonstrates, first, that neither subset is critically involved in the pathogenesis of classical rabies and, second, that the CD4 subset is obligatory for control, whereas the CD8 subset is dispensable. This conclusion is in accordance with a recent report demonstrating that *in vivo* depletion of the CD4 subset but not of the CD8 subset ablates the natural resistance of mice to street rabies virus (17). The protective function of antibodies to rabies virus and the triad of lower antibody titers in serum, increased virus spread in the brain, and mortality from encephalitic disease in the immunocompromised host are well established (7, 8, 13, 14, 17), and there is no doubt that the beneficial contribution of CD4 cells is to provide help to B cells.

That RVNP fails to develop in CD8-depleted mice excludes a direct immunopathogenic role of the CD4 cells in RVNP and strongly suggests that the CD8 cells are the immunopathogenic effector cells. It is of interest that intraplantar ERA infection indeed effectively primes CD8-positive cytolytic T lymphocytes that recognize an antigen common to rabies viruses and the serologically distant rabies-related Mokola virus (19), putatively the N protein. It clearly would have been desirable to verify the effector subset by a second experimental approach, the transfer of

RVNP to infected, immunodeficient recipients with primed CD8 cells derived from infected, immunocompetent donors that are at a 60% risk of getting RVNP. However, this approach failed when up to  $10^7$  popliteal lymph node T lymphocytes or selected CD8 cells derived from immunocompetent donors were infused intravenously into irradiated or CD4 subset-depleted recipients (not shown). Transfer into CD8-depleted recipients was technically not feasible because of the presence of the depleting antibody. However, one must consider that even in immunocompetent mice, the incidence of escaping RVNP is about 40%, which indicates that the induction of RVNP is delicately dependent on the timing of local presentation of antigenic peptides and infiltration of a sufficient number of effector cells. It is therefore not surprising that these conditions are not easily met by a transfer approach.

The mechanism by which CD4 cells contribute to RVNP is open to question. Even though it is established that, in general, CD4 cells are not obligatory for the induction of a CD8 response (18), CD4 cell-derived lymphokines may accelerate the clonal expansion of CD8 effector cells and aid their timely infiltration to the tissue site of action. Another idea is that gamma interferon produced by CD4 cells of the T-helper type 1 (15) is critical for antigen presentation in infected neurons by enhancing the expression of major histocompatibility complex class I molecules (12). An essentially different explanation is suggested by previous work, which has demonstrated that disseminated rabies virus replication suppresses cell-mediated immunity (25). Owing to the ablation of antibody production, depletion of the CD4 subset results in disseminated infection (Fig. 2B and D), which could prevent the immunopathogenic CD8 response.

At first glance, RVNP as a rabies-associated demyelination is reminiscent of rabies postvaccination encephalomyelitis, an autoimmune complication elicited by myelin basic protein contained in inactivated Semple-type adult animal nerve tissue vaccines (10). It is therefore important to stress the differences. First, RVNP is elicited by purified virus derived from cell culture. Second, virus inactivation prevents RVNP (not shown). Third, RVNP remains local. In agreement with the view that the immunopathogenic CD8 cells are directed against a viral antigen, neither RVNP nor encephalomyelitis could be induced in uninfected mice by transfer of T lymphocytes from infected donors (not shown).

In conclusion, RVNP is a new example of an antiviral immunopathological process.

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