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Transmissible spongiform encephalopathy in the gray tremor mutant mouse

(unconventional transmissible agent/slow virus/neurological mutant mouse/myelination disorder/pigmentation disorder)

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ABSTRACT **Gray tremor (*gt*) is an autosomal recessive mutation in the mouse linked to caracul (*Ca*) on chromosome 15. The complex mutant phenotype includes pigmentation defects, tremor, seizures, hypo- and dysmyelination in central and peripheral nervous systems, spongiform encephalopathy, and early death. The heterozygote (+/*gt*) is phenotypically normal but develops a mild spongiform encephalopathy from 2 months of age onward. The pigmentation and myelination disorders indicate that the *gt* genetic locus is active neonatally and probably earlier. This report focuses mainly on the later-expressed vacuolating disorder, which most closely mimics in tissue distribution, histopathology, and ultrastructure the spongiform encephalopathies caused by unconventional transmissible agents. This lesion was produced in genetically normal mice in a transmission experiment: of 99 neonatal mice inoculated intracerebrally with *gt/gt* brain homogenate, all 7 mice of three strains (BALB/cBy, C3HeB/FeJ, and C57BL/6J) allowed to survive for the unusually long interval of 682–721 days after inoculation, developed spongiform changes distributed as in the mutant phenotype. The gray tremor mutant presents a naturally occurring spongiform encephalopathy whose expression is determined by the interaction of genetic factors and a transmissible agent.**

Gray tremor (*gt*), an autosomal recessive mutation in the mouse (1), features a complex phenotype including pigmentation defects, tremor, seizures, central and peripheral myelin abnormalities, and early death (2). The lesion of greatest relevance to human disease is a spongiform encephalopathy in the mutant's central nervous system (CNS) characterized by noninflammatory vacuolation with rare neuronal loss and mild gliosis (3). The spongiform lesion is the morphological hallmark of the recognized spongiform encephalopathies of Creutzfeldt–Jakob disease and kuru in humans, scrapie in sheep, and transmissible mink encephalopathy, all caused by unconventional transmissible agents or "slow viruses" (4). These agents share the unusual properties of relative resistance to DNA inactivating procedures, sensitivity to protein denaturation, and elusiveness to ultrastructural detection; their exact molecular nature is controversial (4–8). Another cause of spongiform lesions appears to be certain ecotropic murine leukemia viruses (9–12). All the known spongiform encephalopathies are usually sporadic, although host susceptibility and length of incubation period may be under genetic control (13–16). This report describes the phenotype and genetics of the gray tremor mouse and a preliminary inoculation experiment indicating transmissibility of the spongiform encephalopathy.

The *gt* mutation appeared spontaneously in the inbred (HYIII/Le) strain carrying the hydrocephalus 3 (*hy*-3) mutation in the Mouse Mutant Stocks Center at The Jackson Laboratory in 1977 (1). The new mutant's whole body tremor and frequent convulsive seizures are characteristic of mice with myelin deficiency in the CNS (17), and its nervous system was examined in Boston with the expectation of similar findings. A myelin disorder was indeed discovered, though not the expected one, and additional important abnormalities were found that had not previously been observed among the more than 100 mutant disorders of the nervous system in mice (see, e.g., refs. 18 and 19).

Phenotype

Pigmentation defects identify the mutant individuals in the first week after birth: light ear pinnae at postnatal day 3 (P3), white blaze on head at P4, and extensive white belly spot, white feet and tail, and uniform gray agouti coat color evident thereafter. Eye color is not affected. On P8 the homozygotes develop a whole body tremor when moving about in the cage, and seizures appear subsequently. Mutant weanlings often develop a gastrointestinal illness with abdominal distension and watery fecal material with gas bubbles. Death usually occurs by P90, though on a heterogeneous background, some mice survive to reproduce. Heterozygotes (+/*gt*) have normal pigmentation, display normal behavior, and live a normal lifespan.

Genetics

The *gt* mutation was first recognized in one female weanling of a litter of four born to +/*hy*-3 parents. The original male parent +/*gt*, +/*hy*-3, when mated to a C3B6-$A/A^{w\text{-}J}$ (C3HeB/FeJ × C57BL/6J-$A^{w\text{-}J}/A^{w\text{-}J}$) hybrid female, produced all normal progeny. *Inter se* matings of these F_1 progeny resulted in the reappearance of affected (*gt/gt*) animals in the F_2 generation. Of 225 offspring born to known carriers, 67 were classified as *gt/gt*, a frequency not significantly different from the theoretical value of 0.25 for an autosomal recessive mutation (67/225 = 0.2978, $\chi^2 = 2.73$, $P > 0.05$). A mating between two homozygotes (*gt/gt*) produced six affected offspring. The present colony is derived from continued brother × sister matings from the original outcross of the +/*gt* male to the C3B6-$A/A^{w\text{-}J}$ hybrid.

Linkage tests were made with several chromosome markers before linkage was found with caracul (*Ca*) on chromosome 15 (Table 1). The estimates of recombination values, calculated using Finney's scores (20), give a recombination value for all crosses of 19.22 ± 2.63%. Although the order is not known the position of caracul toward the distal end of chromosome 15 suggests that *gt* is proximal to *Ca*.

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Abbreviations: CNS, central nervous system; P*x*, postnatal day *x*.

Table 1. Results of data showing linkage of *gt* with *Ca* on chromosome 15

Mating (♀ × ♂)	Progeny				Total	% recombination*
	Ca +	+ *gt*	+ +	*Ca gt*		
Ca +/+ *gt* × + *gt*/+ *gt*	15	7	4	2	28	21.42 ± 7.75
Ca +/ *gt* × + +/+ *gt*	111	41	65	8	225	15.31 ± 4.38
+ +/ *gt* × *Ca gt*+ +	63	9	78	30	180	23.97 ± 4.90
Ca gt/+ + × + +/+ *gt*	49	3	49	24	125	17.73 ± 5.88
Combined					558	19.22 ± 2.63

*± standard error. Recombination estimates were calculated using Finney's scores (20).

Pathologic findings in *gt*/*gt* and +/*gt* mice

The nervous system was examined in 10 homozygotes, ages P7 to P238, 10 heterozygotes, P59–P578, and 12 wild-type mice, P12–P425, all on the HYIII/Le × C3B6 and C3B6F$_1$ genetic backgrounds. In the gray tremor mutant (*gt*/*gt*), CNS myelination is delayed and reduced in amount. Axons are continuously myelinated along their lengths, as in normal animals, but the myelin sheaths on the average are thin relative to the caliber of the enclosed axons. In the mutant's peripheral nervous system, myelin abnormalities include delayed myelination, hypomyelination, especially at the root entry zones, and dysmyelination—for example, a whole bundle of axons of various calibers enclosed within a single myelin sheath (Fig. 1). These findings are in contrast to the normal relationship of a single axon enclosed within a single myelin sheath, with a constant ratio of myelin sheath thickness to axon diameter in the mouse (21).

The most distinctive finding in the homozygote's CNS is vacuolation of gray and white matter. In gray matter the vacuoles are conspicuous in the neuropil, where they are round or irregularly oval and occur in a range of sizes up to 20 μm in diameter (Fig. 2). By electron microscopy the vacuoles are membrane-bound and contain various amounts of granular material, wisps of membrane and vesicles (Fig. 3). Dendrites are focally swollen and contain irregular vacuoles of uncertain origin. Small vacuoles are also present in neuronal perikarya, axon shafts, and presynaptic terminals. Vacuoles in white matter are formed by interlamellar splitting of myelin sheaths. Astrocytes lack vacuoles.

Vacuolation is found initially at P7, predominantly in white matter of spinal cord, but within a week it involves gray matter of brainstem, thalamus, and, to a lesser extent, spinal cord. By the end of the first postnatal month, virtually the entire neuraxis is involved and vacuolation thereafter is consistently more severe in CNS gray than white matter. The superficial cerebral cortex, cerebellar cortex, and retina are uniformly spared at all ages. Astrocytic proliferation is mild and neuronal loss is inconspicuous. Inflammatory cells, congophilic angiopathy, neurofibrillary tangles, and senile plaques are not observed.

An intensive search for conventional viruses by electron microscopy resulted in the detection of 83-nm intracisternal particles consistent with type A retrovirus in a damaged, unidentifiable cell in the anterior horn of the lumbar spinal cord in one mutant individual at P31. Budding viral particles from plasma membrane or extracellular particles were not seen. "Scrapie-associated fibrils" (22) were not detected in the brains of five homozygotes at P18–33, three heterozygotes

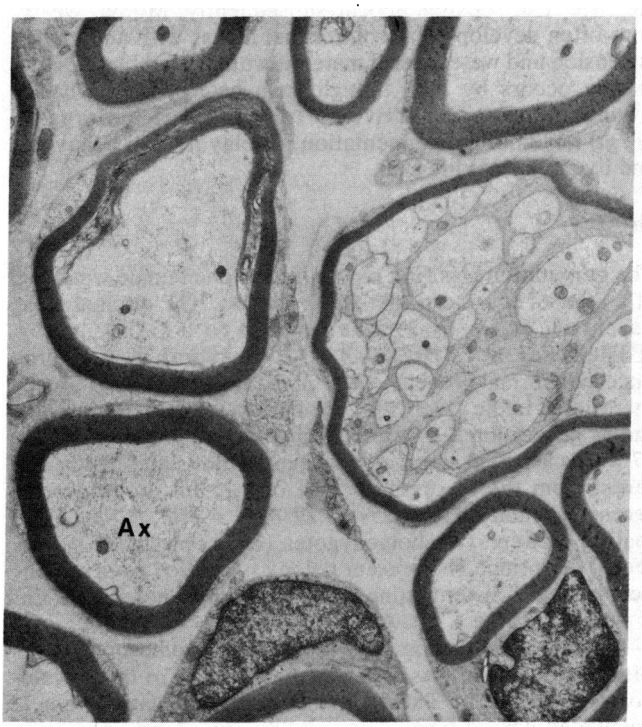

FIG. 1. Sciatic nerve, P238 *gt*/*gt* mutant. A bundle of axons of different calibers is enclosed within a single myelin sheath (right center), in contrast to the normal 1:1 relationship of axon (Ax) to myelin sheath. (×3000.)

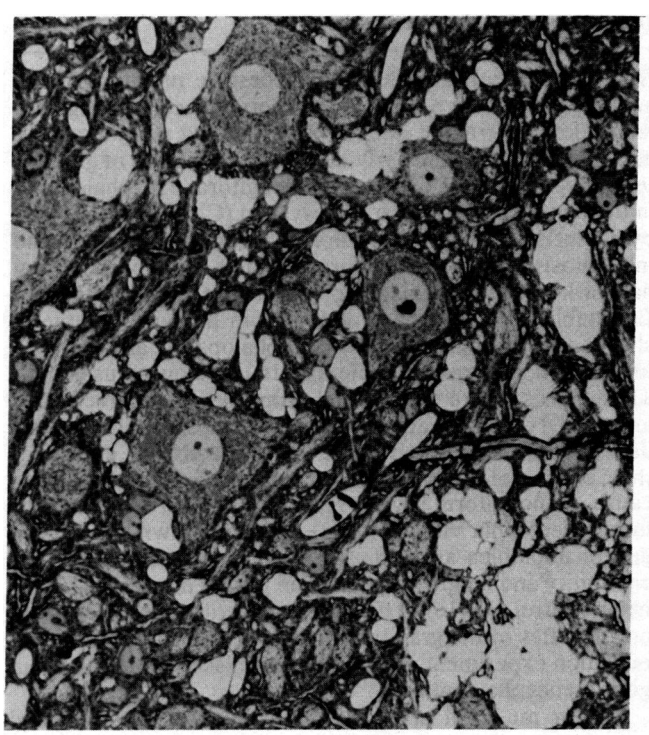

FIG. 2. Ventral horn of lumbar spinal cord, P18 *gt*/*gt* mutant. Gray matter vacuoles are irregular, often confluent, and located in the neuropil. (×530.)

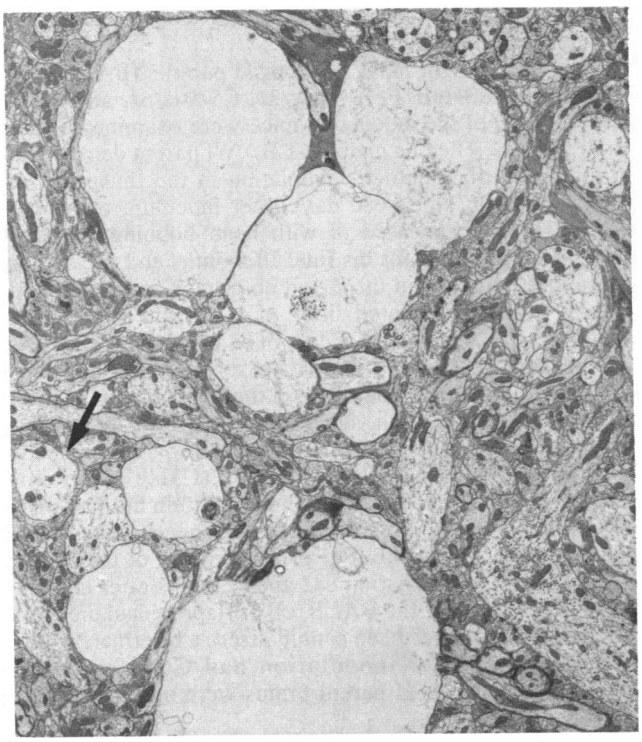

FIG. 3. Ventral horn of lumbar spinal cord, P31 *gt/gt* mutant. Vacuoles in the neuropil are membrane-bound and contain granular material, membrane fragments, and vesicles. Irregular vacuoles are present in swollen dendrites (arrow) with intact synapses. (×2000.)

at P450–P640, eight wild-type (+/+) mice of the background strain at P19–P420, and eight C57BL/6J and DBA/2J mice at P19–P420 (P. Merz, personal communication).

General autopsy, including bone marrow examination, of five mutants at P18, P25, and P60 was unremarkable. Aganglionosis or other abnormalities of the gastrointestinal tract was not detected in random histological sections, but quantification or subclassification of enteric system neurons has not been attempted. In the eye, choroidal melanocytes are not distinguished until P32, after which time their numbers appear decreased in comparison with wild-type controls. Pigment granules are present in the retinal pigmented epithelium.

In +/*gt* mice, qualitative examination of central and peripheral myelin and of eye has revealed no abnormalities up to P578, the oldest age studied. However, mild to moderate vacuolation is seen from P59 onward in the same gray matter sites affected in *gt/gt* mice and is ultrastructurally indistinguishable. In contrast to the homozygotes, white matter vacuolation is minimal. Such CNS vacuolation is not present in wild-type (+/+) mice of the background strain or any other strain in our colony at any age examined, with the exception of one female from the inbred colony examined at P341 and found to have minimal vacuolation in white matter of spinal cord and rostral brainstem. Her classification as +/+ was based on generation of 0/17 affected progeny when she was mated to a known +/*gt* male. The degree of vacuolation and sites of involvement were far less than in +/*gt* mice of similar age.

Transmission study

Ninety-nine mice of seven strains obtained from The Jackson Laboratory were inoculated with homogenized *gt/gt* brain on P0 to P6 (Table 2). We used three strains homozygous for the *Fv-1*^{n} allele (DBA/2J-*cri*, DBA/2J, and C3HeB/FeJ) and three homozygous for *Fv-1*^{b} (BALB/cBy, BALB/cWt, and C57BL/6J). These alleles restrict replication of B-tropic and N-tropic ecotropic murine leukemia viruses, respectively. The inoculum was prepared by light homogenization of the brains of two mutants at P30 in lactated Ringer's solution followed by dilution of the suspension to approximately 5% (estimated, wt/vol); 0.01 ml of the supernatant (gravity sedimentation) was injected intracerebrally. The inoculated mice were kept in isolation in a quarantine facility with their parents until weaning (P18–P22) and then were separated into cages with littermates of the same sex. The parents and inoculated mice were housed for life in the quarantine facility, where they were fed standard laboratory chow and water *ad lib* and maintained on a 12-hr:12-hr light/dark cycle. Noninoculated control mice of the same strains were housed separately in nonquarantine animal rooms but were otherwise maintained similarly to the inoculated mice and their parents.

Of the 99 mice inoculated with *gt/gt* brain homogenate, 81 mice survived to weaning and, of these, 73 mice were examined histologically at serial time points 17 to 721 days after inoculation (Table 2). None developed hindlimb paralysis, overt tremor, or seizures. Ruffled fur and mildly unsteady gait were present in aged inoculated and noninoculated control mice. CNS vacuoles were present in 7 of 13 mice of the HYIII/Le × C3B6-*A*/*A*$^{w\text{-}J}$-*gt*/+ genetic background from 88 days after inoculation onward. Progeny tests to distinguish +/*gt* from +/+ genotypes among these mice were unsuccessful but it is likely that several of these mice (statistically, two-thirds of them) were heterozygotes, shown after initiation of this experiment to have spontaneous CNS vacuolation (see above). We noted no difference in the rate of progression of the spongiform lesion between these mice and proved heterozygotes.

No definitive CNS vacuolation was detected in non-*gt*

Table 2. Summary of transmission study

Strain	Survivors at weaning/mice inoculated	Age at inoculation	Mice with CNS vacuolation/mice examined histologically					
			17	88	192	233–488	682–721	Total
C3B6-*A*/*A*$^{w\text{-}J}$-*gt* (+/−)	14/22	P2, P4	0/2	1/2	2/4	2/3	2/2	7/13
DBA/2J-*cri*	4/7	P1	0/1	0/1	0/1	0/1	NA	0/4
DBA/2J	7/8	P2, P4	0/2	0/1	0/2	0/1	NA	0/6
C3HeB/FeJ	11/11	P0	0/1	0/1	0/2	0/3	4/4	4/11
BALB/cBy	6/11	P3	0/1	NE	0/2	0/1	1/1	1/5
BALB/cWt	8/8	P2	0/1	0/2	0/2	0/3	NA	0/8
C57BL/6J	31/32	P2–P6	0/4	0/4	0/8	0/6	2/4	2/26
Total	81/99		0/12	1/11	2/21	2/18	9/11	14/73

Mice were examined at serial time points from 17 to 682–721 days after inoculation. NA, not available; NE, not examined.

strains until 682 days after inoculation. At 682–721 days, the termination of the study, all four C3HeB/FeJ mice, the one surviving BALB/cBy mouse, and two of four C57BL/6J mice had CNS vacuolation (Table 2). Of the C57BL/6J mice, two were negative at 682 days and two were mildly positive (less severe than the C3HeB/FeJ and BALB/cBy mice) at 721 days. Vacuolation in all three strains was most prominent in brainstem neuropil with mild to minimal involvement of thalamus and spinal cord; cerebral and cerebellar cortex, basal ganglia, and hippocampus were spared, as in the early stages of the naturally occurring disease. The three affected BALB/cBy and C3HeB/FeJ brains examined ultrastructurally contained no recognized viral particles.

All seven C3HeB/FeJ mice present 233 days after inoculation appeared slightly tremulous and were considered abnormal; however, there was no progression of signs, and vacuolation was not recognized until about 13 months later (Table 2). One BALB/cBy mouse was slightly tremulous about 375 days after inoculation but also showed no progression; its brain was extensively vacuolated 711 days after inoculation. The C57BL/6J mice were behaviorally normal at all times.

At 682 days one inoculated C3HeB/FeJ mouse appeared chronically ill with weight loss, generalized weakness, and reduced activity. At autopsy the brainstem was moderately vacuolated (Fig. 4), with thalamus and spinal cord less involved. This animal also had a chronic meningitis with lymphocytes and rare plasma cells but no intraparenchymal inflammation or viral inclusions. The liver showed chronic inflammation and nodular regeneration; with diligent searching, two extracellular, immature C type retroviral particles were detected in a single electron microscopic field. In no other affected inoculated mouse was there evidence of inflammation or viral inclusions intra- or extracranially. CNS vacuoles or inflammation were not present in C3HeB/FeJ, BALB/cBy, and C57BL/6J control brains. In other studies, hundreds of mice inoculated with homogenates from the brains of control animals did not develop a neurological disorder (see, e.g., ref. 6).

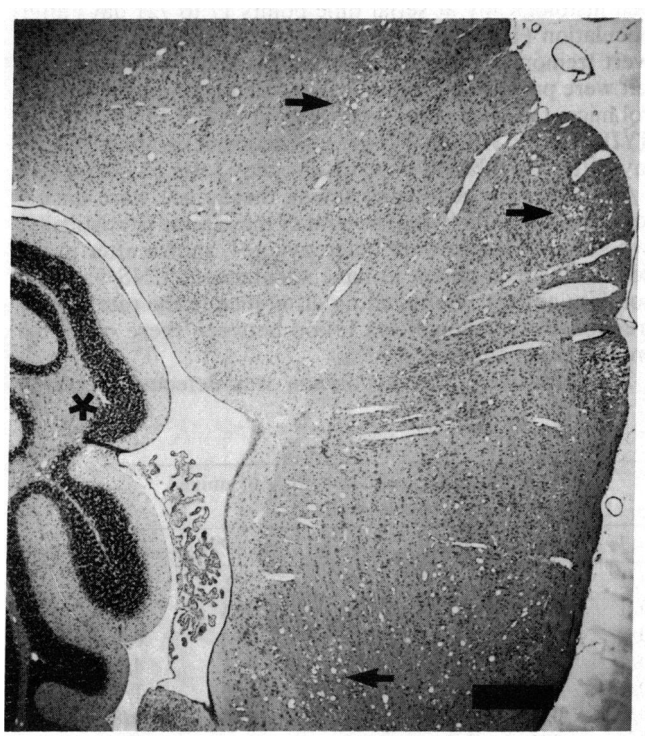

FIG. 4. The spongiform lesion consists of irregular, fine vacuoles distributed nonuniformly through the neuropil of the brainstem (arrows) and the cerebellar white matter (*) of an inoculated C3HeB/FeJ mouse 682 days after inoculation. (Bar = 250 μm.)

The CNS of eight male and female parents (BALB/cWt, BALB/cBy, C3HeB/FeJ, DBA/2J, C57BL/6J, and C3B6-$A/A^{w\text{-}J}$-*gt*/+) of the inoculated mice were examined at ages 13–27 months. The one male BALB/cWt parent demonstrated mild noninflammatory vacuolation in the thalamus and brainstem when killed 686 days after inoculum of his offspring. He had appeared ill with head bobbing, hunched back, and ruffled fur for his final 10 months and was hyperactive for 2 months but displayed no tremor or seizures. All progeny in his inoculated litter of eight had survived to weaning, so that cannibalism cannot be invoked as a route of transmission to account for his disease; he could, however, have licked inoculum leaking from the injection sites. None of his offspring had brain vacuoles but all were killed before 1 year of age. Of five other noninoculated parents killed at 13–20 months of age, two females (C3HeB/FeJ and BALB/cBy) had mild vacuolation in the brain but had demonstrated no overt illness. There had been no neonatal deaths among the C3HeB/FeJ offspring; four of the 11 offspring had CNS vacuolation 682 and 721 days after inoculation. One of seven of the BALB/cBy offspring had died as a neonate and may have been cannibalized; a littermate examined 711 days after inoculation had CNS vacuoles. C57BL/6J and DBA/2J parent brains were unremarkable.

Interpretations and conclusions

1. The pigmentation defects and the unusual abnormalities of peripheral nerve myelination suggest that the gray tremor locus affects neural crest derivatives prior to birth, with ongoing effects on neural-crest derivatives into the neonatal period.

2. The gene dose dependency of the spongiform change (early, severe, and symptomatic in the homozygote and late, mild, and asymptomatic in the heterozygote) suggests that this lesion may be close to the primary action of the altered gene product. No information is available as to whether a common fundamental abnormality might underlie the developmental disorders of pigmentation and myelination as well, or alternatively, whether *gt* is a mutation involving more than one gene.

3. Gray tremor's spongiform encephalopathy and the transmitted disease in genetically normal mice share many morphological features with disorders known to be due to the unconventional transmissible agents (4, 23), particularly experimental Creutzfeldt–Jakob disease expressed in mice (24, 25). However, the transmissible agent in gray tremor has not yet been identified, and certain strains of ecotropic murine leukemia virus have been reported to cause a noninflammatory spongiform encephalopathy in wild mice (9–12). In this entity, abundant type C virus particles (9, 11, 15), as well as occasional type A particles (9), are readily identified ultrastructurally. Susceptibility to type C retrovirus expression is influenced by the Fv-1 genetic locus (15, 16). The transmission of disease to both *Fv-1*^{n} and *Fv-1*^{b} strains of mice suggests that, if ecotropic murine leukemia viruses are involved in the disease process, they may have unusual host range characteristics that distinguish them from the endogenous N-tropic ecotropic leukemia viruses of C57BL/6 and C3H mice. The significance of the rare type A particles in one degenerating cell in the spinal cord of a P31 *gt*/*gt* mutant and type C particles in the abnormal liver of one inoculated C3HeB/FeJ mouse is unknown; their presence may be incidental, however, since retroviruses are endogenous in the murine genome (26).

4. The results of the transmission study indicate that the gray tremor mutant represents a naturally occurring spongiform encephalopathy whose expression is determined by the

interaction of genetic factors and an unconventional, retroviral, or hitherto unrecognized transmissible agent. Like the known unconventional transmissible agents, the agent in gray tremor is nonimmunogenic, as suggested by absence of inflammation, and has an unusually long incubation period in the genetically normal host. (This incubation period may, in future studies, change with route and dose of inoculum and age of recipient.) Similarity is further emphasized by the corresponding distribution of the initial lesions. The gray tremor agent is unusual, compared with known unconventional agents, in showing horizontal transmission as evidenced by the finding of vacuolation in brains of parents of inoculated newborns. Also unusual would be affection of the nervous system during its developmental phase, if this also proves referrable to the transmissible agent.

5. It is unknown whether *gt* is a genetic locus controlling host susceptibility or length of incubation period of the transmissible agent or represents the integration site of the agent in the host's genome with vertical transmission through the germ line. Identification of the infectious agent and of the genetic mechanisms governing expression may provide further insight into the general class of unconventional transmissible agents.

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