TRANSTHYRETIN AMYLOIDOSES OF FAMILIAL AMYLOIDOTIC POLYNEUROPATHY AS A PARADIGM FOR THE GENETIC CONTROL OF SPONTANEOUS GENERATION OF INFECTIOUS AMYLOIDS BY PATTERNED CONFIGURATIONAL CHANGE IN HOST PRECURSORS IN CREUTZFELDT-JACOB DISEASE

D. CARLETON GAJDUSEK
National Institutes of Health, Bethesda, Maryland. U.S.A.

SUMMARY

Free tematic index (added by the Editor)

Transthyretin amyloidosis. Infectious amyloid proteins. Unconventional slow virus diseases. Spongiform encephalopaties. Genetic control and point mutations.

RESUMO

Índice temático genérico (preparado pelo Editor)

Transtiretina e Amiloidose. Proteínas amiloides infecciosas. Doenças por vírus lentos. Encefalopatias amiloides. Controlo genético e mutações pontuais.

Familial amyloidotic polyneuropathy (FAP) caused by conversion of transthyretin, a normal serum prealbumin involved in the transport of thyroid hormones, to polymerized beta-pleated sheet structured amyloid fibers has provided an elegant paradigm for predicting the genetic control of the generation of infectious amyloid proteins in familial Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler syndrome (GSS). From the moment we have recognized that the unconventional virus infections of kuru-CJD-GSS--scrapie-BSE are transmissible amyloidoses of brain (Gajdusek 1988a, b, 1989, 1990, Gajdusek and Gibbs 1900) with a different precursor protein from the β/A4 amyloid protein precursor of the non-transmissible brain amyloidoses of normal aging and Alzheimer's disease, we seem to have been asking the right question for elucidating the molecular chemistry and genetics underlying the pathogenesis of the spongiform encephalopathies. As with all systemic amyloidoses, the first problems are to define the host precursor molecule and its function, and to elucidate the genetic, toxic and metabolic factors-even infectious-which precipitate the configurational crossed β-pleated change and polymerization of non-fibrillary precursor molecules into. insoluble amyloid fibrils. To understand the quantum mechanics of the polymerization resulting in amyloid fibril formation requires crystallographic analysis of the secondary, tertiary and quaternary fine structure of the precursor protein and its amyloid fibrils. With our hydrophobic molecules, even cloned in baculovirus, we have not succeded in obtaining the necessary crystals for x-ray crystallographic structural analysis. We hope to achieve this analysis with NMR comparison of the non-infectious full-length precursor with its configurationally altered infectious form.

Of most pertinence to our problem of the unconventional viruses, wich are infectious amyloids, have been the transthyretin (TTR) amyloidoses or familial amyloidotic polyneuropathies (FAP) (Costa et al, 1991). Patients are members of several dozen families scattered around the world in which the disease appears as an autosomal dominant trait. The onset of the clinical disease may occur at different ages and leads to the destruction of peripheral nerves by progressive deposition of amyloid in the perineurium. The human TTR gene has been cloned and its full sequence of 6.9 kb composed of four exons and three introns is known. Its enconding gene is located on chromosome 18 (Sasaki et al, 1985, Tsuzuki et al, 1985). Transthyretin in its pure and crystalline form is a soluble prealbumir of 14 kDa molecular weight with 127 amino acids. Its secondary, tertiary and quaternary structures have been determined by x-ray crystallography. It is a symmetrical tetramer of 55 kDa made of four subunits showing extensive β-pleated sheet structure (Blake et al. 1971, 1974, 1977, 1978). Thus, it is amyloidogenic by structural chemical considerations, but amyloid formation does not apparently occur *on the hoof* no sporadic cases with no mutation in the TTR gene are known.

Members of different affected families have a mutation resulting in a one amino acid substitution in the precursor that increases the statistical mechanical likelihood of the moleccule falling into the amyloid form by a factor of about 10^4 to 10^6. There is no one specific mutation causing the disease in all families. Thus, in over two dozen investigated families, 17 diferent mutations have been detected (see Figure 1). The FAP is thus caused by precipitation of amyloid formed from the transthyretin precursor, with any of a set of point mutations each causing a single amino acid

replacement which increases the statistical likelihood of amyloid formation. This amyloid is not a replication infectious molecule. Without one of these point mutations it is difficult to change the trasnthyretin polypeptide by concentration and nucleation into the amyloid configuration. With these single amino acid substitutions amyloid formation occurs as a much more likely stochastic event, even *in vitro* and extracellularly. There are also several silent polymorphisms in the population with point mutations causing non-pathogenic single amino acid substitutions (Figure 1).

The codon 30 point mutation with proline replaced by methionine has been expressed in transgenic mice which develop deposits of human amyloid containing the methionine-30 mutation similar to depositions in FAP, but also in the intestine and other tissues, and pass this trait to their offspring (Wakasugi et al. 1988, Yi et al. 1990).

Parallels to familial CJD and its familial GSS variant are obvious (Figure 2). Most GSS families display an amino acid replacement of proline by leucine at codon 02 (Goldgaber et al. 1989, Hsiao et al. 1989, 1990c). In a family with atypical GSS there is instead a replacement of alanine by valine at codon 117 (Doh-ura et al. 1989, Hsiao et al. 1990b). Other GSS families have none of these mutations (Hsiao et al. 1990a). The more common type of familial CJD has a codon 200 mutation which replaces glutamic acid with lysine (Goldgaber et al. 1989, Goldfarb et al. 1990a, b, c, d). This has now been found in 14 families. However, in a large Finnish kindred with CJD (Haltia et al. 1979) there is a replacement of aspartic acid by asparagine in codon 178 (Goldfarb et al. 1991) and there are Dutch , French, Hungarian and American CJD families also with codon 178 point mutation (Nieto et al. 1991). British colleagues investigating a pedigree with CJD patients in America and England have found a 48 amino acid insert, an octapeptide six-fold repeat, between

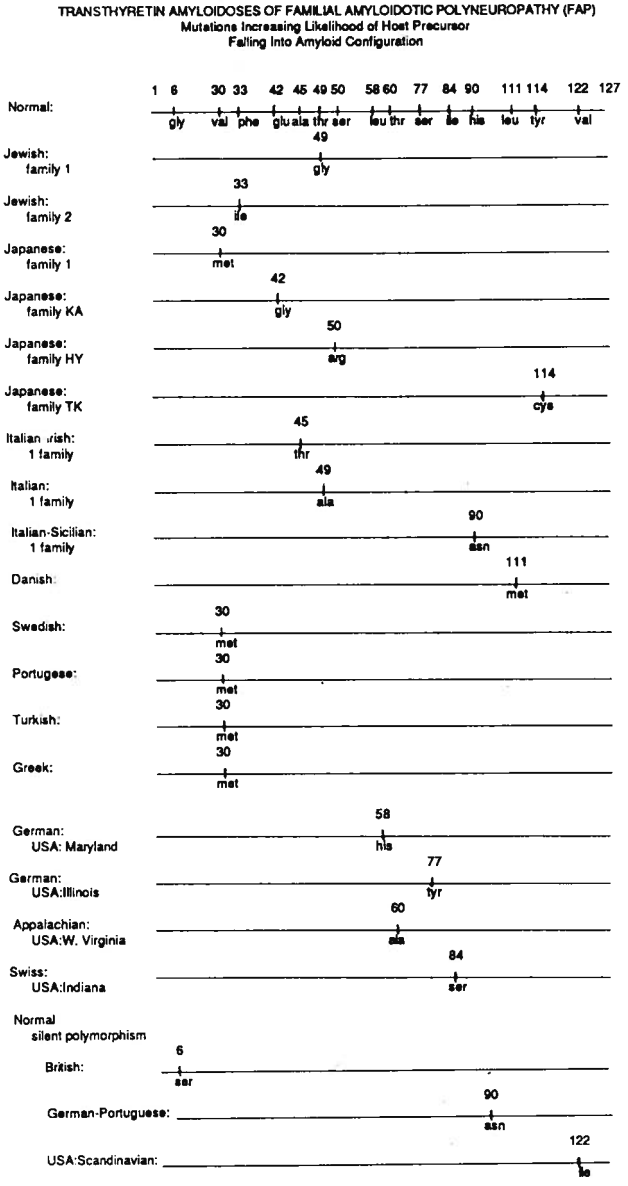

Fig. 1 — Sixteen different amino acid substitutions caused by point mutations in the gene specifying the transthyretin prealbumin precursor molecule in over twenty families of various ethnic origin are shown. Four of these families are normal without FAP and the mutation is a silent non-pathogenic polymorphism in these. On Codon 49 are two different amino acid substitutions, in a Jewish and Italian family, respectively. Codon 90 (his→ asn) mutation has apparently caused FAP in the Italian: Sicilian family, but not in Garman, Portuguese families.

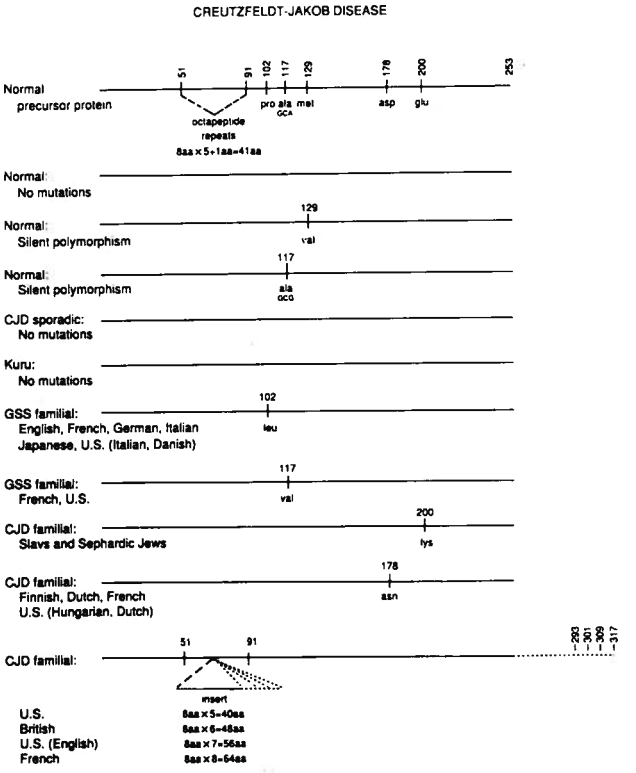

Fig. 2 — Five different amino acid substitutions caused by point mutations in the gene specifying the host precursor molecule CJD amyloid. Four are found in families of diverse ethnic origin with familial CJD and its GSS variant. The fifth is the codon 129 substitution of valine for methionine, which is a silent polymorphism found in about 20% of the normal population. Four additional mutations in families with CJD are insertions of octapeptide repeats into a region where there are already five copies of the same repeat. In a U.S family there are five copies of the octapeptide (8aa × 5=40aa) inserted, bringing the total to 10 copies; in a Dutch family there are six copies (6aa × 8=48aa) inserted, bringing the total to 11 copies in U.S. family from England there are seven copies (8aa × 7=56aa) inserted, brinding the total to 12 copies; in a French family, there are eight copies (8aa × 8=64aa) inserted, brinding the total to 13 copies. There are also families of familial CJD and GSS without any point mutations.

codons 51 nd 91 (Owen et al. 1989, 1990a, b). Thus we know five different mutations which are responsible for the alteration of the normal precursor protein into the infectious amyloid form.

At codon 129 we have a nonpathogenic point mutation with substitution of valine for methionine which is a silent polymorphism in the general population (Goldfarb et al. 1990a). Another silent polymorphism is a point mutation of codon 117 of GCA to GCG but causes no amino acid change. Finally we find some normal subjects carry four instead of five copies of the octapeptide normally at codon 41 to 91 (Goldfarb, et al. 1991c).

At present the best explanation for the regular incidence of sporadic non-familial CJD around the world is the *de novo* creation of the CJD amyloid infectious agent by a rare, spontaneous event occuring at a frequency of one per million population per annum, the surprisingly uniform world-wide incidence of CJD (Gajdusek 1990). If one of the point mutations of familial CJD is present this configurational change occurs with about a million-fold higher likelihood. One corollary of this paradigm that has already been proved is that the replication of the infectious amyloid caused by inoculations of a different individual does not *breed true*. The point mutation is not copied in the amyloid formed in the new host, although it, in turn, is also infectious. The new CJD amyloid has the amino acid sequence of the newly inoculated individual. CJD from patients with the 102, 117, 178 and 200 codon mutations have all been transmitted to monkeys or chimpazees wich do not carry these point mutations, nor do the infectious proteins made in these experimentally infected host contain those point mutations. The process of conformational change may well be an induced nucleation and homotaxic pattern setting for crystalline or fibril growth. The further elucidation of this transformation to β-pleated insoluble, protease resistant anf infectious configuration will require the full structural comparison of infectious and non-infectious forms of the molecule, probably by NMR.

Therefore the agents of these rare, so-called unconventional slow virus diseases present several heresies to those with implicit faith in the central dogma of modern molecular biology. First, these infectious amyloid proteins spontaneously create themselves; second, they contain no nucleic acid and thus new copies of themselves are made post-translationally from a host specified precursor; third, in their replication in a new individual they do not copy mutation in the nucleating and pattern setting agent. One implication of this is that if one inoculates goats with human brain material infected with CJD, GSS, or kuru, the goat will develop goat scarpie, with the sequence of its infectious amploid *virus* not that of man, but of the goat. If the agent is transmitted from goat to sheep, the sheep sequence will be found in the induced sheep scrapie agent. The goat is susceptible to kuru, to CJD and also to mink encephalopathy. If a mink is inoculated with sheep scrapie, the resulting mink virus has the amino acid sequence of the scrapie precursor protein of mink, not that of the sheep. The mink virus, however, no longer is infectious in the mouse, even if it originally came from a mouse passage of sheep scrapie. But, after passage through a goat, it will infect the mouse. These changes of host range appear to be mutations, and five years ago everyone would have considered them mutations, but they are, in fact, induced post translational configuracional changes in the presursor of the new host with the amino acid sequence of the normal precursor protein unaltered. In the case of BSE, whether it were induced by goat, sheep, mouse or hamster scrapie, mink encephalopathy or human CJD, GSS or kuru the infectious amyloid of the cow would all have the same amino acid sequence.

Dependence in Scrapie of Host Range, Incubation Period, Disease Duration, Plaque Production, and Lesion Distribution on Mutation in the Precursor Protein Gene

Primary transmission of any of these infections to a new host usually only affects a minority of the infect animals and only after a prolonged incubation period. This species barrier may disappear on one further passage in the new host, and incubation period is usually shortened. Incubation period may vary greatly in different breeds in mice, hamsters or sheep, as does also the apeearance of amyloid plaques.

These breed differences are associated with different sequences in the scrapie precursor protein.

In mice point mutation in the scrapie precursor protein determine the incubation period (Carlson 1986, 1988, Westaway et al. 1987). In different breeds of hamsters incubation periods are similary determined. Chinese and Syrian hamsters which develop amyloid plaques have mutation in codon 102 and 107, respectively, wich are not found in Armenian hamsters which do not develop plaques (Lowenstein et al. 1990). Transgenic mice expressing hamster scrapie precursor gene develop on hamster scrapie inoculation, hamster-specific amyloid plaques and hamster-like distribution of lesions. They express mostly hamster scrapie infectious amyloid protein and much less infectious mouse amyloid protein. When inoculated with mouse scrapie they express only mouse scrapie infectious amyloid and no plaques (Prusiner et al. 1990, Scott 1989). In different breeds of sheep the incubation period depends similary on the amino acid sequence in the precursor protein (Hunter ek al. 1989).

Expression of the CJD Precursor Protein with These Point Mutations in Transgenic Mice and Baculovirus Systems and Synthetic Polypeptides with These Mutations

Similarly to the deposition of human transthyretin (TTN) amyloid in transgenic mice wich have received the human TTN gene containing a point mutation causing FAP (Wakasugi et al. 1988, Yi et al. 1990), the GSS codon 102 point mutation (leucine replacing proline) implanted into transgenic mice with results in spongiform pathology similar to that in the human disease (Hsiao et al. 1990d, e). If the configurationally altered human precursor in these mice is an infectious amyloid our understanding will be enormously advanced.

We have now expressed these points mutations in the human scrapie precursor molecules cloned in baculovirus (unpublished data from this laboratory and Dimitry Golgaber; personal communication). We had also synthesized large portions of the human homologue of the scrapie precursor with the appropriate CJD — or GSS — inducing point mutations. These cloned proteins and the synthetic polypeptide have been inoculated in the CJD-susceptible monkeys held in scrapie-free facilities. If these synthetic polypeptides or the baculovirus-cloned proteins do serve as nucleants to produce the infectious transmission, the quick unravelling of the molecular event at an atomic level that produce this infectious change may be anticipated.

What Does *De Novo* Generation of an Infectious Protein Imply?

The full lenght 35 kDa infectious form of the scrapie precursor protein displays no amino acid change as compared to the naturally occurring form (Chesebro et al. 1985, Basler et al. 1986). The difference between the infectious form of the scrapie precursor protein and its non-infectious host precursor is not known and must eventually be resolved by x--ray crystallography or NMR techniques — but it is clearly a post-translational modification of the conformation of the

normal host precursor proteins. It may even involve a covalent or coordinate covalent chemical bond formation, but not with any change in amino acid sequence (Diringer et al. 1991, Safar et al. 1990a, b, c, 1991) (Figure 3).

Protein	*Process*	*Synonymy*
scrapie precursor		PrP^{c} 33-35
↓	induction by autopatterning of configurational change	↓
infections form of scrapie precursor		PrP^{Sc} 33-35
↓	proteolytic cleavage	↓
scrapie amyloid		PrP 27-30
↓	autonucleation of fibril polymerization	↓
scrapie-associated fibrils		prion rods

Fig. 3 — De novo generation of infectious scrapie amyloid proteins from the normal host scrapie precursor protein.

Nucleation of protein crystallization can be triggered by pulverized or ground mineral, as shown by McPherson and Shlichta (1988) for beef liver catalase, lysozime, canavalin, and concanavalin B. Different minerals cause different patterns of crystal growth in the same protein (Shlichta 1991). As many crystalline forms may be laid down as the different patterns of snowflakes or frost on a window or stalagmites or stalactites in a cavern. Any particicles of the grown crystal are themselves able to act as pattern-setting nucleants for further crystal formation. In scrapie infection it is likely that by a related process of pattern induction the infectious protein induces a change in structure on the normal precursor. The atomic, molecular and crystallographic details of new such a process of auto-induced and patterned configurational change occurs remain to be elucidated. Whether chemical covalent or coordinate covalent bonding is catalyzed or only hydrogen bonds are altered to cause configurational change and polymerization will require elucidation at the quantum mechanical level. It is very unlikely that most neurons should undergo an alteration of biosynthesis of the precursor protein from altered gene transcription (Caughey et al. 1988).

Epidemiological Evidence for *De Novo* Generation as Infectious Protein Rather Than a Chain of Infection

When Kirschbaum reviewed CJD in 1968 there had been only about 100 cases reported in the world since the first description in the 1920s. Our laboratory has now had a most 4000 cases of CJD brought to our attention. Interestingly, whenever we stimulated our colleagues in neurology to hunt for CJD patients in cities throughout the world, they usually quickly reached an incidence rate of one case per million population per annum. This has been the case for Japan and China through Chile and Argentina, Australia and New Zealand. Intensive CJD surveillance in over a dozen countries has found incidence everywhere of about one per million inhabitants per annum (Brown et al. 1987, Masters et al. 1979). There is no other infectious disease with so uncannily a similar prevalence and incidence in all races in all climes from the arctic to the tropics. One certainly wonders how an infectious disease should have the same incidence everywhere in the world. This strange findings first stimulated the conjecture over 30 years ago that the disease might result from a spontaneous somatic mutation (F.M. Burnet, personal communication, Gajdusek 1990).

Truly sporadic cases of CJD account for over 85% of all cases. They never have had known contact with other CJD patients. Intensive effort to track down a possible source of infection has failed (Brown et al. 1987, Masters et al. 1989). Strikingly, in Sephardic Jews of Libyan origin in Israel the disease was found at over 20 times the incidence in Ashkenazi Jews in Israel. Foci of high incidence and local clustering of a few cases of CJD have been observed but only those of Sephardic Jews of Lybian origin in Israel and in the Lucenec and Orava areas of Slovakia have been acceptable as truly significant increased incidence (Cathala et al. 1985, Kahana et al. 1974, Mitrová 1980, 1990, Neugut et al. 1979). Among Sephardic Jews in Israel the familial occurence could account for at least half of this high incidence; Sephardic Jews all belong to an extended inbred family tree. As generally found in familial CJD, these Sephardic families demonstrated an autosomal dominant pattern of expression. Several dozen expanded pedigrees all showed a Mendelian autosomal dominant pattern as cleary as that found in Huntington's disease. On the other hand, all affected individuals have the virus, as we proved by transmission to monkeys or other laboratory animals (Masters et al. 1981). This, to my knowledge, is the only infectious disease of man in which expression of disease is proved to be controlled by a single autosomal dominant gene.

Attempts have been made to correlate the high incidence of CJD in these population groups with their habits of consuming sheep meat, especially brain and even eye balls. On the other hand, the prevalence of CJD in occupational groups such as shepherds, abattoir workers, and veterinarians does not exceed that amog the normal population (Brown et al. 1987, Chatelain et al. 1981, Gajdusek 1976, 1977, Masters et al. 1979).

Extensive study of sporadic cases of CJD has failed to reveal a source of infection in the life time of the patients. They have never been known to have encountered another CJD patient. Thus, we have two unusual epidemiological findings in CJD not found in any other infectious disease: 1) the strangely uniform one per million per year incidence and prevalence everywhere, and 2) the lack an infectious chain for the sporadic cases except in the few iatrogenic cases where the virus has been inoculated parenterally and thus provided an established chain of infection. These two findings have led us to conjecture that the sporadic event may generate spontaneously their infectious amyloid by a rare stochastic event of their host precursor protein falling into the infectious β-pleated configuration at an incidence rate of one per million population, per annum, the world-wide incidence of CJD. In the genetically determined cases of familial CJD and GSS the single amino acid substitution increase the likelihood of this rare event by about one million-fold (Gajdusek 1990). This would be in complete parallel with the familial amyloidotic polyneuropathy paradigm of point mutation greatly increasing the likelihood of amyloid fibril polymerization by altered chain interaction for a precursor that has a β-pleated structure.

Oravske Kuru

We now have a new focus of CJD in high incidence in man reported in Slovakia by Mitrová (1990). She has been following the incidence of CJD throughout Slovakia for more than two decades. In 1980 she identified an unusually high incidence of CDJ in the rural Lucenec area of south central Slovakia with many cases also across the border in Hungary (Mitrová 1980). During the past decade cases have been found in increasing frequency from the most sparsely

populated area of Slovakia, Orava, to the west of the High Tatra mountains on the Polish border (Mitrová 1990). Here an epidemic of CJD has developed during the 1980s with some 30 cases occurring in patients born and reared in a dozen small rural villages with a total population of under 15,000. This yields an incidence over 1,000 per million population per year in contrast to the worldwide incidence of one per million per year. The most intensely involved villages of Zuberec and Habovka with a total population of under 2000 have had over 20 cases of CJD in the past three years. The incidence in the villages has thus reached over 3000 times higher than in the rest of the world in such cities as Paris, Berlin, Boston, New York, Sydney, Santiago or Beijing, or any other large cities, and it is still 100 times higher than that among the Sephardic Jews in Israel. Members of the same family who were 20 to 30 years different in age become sick at nearly the same time. This suggested a common source infection rather than genetic determined etiology, as also did the new *epidemic* of appearance of CJD in the 1980s. For these reasons at first we believed that this outbreak may not be explained genetically.

However, we have now sequenced DNA from nine of the Orava and six of the Lucenec CJD brain and all have shown the substitution of lysine for glutamic acid in codon 100. Four of the 11 studied healthy adult first-order relatives have the same mutation (Goldgaber et al., 1989). CJD had not been know in Orava before the 1970s (Mitrová 1990). The epidemic started with a few cases in the late 1970s and has developed into an escalating epidemic in the late 1980s. We have found some family members with the mutation although they are healthy and over 70 years of age. We are now looking for the cofactor that turns on the expression of the mutation, a factor which in the past inhibited the posttranslational configuration change of the precursor to amyloid. Thus, the new question is not what has caused the Orava outbreak — it is the codon 200 glutamic acid to lysine point mutation — but rather what has prevented its expression in previous generations so that it has accumulated as a frequent silent nonpathogenic polymorphism, only expressing itself as a pathogenic mutation in these people in the past 15 years?

The CDJ Genetic Marker for the Wandering Jews of the Diaspora

On discovering the codon 200 glutamine to lysine point mutation responsible for the high incidence foci of CJD in both the Lucenec and Orava regions of Slovakia and widely disseminated in Slavic peoples of Eastern Europe, we screened a large number of sporadic and familial CJD brain specimens from our archive of frozen brain accumulated over the past 30 years (Goldfarb et al., 1990c). This led us to discover the mutation in Greek CJD patients who were Sephardic Jews and quickly we found the mutation in Sephardic Jews who had come for diagnosis of CJD in France from Tunisia and in Sephardic Jews with CJD in Israel, both Libyan-born and Israel-born. Ashkenazic Jewish CJD patients did not have the codon 200 glutamic to lysine point mutation (Goldfarb et al. 1991).

We are thus now investigating other Circum--Mediterranean Sephardic Jews with CJD and with particular attention to the Iberian Peninsula, particulary Spain where in 1492 the Catholic monarchs, Ferdinand and Isabella, forced the quick conversion of large numbers of Sephardic Jews to Catholicism. Many of the remainder fled and gave rise to the large Sephardic Jewish group in Greece where we have found the mutation.

The CJD Point Mutation for the Large Finnish Pedigree of Familial CJD

One the largest familial CJD pedigrees is that published by Haltia et al. in Finland (1979) which we have now investigated and found therein none of the point mutations previously know in familial CJD or GSS, but instead a codon 178 replacement of aspartic acid by asparagine (Goldfarb et al., 1991). We have now found this codon 178 mutation in Dutch, French, Hungarian, and American cases of familial CDJ (Nieto et al., 1991).

Thus, our use of the paradigm of multiple point mutations causing the enormously increased likelihood of a posttranslational conversion of the host precursor molecule to an amyloid configuration and deposition of insoluble amyloid in various tissues that we found in the familial amyloidotic polyneuropathy (FAP) literature has proved amazingly predictive in our unravelling of the familial CJD and GSS pathogenesis.

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