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A transmissible Creutzfeldt–Jakob disease-like agent is prevalent in the human population

(scrapie/spongiform encephalopathy/buffy coat/dementia/Alzheimer disease/slow virus)

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ABSTRACT **The etiology of most human dementias is unknown. Creutzfeldt–Jakob disease (CJD), a relatively uncommon human dementia, is caused by a transmissible virus-like agent. Molecular markers that are specific for the agent have not yet been defined. However, the infectious disease can be transmitted to rodents from both brain and infected buffy coat (blood) samples. To determine whether human CJD infections are more widespread than is apparent from the low incidence of neurological disease, we attempted to transmit CJD from buffy coat samples of 30 healthy volunteers who had no family history of dementing illness. Primary transmissions from 26 of 30 individuals produced CJD-like spongiform changes in the brains of recipient hamsters at 200–500 days postinoculation. This positive evidence of viremia was found for individuals in all age groups (20–30, 40–50, and 61–71 years old), whereas 12 negatively scored brain samples failed to produce similar changes in hamsters observed for >900 days in the same setting. We suggest that a CJD agent endemically infects humans but only infrequently produces an infectious dementia. Disease expression is likely to be influenced by several host factors in combination with viral variants that have altered neurovirulence.**

Creutzfeldt–Jakob disease (CJD) is most rigorously defined by the demonstration of a biologically infectious and replicating agent. This is accomplished by transmission to susceptible hosts. In this laboratory, inoculation of 23 typical human CJD brain samples to rodents has resulted in characteristic spongiform changes in the brain after a prolonged incubation period (reviewed in refs. 1–3). As in other viral diseases, serial passages lead to a reduced incubation time and more obvious clinical and pathological sequelae (1, 2). Presumably more virulent agent strains are selected in this process. The spongiform changes observed in experimental CJD are similar to those seen in the human host and to those produced by scrapie, a more common transmissible disease of sheep. Nonetheless, most strains of scrapie are demonstrably different from CJD, and the degree and pattern of vacuolization (spongiform change) and clinical symptoms can be quite distinct with different strains or isolates of these infectious agents in the same host (2, 4, 5). Other modifying factors for experimental disease expression include the titer of the inoculum, the route of inoculation, and the genetic characteristics of the recipient host.

We had previously considered that human CJD infections might be more widespread than is apparent from the incidence of neurological disease and might also be implicated in some cases of Alzheimer disease (AD) or other dementias of unknown etiology (1, 2). The descriptive classification of AD may camouflage a heterogeneous group of disorders with respect to underlying causation, and end-stage pathological stigmata of AD can sometimes be accompanied by CJD-like spongiform changes. In addition, AD and CJD can afflict members of the same family (6). We therefore initiated a pilot study to test for the presence of a transmissible CJD-like agent in the leukocytes (buffy coat) of healthy individuals who had relatives with sporadic AD (7). We supposed this group of people might be preferentially infected with this type of slow virus, known from iatrogenic infections to take as long as 30 years to elicit neurological disease (8). In such prolonged iatrogenic infections, the CJD agent is presumably hidden (latent) or restricted from replication in the brain.

Our original demonstration of viremia in experimental rodent (9) and human CJD (10) has been confirmed in other laboratories working with either CJD (11) or animal agents of this same nosological class such as scrapie (12). Buffy coat cells could serve as one peripheral reservoir for the infectious agent. Moreover, because CJD and scrapie are present in the blood at early stages of infection (9, 12), it was pertinent to evaluate healthy individuals without neurological symptoms. Viremia is a classical finding in many viral infections, including poliomyelitis, where only a few of many infected individuals develop neurological sequelae. In poliomyelitis, large numbers of people could be studied for antibodies to a viral antigen. Unfortunately in CJD (and scrapie), no specific viral antigens or nucleic acids have yet been identified, and the ultimate proof of these infections still rests on lengthy transmission experiments.

Our initial pilot study showed that 5 of 11 healthy individuals with AD relatives carried a transmissible CJD-like agent in their leukocytes (7). Potential contamination problems, one of our acknowledged and reasonable concerns, were addressed in a repeat experiment on these same individuals, using fresh blood samples processed in virgin homogenizers. Remarkably, every available buffy coat (seven tested) yielded the same positive or negative transmission data for each individual as was found in the pilot study (13). At the same time, we also initiated a larger study of healthy volunteers who had no family history of dementing illness in order to assess the prevalence of these infections in a more representative population. Individuals in three age groups—20–30, 40–50, and 61–71 years old—were tested for a transmissible viremia with the premise that older individuals might show a higher incidence of infection. Contrary to our expectations, we report here that most samples from all the age groups showed evidence of a CJD-like agent. Control animals were inoculated with negatively scored brain samples and housed with the experimental group to test for both instrument and horizontal contamination.

MATERIALS AND METHODS

LVG hamsters (6–8 weeks old) were inoculated with well-suspended buffy coat homogenates from 30 volunteers as

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Abbreviations: CJD, Creutzfeldt–Jakob disease; AD, Alzheimer disease.

*Deceased November 11, 1992.

described (7) using six hamsters for each sample. Brand new homogenizers (two for each of the three age groups) and sterile disposable (single use) syringes and collection tubes were used. For control studies, 12 brains scored as negative in pilot buffy coat transmission studies (7, 13) were prepared with instruments recycled from standard high-titer brain transmission experiments to test for spurious contamination. Both sets of animals were housed together in common cages that were randomly recycled after autoclaving. Randomly selected animals were sacrificed at periodic intervals >200 days postinoculation to minimize the number of animals found dead that could not be scored histologically. Hamsters were observed daily, and at sacrifice the uninoculated left half of the brain without the needle tract was used for histological scoring. The left half of the brain was fixed in buffered formalin for 2–3 weeks, and then 3- to 4-mm coronal sections were embedded in paraffin to ensure systematic sampling. Slides coded by number were scored blindly for spongiform changes alongside comparably numbered inoculated controls. In some cases, serial sections were used to evaluate the extent of the lesions. Visceral organs were routinely checked for manifestations of unrelated diseases and other spurious causes of death. Very mild diarrhea was found in <2% of our hamsters. Diarrhea due to *Clostridium* typhilitis, usually triggered by stress or spread by fecal contamination of animal technicians' gloves (14), extinguished one scrapie colony elsewhere in a short period (15) but resulted in no neuropathological changes (16).

RESULTS

Careful neuropathological evaluation was essential because clinical signs of disease can be very subtle, or completely absent, in primary cross-species CJD transmissions (1). This parallels the large primary transmission study of Icelandic sheep scrapie (RIDA) where clinical signs of disease were ambiguous or inapparent and >40% of the mice were found dead (17). Random sacrifice of hamsters at appropriate incubation times for CJD (>200 days) minimized the number of animals found dead in the current experiment and allowed us to histologically score a large number of hamsters for up to 950 days. Tabulation of blindly scored animals showed positive and unambiguous CJD neuropathology only in the buffy coat and not in the control inoculated brains. In these positive brains, spongiform changes in the neuropil were identified as early as 210 days postinoculation and typically were visible at both low and high magnification (Fig. 1 *A* and *B*). Such positive changes were not observed in very old hamsters inoculated with negatively scored brains (Fig. 1*C*). Vacuolization was focal, as assessed by serial section and systematic evaluation of all coronal blocks. The distribution of lesions, predominantly in the cortex or Ammon's horn, and less frequently in the basal ganglia, and the noninflammatory vacuolar changes are characteristic of rodent CJD. Identical focal changes, unassociated with prodromal signs, have similarly been observed in low-titer intracerebral (9) and intraocular (18) CJD inoculations, as well as with peripheral routes of injection (1, 2). In contrast, serial hamster passages from such specimens produce more widespread cortical lesions with a prominent astrocytic response at reduced incubation times (1, 2, 7, 19). Even more subtle spongiform changes have been noted by using high dilutions of scrapie and/or particular scrapie strain–host combinations (17, 20). Thus, the focal changes observed in the present study are likely to be a consequence of the known low titer of agent in buffy coat samples (9) as well as the reduced rodent susceptibility to this human CJD-like agent.

Control animals inoculated with brains that had been blindly scored as negative in the pilot studies (7, 13) were used to assess horizontal and instrument contamination as well as nonspecific aging changes. Because we have previously found occasional dropout of neurons in the brainstem of older uninoculated animals, only hamsters with more severe focal spongiform changes in the brainstem were scored as positive. Such brains have previously been shown to serially transmit CJD (21). There was no obvious difference in the distribution or severity of lesions in buffy coat inoculated animals sacrificed at different times. For positive scoring we completely ignored white matter and cerebellar changes that can be age related or artifactual. Inconspicuous telencephalic vacuoles were marked questionable and were not counted in the final positive score. Table 1 shows a detailed summary of the scored data for every hamster inoculated with the buffy coat samples from the three age groups. The microscopic changes were comparable for all three age groups.

The significance of even a single positive animal becomes apparent when the cumulative data from these buffy coat samples (Fig. 2*A*) are compared with attempted transmissions from brain samples scored as negative for CJD (Fig. 2*B*). None of these control hamsters evaluated for up to 850 days showed the above spongiform changes, and only two control brains were scored as questionable. Moreover, these 12 negative brain samples were prepared in homogenizers previously exposed to high-titer CJD material. Additional protein and passage data (51) contradict the speculation that our previous positive serial passages from human buffy coat samples were due to contaminated homogenizers (15). Lat-

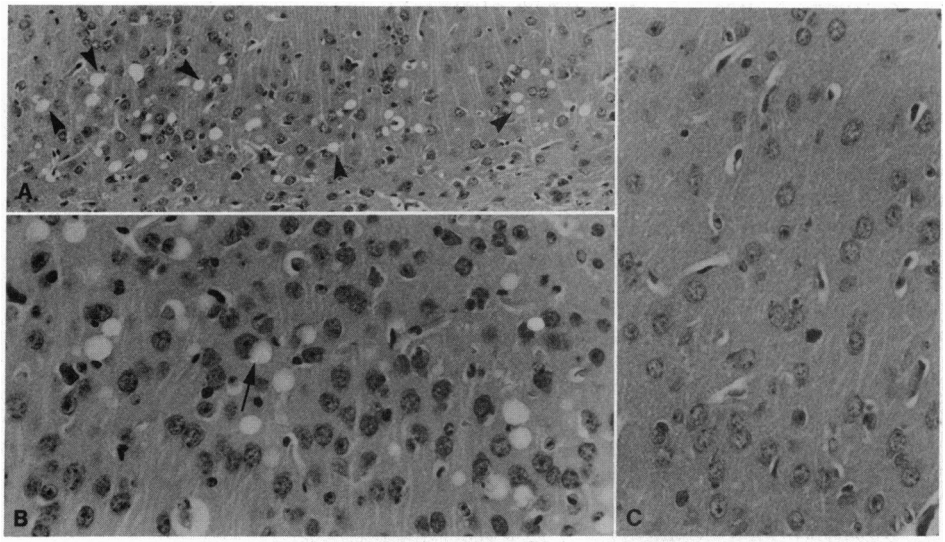

FIG. 1. (*A*) Characteristic focal vacuolization (as at arrowheads) in the cortex of a hamster sacrificed at 231 days postinoculation from age group A (Table 1, A10, Ham2) is obvious at low magnification. (×55.) (*B*) Similar cortical changes at 232 days from group C (Table 1, C3, Ham1) at higher magnification. (×220.) This representative photomicrograph shows vacuolization in the neuropil, with one vacuole impinging on a neuron at arrow. (*C*) Comparable and representative (negative) region of cortex from a hamster inoculated with a negatively scored brain (E 2844, Ham3). Sacrifice was at 761 days. (×320.)

Table 1. Buffy coat samples from healthy volunteers in each age group (group A, 20–30 years old; group B, 40–50 years old; group C, 61–71 years old) inoculated into hamsters

Group	Sample	Age/ sex	E	Ham1	Ham2	Ham3	Ham4	Ham5	Ham6	Score
A	1	20/F	2889	65: FD	284 −	294 −	304: FD	362 −	791 −	0 (4)
	2	26/F	2890	115: FD	284 +	346 −	389 −	368: FD	826: ?	1 (4)
	3	30/F	2891	284 +	313 ?	389 +	427: FD	578 +	622 −	3 (5)
	4	26/F	2892	258: FD	284 +	294: FD	346 +	687 −	826: ?	2 (4)
	5	22/F	2896	239 −	259: FD	341 +	397: FD	406 +	761 +	3 (4)
	6	24/F	2897	215: FD	239 −	268: FD	298 +	334 +	472 +	3 (4)
	7	24/F	2899	232 +	334 +	385 −	471 −	571 −	603 −	2 (6)
	8	30/M	2900	232 −	298: FD	315: FD	334: ?	472 +	619 −	1 (4)
	9	29/F	2901	51: FD	232 +	334 +	449 −	641 +	680: FD	3 (4)
	10	24/F	2907	15: FD	231 +	318 −	374: FD	471 +	651 −	2 (4)
	11	30/F	2920	210 −	308: ?	353: FD	407 −	660 −	721 −	0 (5)
	12	28/F	2921	175: FD	210 −	308 +	350 −	394 −	631 −	1 (5)
B	1	50/M	2883	283 −	336 −	336 −	661: FD	778 −	ND	0 (4)
	2	48/F	2884	283 −	346 −	441 −	465 +	826 +	898 −	2 (6)
	3	42/F	2886	283 −	346 −	441 +	693: FD	798 −	798: ?	1 (5)
	4	49/F	2887	231: ?	294 −	389 +	631: FD	826 +	946 −	2 (5)
	5	41/F	2888	283 +	294 −	389 −	593 −	629: FD	766 −	1 (5)
	6	44/F	2893	115: FD	284 −	328 +	338: FD	346 +	723 +	3 (4)
	7	40/F	2902	232 +	284 −	334 −	371: FD	385 −	472: ?	1 (5)
	8	45/F	2905	231 −	171: FD	359 +	471 +	524: FD	548 −	2 (4)
	9	45/F	2906	231 +	359 +	452 −	557 −	589 −	ND	2 (5)
	10	42/F	2908	1: FD	160: FD	236 +	258: FD	359 −	471 +	2 (3)
	11	40/F	2917	75: FD	113: FD	121: FD	131 −	210 −	308: ?	0 (3)
C	1a	71/M	2882a	202: FD	241: FD	257 +	293: FD	344 −	414 +	2 (3)
	1b	71/M	2882b	188: FD	332: FD	351 −	463 −	490: ?	518 +	1 (4)
	2	63/F	2885	283 −	346 +	375: FD	457: ?	791: FD	826 +	2 (4)
	3	63/F	2898	232 +	238 +	334 +	438 −	533 −	737 −	3 (6)
	4	65/F	2903	231 −	359 +	454 +	589 −	788 −	878 −	2 (6)
	5	64/F	2904	231 −	276: FD	303: FD	359 +	470 −	482 +	2 (4)
	6	64/M	2918	1: FD	1: FD	210 −	261 +	399 −	691 +	2 (4)
	7	66/F	2919	1: FD	210 +	243: FD	308: ?	406 −	538 −	1 (4)

Detailed data for each recipient hamster (Ham1 to -6) show the days and the histological score: positive for spongiform CJD changes (+), negative (−), questionable (?). Animals found dead (FD) were not used for scoring. ND, not done. The tabulated score (last column) shows the number of positive animals in each case, with the total scored hamsters in parentheses. In group C, one sample (1b) was reinoculated at a 1:2 dilution to increase the total number of scored animals for this sample. Second passages have been started and one brain (C3, Ham1; see Fig. 1*B*) has already shown positive serial transmission.

eral transmission was also very unlikely because the negative control animals were housed with the positive animals. In addition, previous experiments here have shown no horizontal spread of CJD, even in attempts to demonstrate maternal transmission of CJD to offspring (22). Likewise, human CJD is not detectably contagious, even to a spouse. The oral route of spread is also known to be inefficient in experimental CJD and requires sustained feeding, and/or ingestion of large amounts of infectious material, an unlikely source of contamination with the present buffy coat inoculations. In addition to the 40 negative hamster control brains depicted in Fig. 2*B*, >40 other well-preserved brains were similarly scored as negative during this same period. Inoculated samples included human brain specimens diagnosed as CJD at the National Institutes of Health that were not transmissible in primates as well as some, but not all, tumorigenic CJD brain cultures passaged *in vitro* for >2 years (23, 24).

The cumulative data in Fig. 2 clearly show that most animals inoculated with buffy coat were scored positive between 200 and 500 days, with approximately every other hamster exhibiting CJD spongiform changes. This is typical incubation time for human to hamster transmissions that have a mean of 425 ± 12 days (3). Although confirming serial passages will take >3 years, one positive brain from an animal sacrificed early (Fig. 1) has already been positive in serial passage. Prior serial passage experiments have also confirmed our diagnostic accuracy in primary CJD transmissions (e.g., see refs. 1, 13, and 21). Nonetheless, it is entirely possible that some brains we judged to be positive histologically will not be verified by serial passage. As noted, local toxic or direct effects of the inoculum were ruled out by restricting the histological diagnosis to the uninoculated half of the brain, and the neuropathology makes the involvement of conventional inflammatory viruses, such as Epstein–Barr virus, which persistently infects human lymphocytes, quite remote. The picture is most consistent with propagation of either a CJD-like agent or another noninflammatory slow virus of humans.

DISCUSSION

These substantial positive data, and the corresponding negative control data, strongly suggest that these buffy coat samples contain an infectious CJD-like agent. The preponderance of volunteers were female, but there is no reason to believe that samples from healthy male volunteers would not be similarly positive. The apparent incidence of viremia in this study is clearly much higher than the incidence of neurologically expressed CJD. Moreover, the transmissible infection shown here is not restricted to families with sporadic AD. Rather, it appears to be a common infection in our asymptomatic volunteers. In a more limited transmission study of one Japanese AD family, buffy coat samples from two afflicted and one healthy relative all produced massive

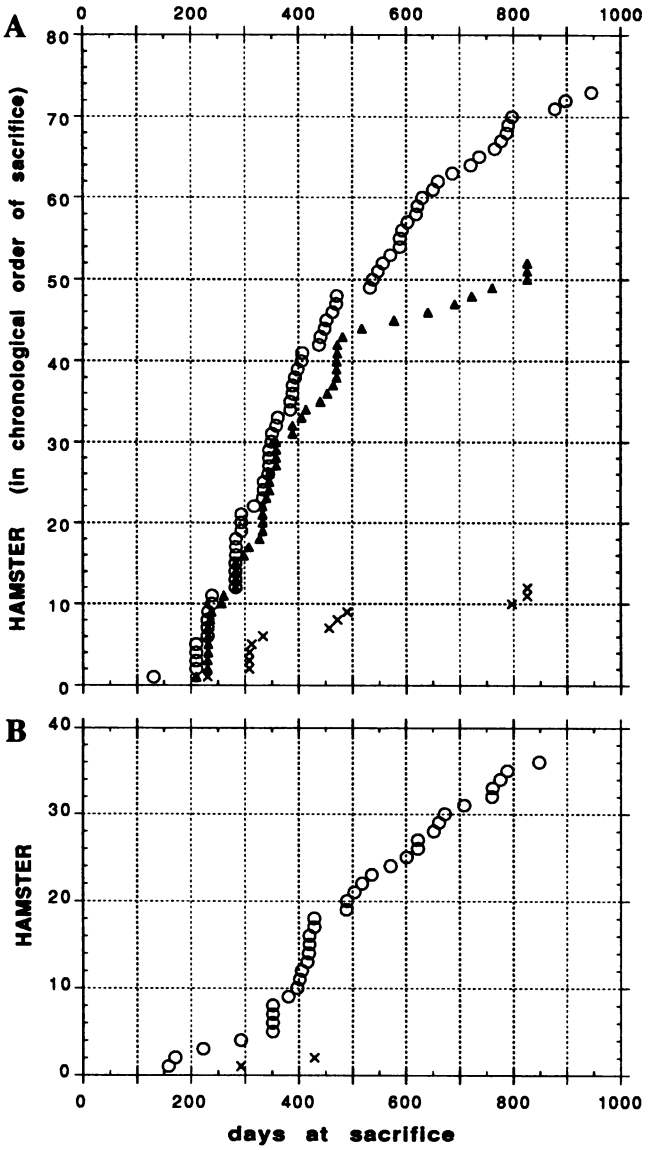

FIG. 2. Cumulative data for every histologically scored hamster inoculated with buffy coat (*A*) compared with all similarly scored control animals inoculated with 12 negative brain samples (*B*). Each point represents a single hamster in chronological order of sacrifice by diagnostic group. ▲, Positive animals; ○, negative animals; ×, questionable animals. In *B* (as in *A*) three to five hamster brains were scored histologically for each inoculum (E numbers were 2841, 2842, 2843, 2844, 2870, 2927, 2931, 2933, 2938, 2940, 2941, and 2942) and unscored brains (found dead) were collected at times comparable to those detailed in Table 1. No visceral pathology was seen in the positive animals.

intracytoplasmic neurofilament accumulations in brainstem neurons but no clear spongiform changes (25). Although it is not yet clear whether this pathology was caused by a transmissible agent or other factors, different agent strains are known to produce different neuropathological sequelae (cf. ref. 2). Japanese variants of CJD cause a highly unusual human leukoencephalopathy (26) and unlike western CJD isolates readily transmit to mice (3). Thus, the changes in that study could be the result of an unusual agent strain, where neurofilament accumulations signify a more AD-like infectious agent. Some CJD cases show little vacuolization (2) and fatal familial insomnia without spongiform lesions have been identified by host prion protein changes (27). This "prion disease" is presumably infectious, although it has not been transmissible from brain samples of symptomatic individuals (27, 28). Interestingly, the healthy individuals without any family history of dementia in our current study yielded more positive takes than those from our pilot AD studies (7, 13). It is conceivable that a common and protective CJD-like variant of low virulence might be less prevalent in some AD families. Alternatively, if a CJD-like variant can cause some cases of AD, it could function by a hit and run mechanism, as in postencephalitic Parkinson disease, or it could continuously and subliminally damage neurons by a persistent low-level infection. In both cases AD-specific pathological sequelae might be evoked in the human host, and the infection would be difficult to transmit from brain samples.

Precise numbers for the incidence of CJD viremia in the general population, as well as an absolutely unequivocal answer to any questions of contamination, will ultimately depend on an agent-specific marker. A host membrane glycoprotein of 34 kDa, commonly known as "prion" protein, has been associated with scrapie and CJD (29, 30). Some investigators believe this host protein is modified so that it resists limited proteolysis and simultaneously acquires attributes of infectivity (31), whereas other investigators consider such changes to be part of the cellular or pathological response to a virus (32–34). Although this protein is a useful marker in ≈75% of clinical or late-stage human CJD specimens (35), it is not detectable in brain samples with significant scrapie titers (e.g., see refs. 34 and 36) and cannot be detected in rodent CJD until titers are $\geq 5 \times 10^6$ infectious units, even with very sensitive chemiluminescent techniques (51). Therefore, an independent assessment of low-titer CJD samples, such as buffy coats from CJD patients, or contaminated lots of growth hormone, are currently not viable with this prion protein assay. Biological evidence for 15 distinct agent strains in scrapie alone underlies the assumption of an agent genome (4, 5, 17), and purification experiments show the infectious CJD agent is separable from the majority of prion protein as a core-like nucleic acid–protein complex of $\approx 10^7$ Da and 30 nm (37–39). Therefore, more sensitive agent-specific markers should be sought for rapid and very large scale studies of the population, as well as for elucidation of variant viral genomes.

The transmission of CJD from many human buffy coat samples, but only infrequently from brain specimens, strongly implicates a natural resistance to neurological involvement over many years. If people commonly harbor a CJD-like agent, as indicated by the above data, it must originate from a widely prevalent endemic infection or be of endogenous origin. In either case, specific host genetic susceptibility features, and possibly developmental, humoral, immunological, or exogenous factors, would be critical for the infrequent expression of neurological disease. Aging itself, a segment of the developmental category, is most relevant because CJD encephalopathies are found almost exclusively in older individuals. The demonstration of viremia in younger individuals (20–30 years old), and the uncommon finding of a CJD encephalopathy in a 2–5-year-old infant (40), is consistent with a late age-related susceptibility factor (51). This end of the age spectrum is rarely considered in progressive viral infections, whereas many viruses have been tested for preferential virulence in early life. These include several neurotropic viruses, such as Sindbis, which shows an age-dependent encephalitis (41). With respect to host genetic susceptibility factors, transgenic and human studies strongly suggest that specific codon mutations in the host prion gene can influence both incubation time and disease expression (31). Remarkably, of the many people who received contaminated growth hormone, the few showing neurological disease have had an unusual preponderance of such mutations (8). Host membrane proteins are often involved in viral susceptibility, and host prion protein has been considered as a potential viral receptor (32, 51).

Neurovirulence can also depend on specific viral features. The history of poliomyelitis is instructive, because attenuated strains of poliovirus initially had to be produced by controlled passage in animals and cultured cells (42), a situation paralleled by the development of variant scrapie strains that are attenuated for their original host (4). Only recently have the molecular determinants of poliovirus attenuation become evident (43). It remains to be seen whether the CJD-like agent transmitted from normal buffy coat samples represents a less-virulent CJD strain. A neurotropic variant of this common strain could arise infrequently and cause neurodegeneration.

Observations in natural sheep scrapie led Dickinson *et al.* (44) to conclude that scrapie was an endemic infection. The sporadic worldwide incidence of neurodegenerative CJD may also be most easily explained by a common but generally asymptomatic infection. There is much precedence in virology for endemic infections that are not expressed except in special circumstances. Latent or persistent human viral infections, such as herpes simplex or JC papovavirus, are present in a large portion of the population but produce disease most often when host defenses are compromised. The JC virus targets the brain to cause multifocal leukoencephalopathy. Furthermore, feline immunodeficiency virus infections are epidemic, as shown by virus isolations from blood mononuclear cells, yet pathological symptoms have been observed only in domestic cats, in sharp contrast to asymptomatic wild feline carriers (45). This widespread retroviral infection has been ascribed to evolving symbiotic interactions between the host and the virus, a concept that can also apply to evolving endogenous retroviruses (46). We have postulated that CJD may contain or utilize retroviral sequences in its life cycle (3, 6, 23, 24, 47, 51), keeping in mind the known unconventional resistance of retroviruses to radiation and their ability to elicit noninflammatory neurodegenerations. Moreover, expression of an endogenous murine leukemia retrovirus is required for a late age-dependent motor neuron degeneration caused by a persistent togavirus (48). The recovery of >6000 contiguous bases of an endogenous retrovirus from highly purified infectious CJD brain samples exhaustively digested with nucleases (ref. 49; unpublished data) makes this precedent a viable scenario in CJD.

In view of the long and intimate relationship between humans and sheep, it might be expected that these two species commonly harbor the similar but distinct CJD and scrapie agents. The natural resistance of humans to scrapie-infected meat may signify a protective or interfering virus, as shown for experimental scrapie strains (50), that is advantageous for survival except in rare instances.

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