Failure To Transmit Disease from Gray Tremor Mutant Mice

GEORGE A. CARLSON,^{1*} SYLVIA BANKS,¹ DIANE LUND,¹ CHERYL REICHERT,^{1,2}
DARLENE GROTH,³ MARILYN TORCHIA,³ STEPHEN J. DEARMOND,⁴
AND STANLEY B. PRUSINER³

McLaughlin Research Institute, Great Falls, Montana 59405¹; Pathology Department, Columbus Hospital, Great Falls, Montana 59401²; and Department of Neurology³ and Pathology,⁴ University of California, San Francisco, San Francisco, California 94143

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Mice homozygous for mutant alleles at the gray tremor (*gt*) locus develop a marked non-intention tremor beginning at 8 days of age. Most homozygous mice die by 3 months. Homozygotes exhibit intense vacuolation of the central nervous system gray matter and vacuolation and hypomyelination of some white matter tracts. Based on neuropathological similarities with scrapie, other investigators inoculated wild-type mice with gray tremor brain homogenates to test the hypothesis of transmissibility. Published reports indicated that spongiform encephalopathy (R. L. Sidman, H. C. Kinney, and H. O. Sweet, *Proc. Natl. Acad. Sci. USA* 82:253–257, 1985) and disease, including hind limb paralysis in NFS mice (P. M. Hoffman, R. G. Rohwer, C. MacAuley, J. A. Bilello, J. W. Hartley, and H. C. Morse III, *Proc. Natl. Acad. Sci. USA* 84:3866–3870, 1987), were transmitted by inoculation of *gt/gt* brain homogenates. In our hands, however, no NFS/NCr animals inoculated intracerebrally with *gt/gt* or *+/+* brain preparations showed any signs of disease or pathological changes in the brain. Positive transmission by other investigators may reflect the microbiological status of their donor or recipient mice.

Severe neurologic disease, in addition to coat-color dilution, results from homozygosity for the gray tremor (*gt*) mutation on chromosome 15 (13). Homozygous mice develop a pronounced tremor that begins at 8 days of age and are subject to convulsions. The mutation is carried on the GT/Le inbred strain, and most *gt* homozygotes die by 3 months of age. On heterogeneous genetic backgrounds, many *gt/gt* mice survive and may reproduce (20). Microscopic examination of the nervous system revealed severe vacuolation in the central nervous system gray matter and in some white matter tracts. Myelin formation was deficient in nerves at dorsal and ventral root entry zones and in the peripheral nervous system generally. Heterozygous mice have no pigment changes, no signs of neurologic dysfunction, and live for more than 2 years. However, mild focal vacuolation is evident in *+/gt* mice by 7 weeks of age, primarily in primitive structures such as the olfactory nucleus and frontal parietal and paraform areas. The spongiform encephalopathy of gray tremor mice is a feature shared with most diseases caused by prions (1, 16), leading to attempts to transmit the disease.

Transmissibility of disease was suggested by the finding that seven mice given intracerebral inoculation of *gt/gt* brain homogenates as neonates showed mild spongiform changes but no behavioral abnormalities 22 to 24 months later (20). No pathological changes were detected in the age-matched uninoculated mice that served as controls. Only a single type A virus particle in a single *gt/gt* mouse was detected by electron microscopy, suggesting, along with the long incubation period and lack of inflammation, that the putative transmissible agent was unconventional. Successful transmission of clinical neurologic disease and spongiform change into NFS/N mice was subsequently reported by Hoffman and coworkers (12).

NFS/N mice were selected because of their sensitivity to

spongiform encephalopathy induced by mouse neurotropic retroviruses (11). All 17 newborn NFS/N pups inoculated with 10 μ l of a clarified 10% brain homogenate from *gt/gt* mice developed neurologic disease characterized by tremors progressing to marked ataxia, weakness, and spasticity predominantly in the hind limbs (12). The tremors were first reported within 60 days after inoculation. Brain homogenates from ill NFS/N mice inoculated with *gt/gt* brain or from aged, but clinically normal, C3H/FeJ and BALB/cBy mice that had received *gt/gt* brain homogenate (20) transmitted disease to both adult and newborn NFS/N mice. These first-passage isolates induced disease within 60 days after inoculation of neonates and between 98 and 150 days after inoculation of adults. Terminal illness developed between 340 and 438 days. Vacuolation and mild gliosis were apparent on histological examination. No infectious mouse retroviruses were detected in *gt/gt* brain homogenates, and there was no accumulation of either murine leukemia virus proteins p30 and gp70 or the scrapie-specific isoform of prion protein (PrP^{Sc}) in diseased NFS/N mice or in *gt/gt* mice (2), possibly suggesting a novel etiologic agent.

Our interest in the gray tremor mutation stemmed from the possibility that the transmissible agent was a prion-like particle replicating infectivity through posttranslational conversion of a benign host wild-type protein to a malignant pathogenic isoform (16).

MATERIALS AND METHODS

Mice. Mice were bred at the McLaughlin Research Institute (MRI). The colonies of mice used in these studies were free of antibodies against any mouse viral pathogens as assessed by Charles River Laboratories and University of Missouri diagnostic services. The serological screen included mouse hepatitis virus, Sendai virus, parvovirus of mice, reovirus 3, Theiler's murine encephalomyelitis virus, ectromelia virus, mouse adenovirus, polyomavirus, lymphocytic choriomeningitis virus, minute virus, and infantile diarrhea virus. The Jackson Laboratory (Bar Harbor, Maine) discontinued maintenance of gray tremor mice as livestock to avoid problems associated with a potential biohazard. GT/Le-*+/gt* breeders and an inbred GT/Le subline that no longer carried the mutation were transferred from the Jackson Laboratory to MRI in September 1988. Gray tremor stock were maintained in Microisolators in an isolation room and han-

* Corresponding author. Mailing address: McLaughlin Research Institute, 1520 23rd St. South, Great Falls, MT 59405. Phone: (406) 452-6208. Fax: (406) 454-6004. E-mail: gac@po.mri.montana.edu.

TABLE 1. Failure to transmit disease from gray tremor mutant mice into adult or newborn NFS/NCr mice

Inoculum ^a	Recipients	No. of mice inoculated	No. of mice with disease
<i>gt/gt</i> (frozen)	Adults	20	0
<i>+/+</i> (frozen)	Adults	16	0
<i>gt/gt</i> (fresh)	Adults	14	0
<i>gt/gt</i> (frozen)	Neonates	15	0
<i>gt/gt</i> (fresh)	Neonates	26	0
<i>+/+</i> (fresh)	Neonates	6	0

^a Each inoculum was a homogenate from a brain from a different mouse.

dled under a vertical laminar flow hood to protect personnel against a potential transmissible agent. NFS/NCr mice were provided from the specific-pathogen-free facility of the Frederick Cancer Research Facility of the National Cancer Institute by Neal Copeland and Nancy Jenkins. NFS/NCr mice were maintained in MRI's research colony until after inoculation with putative gray tremor agent, when they were moved to isolation.

Isolate preparation and incubation time studies. Brains from clinically ill GT/Le-*gt/gt* mice or from the control GT/Le strain were weighed, and 10% (wt/vol) homogenates were prepared in 320 mM sucrose. The homogenate was clarified by centrifugation at $1,000 \times g$ for 10 min. In some experiments, the homogenates were made from brains frozen at -80°C , and in others, preparations of fresh brain were used. Anesthetized mice were inoculated intracerebrally with 10 μl (neonates) or 20 μl (adults) of homogenate diluted twofold in phosphate-buffered saline. Mice were housed in Microisolator cages at MRI and examined for neurologic signs twice a week. Examination included observation for tremors or ataxia, picking up the mice by their tails to detect abnormal front or rear paw clasp and subtle tremors, and placing the mice on their backs as a check for righting ability.

NFS/NCr mice were shipped to the University of California at San Francisco for scrapie incubation time studies with the Chandler prion isolate as previously described (4).

Pathological analysis. Mice were killed by cervical dislocation to minimize anoxic changes in the brain that we observed in preliminary experiments by using CO_2 asphyxiation as the method for euthanasia. Brains were fixed in buffered formalin and hematoxylin-eosin-stained slides were prepared. All scoring was performed in a blind fashion.

RESULTS

Absence of clinical disease and premature death in NFS/NCr mice inoculated with gray tremor brain homogenates. Signs of neurologic disease were not observed in a total of 41 mice inoculated with *gt/gt* brain homogenates as neonates or in 34 mice inoculated as adults. These results are summarized in Table 1. Mice injected with brain homogenate from *+/+* GT/Le mice, rather than uninoculated animals, served as controls. Initial inoculations were done with two preparations of homogenate made by using frozen (-80°C) *gt/gt* brains. After the failure to observe any clinical signs in neonatal or adult mice inoculated with frozen material, fresh-brain homogenates were prepared within 1 h of inoculation to test the possibility that lack of transmission reflected inactivation of the agent by freezing. Mice were killed to determine whether pathological changes were present in their brains throughout the experiments, so that by the time the experiments were terminated, few mice survived. However, cumulative survival for mice inoculated as neonates or adults (Fig. 1) did not differ between mice given mutant or wild-type brain homogenates.

Spongiform degeneration was not induced by inoculation of brain homogenates from gray tremor mice. Microscopic examination of brain sections from NFS/NCr mice inoculated with the putative gray tremor agent did not reveal any consistent pathological changes differentiating them from mice inoculated with wild-type brain homogenates. To test the possibility of subtle differences, a group of brains from adult and neonatal mice inoculated with either mutant or wild-type brain homogenate were scored in a blind fashion. The results from these

FIG. 1. Survival of adult or newborn NFS/NCr mice inoculated with brain homogenates from *gt/gt* or *+/+* mice. Kaplan-Meier survival curves for mice inoculated as adults or neonates were generated by using the StatView (Abacus Concepts, Berkeley, Calif.) statistical analysis program for Macintosh computers. As shown in Table 1, none of these animals showed any signs of neurologic disease. Kaplan-Meier analysis was used because mice were removed for pathological or other analyses throughout the experiment. Solid lines indicate mice inoculated with brain homogenates from gray tremor mutant mice, and dashed lines indicate mice inoculated with wild-type homogenates. Numbers of surviving mice when the experiments were terminated are indicated to the right of each curve.

mice, killed between 137 and 294 days after inoculation, are summarized in Fig. 2. Sections were assigned a vacuolation score as follows: 0, a clearly normal specimen; 1, an equivocal reading; 2, possible vacuolation; 3, definite vacuolation; 4, strong focal vacuolation; and 5, widespread severe changes. No clear pathological changes were observed in any inoculated mice, and scores did not differ between mice inoculated with wild-type or mutant brain. In contrast, brains from *+/gt* mice were readily identified with definite vacuolation observed in

FIG. 2. Absence of pathology associated with inoculation of the gray tremor agent. Brain sections from mice inoculated as adults (white bars) or newborns (stippled bars) with brain homogenates from gray tremor mutant (*gt/gt*) or wild-type (*+/+*) mice were scored for vacuolation in a blind fashion. Also included were brains from five proven GT/Le-*+/gt* retired breeders (hatched bar). The interval between inoculation and sampling ranged from 137 to 294 days, with a median of 229 days. The means, standard deviations, and numbers of mice in each group are indicated. Vacuolation scores: 0, a clearly normal specimen; 1, an equivocal reading; 2, possible vacuolation; 3, definite vacuolation; 4, strong focal vacuolation; and 5, widespread severe changes.

almost all cases (mean score = 2.8 ± 0.2). As described previously in more detail (13), vacuolation in $+gt$ occurred in the regions of the brain most severely affected in gt/gt mice (gray tremor mutant brains score as 4 to 5). For example, spongiform degeneration in gt heterozygous mice was more pronounced in the cortex (primarily in the primitive areas) than in the hippocampus. In contrast, brains that were scored as having possible vacuolation had their putative pathological changes primarily in the hippocampus. Only three mice inoculated with gt/gt brain homogenates showed possible spongiform degeneration—two mice inoculated as adults, both 267 days old, and one 236-day-old mouse inoculated neonatally.

Prion incubation times in NFS/NCr mice. The apparent susceptibility of the NFS/N strain to gray tremor agent was explained as a manifestation of the striking susceptibility of these mice to agents that cause spongiform encephalopathy (12). The sensitivity of NFS/N to neurotropic retroviruses is well-known (11), but in addition to reporting transmission of heritable spongiform degeneration, Hoffman and colleagues (12) reported an exceptionally short scrapie incubation time of 65 ± 3 days after inoculation. In our hands, clinical signs first appeared in scrapie prion-inoculated NFS/NCr mice 148 ± 2 days ($n = 5$) after injection, a typical incubation time for this isolate in mice with the a allele of the prion protein gene (*Prnp*) (3, 21). Interestingly, the NFS/N mice reported to have 65-day scrapie incubation times died approximately 158 days after injection compared with 150 ± 2 days in this study, suggesting no significant alteration in susceptibility to the lethal effects of prions compared to most other strains carrying the a allele of *Prnp*.

DISCUSSION

Following publication of transmissible spongiform encephalopathy in the gray tremor mutant mouse (20), the GT/Le-*gt* strain was maintained as a potential biohazard at the Jackson Laboratory and later at MRI. A subsequent report that clinical disease could also be transmitted to NFS/NCr mice (12) seemed to justify this precaution and also introduced an experimentally tractable model to study this unconventional agent. An incubation period of 2 to 3 months to disease onset in NFS/NCr mice as reported by Hoffman and coworkers would be sufficiently short to begin approaching the biochemical nature of the agent, while a 700-day interval between inoculation and mild spongiform changes in the brain posed a major impediment to transmission studies. Based on the results reported here, we conclude that the *gt* mutation does not cause a readily transmissible disease, and we no longer house GT/Le-*gt* mice in isolation. Although our experiments do not address the question of whether mild spongiform changes can be induced after a very long incubation time, the possibility of unintentional disease transmission to humans or to other rodents seems remote, particularly if good animal husbandry procedures are observed to minimize cross-cage contamination and worker contact with allergens and pathogens.

Ignoring, for the sake of discussion, our failure to reproduce transmission of gray tremor disease, we will consider some previous attempts to transmit other neurodegenerative diseases. The fortuitous observation of neuropathological similarities between the transmissible disease scrapie and the human disease kuru (10) provided the impetus for intracerebral inoculation of chimpanzees with brain homogenates from kuru patients (5). After a long incubation period, the chimpanzees developed neurologic disease with striking clinical and pathological features similar to those of humans with kuru. Importantly, similar disease could be passed experimentally from

chimpanzee to chimpanzee, with a shorter incubation time than the primary passage (6). Although demonstration that prions are a novel class of agents distinct from conventional viruses took many years (16), the discovery that some noninflammatory neurodegenerative diseases were due to a slow infection spurred attempts to transmit other brain disorders. Many attempts to transmit Alzheimer's disease (AD) to laboratory animals by inoculation of brain extracts were unsuccessful, with rare positive transmissions early on traceable to inadvertent inoculation with prions (9).

More recent work (14) claiming positive transmissions to hamsters with blood samples from AD patients and healthy relatives also has implications for the transmissibility of the gray tremor agent. Based on speculation that titer of a putative AD agent in the brain at end-stage disease might be too low to be detectable, buffy coat homogenates were inoculated intracerebrally. Although there were no specific neurological signs of illness, mild spongiform changes were induced by 5 of 11 buffy coat samples. Second-passage material induced neurologic signs similar to those induced by experimental human Creutzfeldt-Jakob disease (CJD) prions along with much more severe spongiform changes. Other investigators were unable to reproduce these results (7, 8). Not surprisingly, given the lack of AD-like pathological changes in primary or secondary recipients of putative AD virus-containing inocula, transmissibility was not specific to AD patient's relatives; buffy coat samples from 26 of 30 healthy volunteers with no family history of dementia induced spongiform changes in hamsters (15). The researchers' conclusion that these transmissions indicated an CJD virus prevalent in the human population does not seem warranted, however.

The initial observation of Sidman and coworkers (20) is intriguing. Although transmission of disease similar to that in gray tremor mutant mice was not achieved, the spongiform changes were similar to those observed in $+gt$ mice. Such results would be consistent with a prion-like, dominant effect of *gt*-encoded protein producing spongiform degeneration. Unfortunately, aged, uninoculated mice, rather than those inoculated with wild-type brain, were used as controls. Physical insults to the brain can induce progressive changes in humans; for example, repetitive head injury can lead to an AD-like, progressive dementing syndrome (dementia pugilistica) (17). However, we should stress that our experiments do not address the transmissibility of spongiform change after an exceptionally long incubation period; the oldest animals in our study were 525 days old, far less than the reported incubation time for spongiform change following inoculation with *gt/gt* brain (20).

In contrast to the similarities in spongiform changes in $+gt$ heterozygous mice and in mouse strains other than NFS inoculated with gray tremor brain homogenates, the disease induced on second passage was unlike that of the mutant mouse (12). Such an observation, similar to that of Manuelidis and coworkers (14), where the pathological changes or disease induced by a putative agent differ dramatically from those of the donor should indicate caution. It is interesting that the only samples reported not to induce disease by Hoffman and coworkers was one from a 12-month-old healthy NFS/N mouse syngeneic with the recipients (12). The frequency with which samples from wild-type mice induced changes may have proved informative; expansion of studies aimed at demonstrating an AD-inducing agent to include individuals expected to be negative for the agent forced abandonment of the hypothesis that AD is transmissible with buffy coat (15). In short, none of the studies discussed above provide convincing evidence for transmission of AD or gray tremor disease.

The reason for the discrepancy between our results and those of Hoffman and coworkers is not known. The sublines of NFS/N mice used in both studies were probably identical, as both were obtained from the National Cancer Institute Facility in Frederick, Md. One possible explanation for the reported enhanced susceptibility to intracerebral inoculation with scrapie and gray tremor brain homogenates is the possibility of intercurrent infection with other mouse pathogens; a similar explanation for positive AD transmissions into hamsters has been offered (18). The microbiological status of mice at MRI is carefully monitored, and no known viral pathogens are present in the colony. The presence or absence of viral pathogens in the experimental colonies was not presented in the reports of gray tremor transmission. The failure to detect virus particles in NFS mice inoculated with gray tremor brain (12) does not eliminate the possibility of a virus-initiated process shortly after inoculation (19).

It is conceivable, however, that transmission of the gray tremor agent may be less efficient under specific-pathogen-free conditions. Positive transmissions in previous studies could reveal the presence of a cofactor in the animal colony necessary for expression of disease. This possibility should be considered when working with gray tremor mice. In summary, in spite of our failure to transmit clinical disease from gray tremor mice, understanding the nature of the mutation is likely to provide clues to understanding neurodegenerative processes.

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