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Evidence suggesting that PrP is not the infectious agent in Creutzfeldt-Jakob disease

L.Manuelidis, T.Sklaviadis and E.E.Manuelidis

Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA

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It has been suggested that the infectious agents of scrapie and Creutzfeldt-Jakob disease (CJD) are 'prions' constituted by a protease resistant glycopeptide, PrP. To analyze the role of PrP in CJD infectivity we re-evaluated the biochemical characteristics of infectivity. First, when the infectious agent is not aggregated, infectivity is exquisitely sensitive to proteinase K treatment, and therefore a proteinase-K-resistant molecule (e.g. PrP) is unlikely to contain information essential for agent replication. Second, removal of sugar residues from Gp34 (the major precursor of the proteolyzed PrP band) failed to reduce infectivity. Third, one-half of the PrP peptides could be separated from significant infectivity using non-denaturing conditions with practical quantitative recovery of infectivity. These studies suggest that PrP in itself is unlikely to be the replicating component of the infectious agent. We suggest that these as yet undefined agents may consist of core protein and nucleic acid that are incompletely assembled in, and protected by, cell membranes. This hypothesis would (i) explain the absence of conventional viral particles in these diseases, (ii) account for observed membrane pathology including altered behavior of endogenous membrane proteins, and (iii) would be consistent with the replication and transforming properties of CJD that indicate there is an agent specific nucleic acid.

Key words: Creutzfeldt-Jakob disease/scrapie/prion protein/ infectivity

Introduction

Partially purified infectious fractions from Creutzfeldt-Jakob disease (CJD) or scrapie infected hamster brain contain an abundant 34-kd glycoprotein (Gp34) which is not present in identically prepared fractions from non-infected hamster brains (Manuelidis *et al.*, 1985; Sklaviadis *et al.*, 1986). Because to date no infectious nucleic acid has been identified for this class of unconventional agents, it has been postulated that these agents may actually contain little or no nucleic acid (Alper *et al.*, 1967; Gibbons and Hunter, 1967; Griffith, 1967; Millson *et al.*, 1976; Hunter *et al.*, 1979; Prusiner, 1982; Manuelidis and Manuelidis, 1986, for review). This concept has been canonized by the introduction of the term 'prion' (for protein infectious agent), and a 27-kd form of Gp34 prepared by limited protease digestion (referred to as PrP or prion protein) has been presented as the major integral component of the infectious agent (Prusiner *et al.*, 1983). However, cDNA studies have shown that PrP is a conserved endogenous normal gene product, transcribed equally in normal and infected tissues, and expressed in several types of tissues and cells (Chesebro *et al.*, 1985; Oesch *et al.*, 1985). It has been suggested therefore that post-translational modifications confer specific physical characteristics (resistance to proteinase K digestion and relative sedimentation differences) coincident with the acquisition of infectivity. The only post-translational modification yet detected on Gp34 or PrP in CJD and scrapie is glycosylation (Manuelidis *et al.*, 1985; Multhaup *et al.*, 1985; Bolton *et al.*, 1985; Sklaviadis *et al.*, 1986).

We here show that in highly infectious triton-solubilized brain fractions, CJD infectivity is exquisitely sensitive to short proteinase K treatments. In more highly purified salt-precipitated fractions, which contain Gp34 only when prepared from infected brain, selective partial or complete deglycosylation has no significant effect on infectivity. In addition, a significant proportion of Gp34 present in infectious fractions is separable from infectivity under conditions where starting infectivity is quantitatively recovered. The present separations were done under mild non-denaturing conditions known to preserve infectivity, and thus contradict the claim that PrP is directly proportional to the infectious titer and is 'inseparable from . . . infectivity' (McKinley *et al.*, 1983; Oesch *et al.*, 1985). It is likely that the critical informational molecules of these infectious agents have not yet been detected.

Results

Infectivity assay and proteinase K digestion

For each determination of infectivity a minimum of six hamsters were inoculated and the presence of symptomatic disease was verified histologically (Manuelidis and Manuelidis, 1979). Figure 1 shows a standard curve of serial dilutions of infected hamster brain versus incubation time. The most reliable portion of the curve, corresponding to dilutions of $10^{-2.5}$ to 10^{-5}, was used to quantitate unknown samples. In our laboratory, infectivity assayed in this way is sufficiently reproducible to detect changes as small as 0.6 logs with statistical significance, although other groups appear to need changes of several logs to achieve equal statistical significance. Thus in the following experiments, enzymatic treatments which were $\geq 90\%$ effective (i.e. represented changes in the majority of the protein molecules) would produce unambiguous bioassay results.

Previous studies have shown that scrapie infectivity was greatly reduced by protease treatment of crude homogenates (Millson *et al.*, 1976) or high speed supernatants (Cho, 1980). In contrast, proteinase K resistance (100 μg/ml for 2-3 h) has been reported to be an intrinsic property both of the infectious agent and of 'prion protein' (Prusiner *et al.*, 1981; McKinley *et al.*, 1983). However, others have reported that similarly prepared scrapie fractions had reduced infectivities with comparable proteinase K treatment (Lax *et al.*, 1983). Protease treatment of CJD fractions prepared from insoluble salt fractions yields a proteinase-K-resistant PrP peptide of 26-29 kd but simultaneously reduces infectivity by more than 90% (Manuelidis *et al.*, 1985).

To rigorously evaluate the intrinsic proteinase K resistance of CJD infectivity, a detergent-solubilized fraction was prepared from crude membranes. Triton X-100 (1%) released 50% of the infectivity from the crude membranes (Figure 2) which is com-

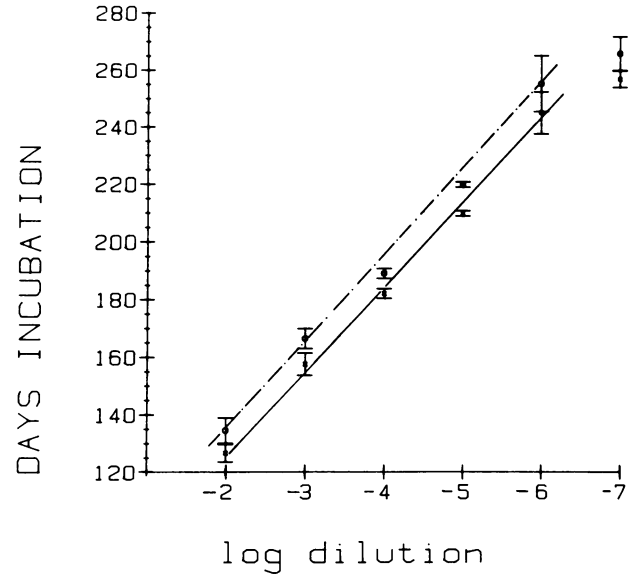

Fig. 1. A 10% hamster brain homogenate (i.e. 10^{-1}) was serially diluted and 50 μl aliquots were inoculated into six hamsters for each dilution point. Onset (in days) of clinical illness (X) or for terminal disease (●) ± SEM were scored. The lower the infectious dose, the longer the incubation time (Kimberlin and Walker, 1977). At a dilution of 10^{-7} only three of six hamsters died of disease before 300 days and therefore the 10^{-7} point was omitted for the best curve fits (lines). An additional one of six hamsters died of disease at 10^{-8} dilution, i.e. the LD_{50} titer was 8.5 logs/g (equivalent to one brain) in this experiment. The minimum incubation time for clinical disease in this model of CJD is 120 days (the same at a 10^{-2} or 10^{-1} dilution). Relative titers of treated unknown samples were calculated using this standard curve, and all unknowns assayed between $10^{-2.5}$ and 10^{-5} or on the most accurate and linear region of the curve. A change of >0.6 logs or a loss of 78% of the starting infectivity was significant, and a reduction of ≥1 log (90%) was completely unambiguous.

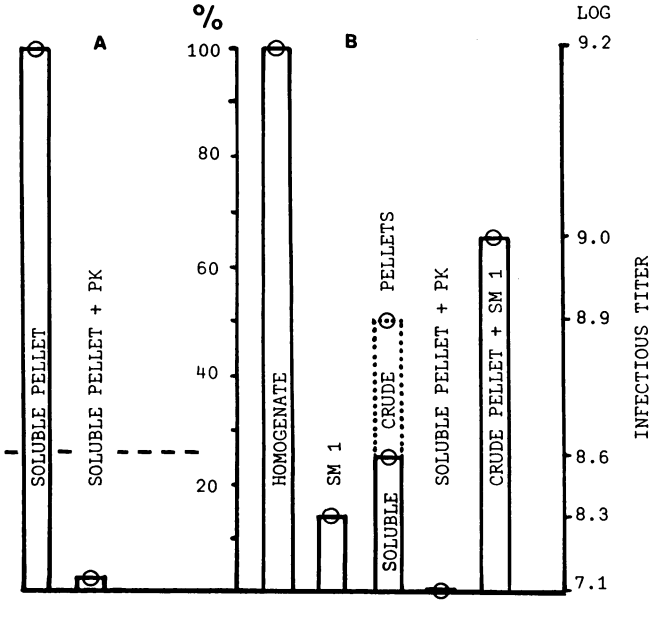

Fig. 2. A Triton X-100-soluble fraction derived from a crude low speed homogenate pellet (3 g starting material) was incubated in the presence or absence of proteinase K **(panel A)**. Proteinase K gave a 97% reduction in titer (% scale) which was clearly significant (dashed lines). **Panel B** shows recovered infectivity in each fraction where SM 1 is the first synaptosome pellet (see Materials and methods).

parable to the 85% solubilization of infectivity previously obtained from cleaner synaptosomal fractions (Manuelidis and Manuelidis, 1983a; Manuelidis *et al.*, 1985). Proteinase K treatment (100 μg/ml for 1 h, 37°C) reduced infectivity 97% compared to control incubations (a 1.5 log change). This study quantitatively shows that the infectivity of the CJD agent is markedly sensitive to protease digestion even in the presence of large amounts of total protein. These data are inconsistent with the concept that an intrinsically protease resistant PrP peptide is responsible for infectivity. The relative resistance of infectivity to proteolysis observed in more insoluble salt-precipitated preparations is likely due to aggregation of the agent, and possibly even fortuitous protection by a protease resistant protein.

Glycosidase experiments

We have previously described three prominent peptides in non-proteolyzed highly infectious CJD and scrapie fractions that are not present in identically derived fractions from uninfected brain (Manuelidis *et al.*, 1985; Sklaviadis *et al.*, 1986). Gp34, a sialoglycoprotein, is the major precursor of the heterogeneous PrP smear obtained with proteinase K treatment, and if it functioned as some conventional viral sialoglycoproteins, it could promote the absorption and penetration of the infectious agent into target cells via its sialic acid residues. Thus glycosidases were used to see if partial or complete removal of sugar residues from Gp34 reduced infectivity.

On one-dimension gels the major glycosylated 34-kd species and the distinct p26 peptides in the infectious fraction could be clearly delineated using lectins for carbohydrate detection, and polyclonal anti-PrP antibodies which detect all major peptides (Figure 3, lane 1, Figure 5B); p26 does not contain sialic acid residues. In some preparations, in addition to the 34-kd and 26-kd species, a minor degradation product of Gp34 is seen at 29 kd (Figures 3 and 5) and a minor p18 band seen is likely to derive from p26, as determined by NEPHAGE analyses (Sklaviadis *et al.*, 1986). Variable 60-kd smears represent aggregates of the major peptides (Figure 5, lanes 1 and 2). Gp34 and p26 also reacted with monoclonal antibodies raised to hamster scrapie PrP, and therefore represent the well characterized 'prion' protein species and corresponding cDNA clones isolated by others.

Sugar residues can be effectively and reproducibly removed from non-proteolyzed Gp34 in the highly infectious p215 salt fraction even though the major proteins in this fraction are no longer soluble, i.e. they quantitatively precipitate at 13 000 *g* (15 min) after they are treated with high salt. It should be noted that in preparations from normal brain, Gp34 is not similarly sedimented or precipitated with salt. Thus the infectious CJD fraction here utilized contained only the supposedly 'infectious' form of Gp34. It has been suggested that any unique properties of PrP are the result of post-translational modifications of this protein (Oesch *et al.*, 1985). Enzymatic deglycosylation of p215 salt fractions were undertaken to evaluate whether the only known post-translational modification of Gp34, via *N*-linked complex sugars (Sklaviadis *et al.*, 1986) contributed to CJD infectivity. Glycosidase cleavages were monitored by lectin binding, and polyclonal antibodies were used to rule out degradation of treated peptides.

Gp34 contains multiple sialic acid residues which produce charge heterogeneity demonstrated by two-dimensional gel electrophoresis. Neuraminidase digestions remove >95% of these residues and produce a single spot of pI 7.8 which binds ricin faster due to the exposure of secondary galactose-like sugars (Sklaviadis *et al.*, 1986). Removal of the multiple terminal sialic acid residues did not diminish infectivity (Figure 4). The completeness of the sialic acid removal in the inoculated sample was confirmed by loss of WGA binding and development of enhanc-

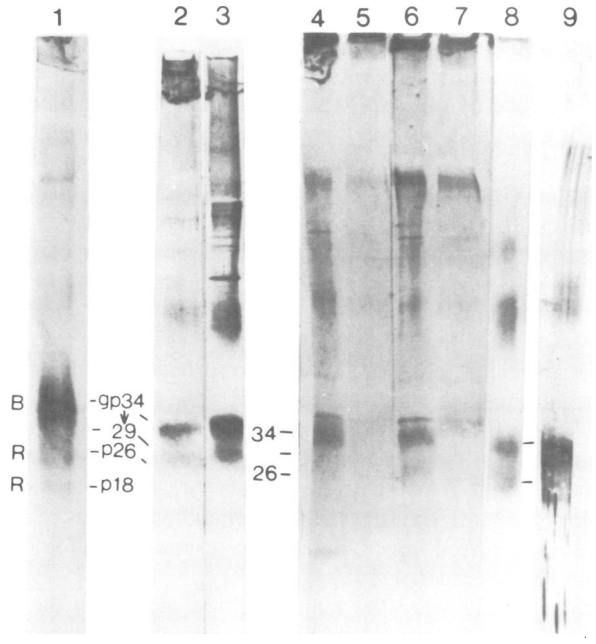

Fig. 3. Lane 1 shows a blot of major peptides in the p215 salt fraction. Gp34 first exposed to biotinylated WGA was stained blue (B) with nitrobluetetrazolium. The same blot was then incubated with anti-PrP antibodies and developed with TRX-fast red which highlighted p26 and p18 peptides in red (R). (Antibodies alone bound both distinct Gp34 and p26 peptides as seen in **lane 8**.) **Lanes 2** and **3** are from the control and neruaminidase-treated p215 salt fraction. These inoculated samples were analysed for ricin affinity. **Lane 3** shows the characteristic strong ricin binding at short development times after the secondary D-galactose-like residues on Gp34 are 'unmasked'. In **lane 3** the additional sharp band at 80 kd reflects added enzyme, and the fainter band at 29 kd represents a degraded or cleaved form of Gp34 (Sklaviadis *et al.*, 1986). **Lanes 5, 7** and **9** are from the inoculated Endo H-treated p215 salt fraction compared with identically inoculated controls (no enzyme, **lanes 4, 6** and **8**). Ricin (**lanes 4** and **5**) and WGA (**lanes 6** and **7**) detected significant sugar residues on Gp34 only in the control fraction not treated with Endo H. The single *N*-acetylglucosamine residue remaining on the peptide after Endo H treatment required higher gel loads for detection. Concanavalin A was more useful for unambiguous detections of this residue than WGA because the latter also can bind sialic acid residues. Antibodies showed equivalent binding to Gp34 and p26 peptides in the control and Endo H-treated fractions (**lanes 8** and **9**, respectively). Stained material at 60 kd represents aggregates of 34- and 26-kd peptides.

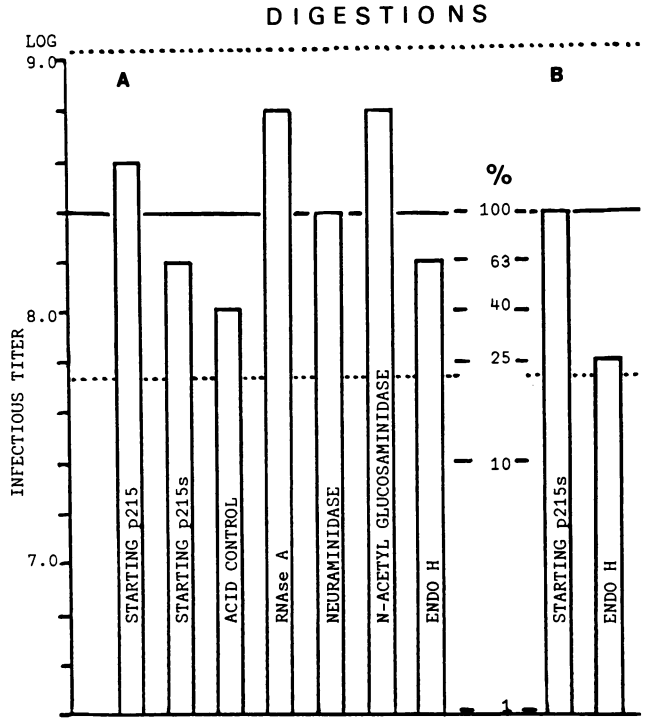

Fig. 4. Aliquots of the p215 salt pellet (p215s) from 3 g of brain (8.4 logs total recovered) was treated with various glycosidases, mild acidic conditions (control for glycosidase cleavages), and with RNase A. No significant decrease in infectivity was seen with any of these digestions (i.e. >0.6 logs difference, dotted lines). **Panel A** also shows the starting p215 fraction (not salt precipitated) used to derive the p215 salt fraction for this experiment. **Panel B** shows an independent repeat experiment with Endo H digestion (residue sugar assays shown in Figure 3).

ed ricin binding (Figure 3, lanes 2 and 3). Similarly, treatment with β-*N*-acetyl-glucosamidase had no effect on infectivity (Figure 4).

Removal of essentially all sugar residues by incubation with Endo H failed to produce any significant reduction of infectivity in two separate experiments (Figure 4). To verify complete removal of carbohydrate, aliquots of the inoculated fraction were run on gels and assayed for lectin bindings (Figure 3, lanes 5 and 7) and were shown to have no detectable residual sugars except for the single most internal sugar residue characteristically left by the enzyme (Tarantino and Maley, 1974) detected with concanavalin A on gels with high protein loads. When parallel lanes were stained with anti-PrP antibodies, it was apparent that the peptide cores of both Gp34 and p26 remained intact (Figure 3, lanes 6 and 8). Although many complex sialoglycoproteins are resistant to digestion with Endo H, control experiments using neuraminidase prior to Endo H digestion yielded an identical profile with lectins and antibodies as depicted above and showed that ≥90% of intact Gp34 could be cleaved by Endo H despite the presence of terminal sialic acid residues. The sugar specificity of lectin binding was further confirmed with glycopeptidase F digestions. This enzyme removes all sugar residues from all *N*-linked sialoglycoproteins including Gp34. In this experiment antibodies stained identical peptide bands as above with apparent mol. wts of 30 and 26 kd, and even with very high gel loads there was no specific or non-specific binding of lectins.

If the glycosylation of Gp34 contributed to infectivity, at least a 1 log reduction in infectivity would be expected with the >90% deglycosylation observed. Therefore, either Gp34 itself is not related to the infectious agent, or glycosylation (its only known post-translational modification) is not required for infectivity. Notably all samples and controls (total = 9) assayed within ±0.6 log units (Figure 4) and indicated the reproducibility of the bioassay described.

Lectin column chromatography

Because the above effects of proteinase K and glycosidase suggested that Gp34 might not be 'intrinsically related' to the CJD agent, we attempted to separate infectivity from this and other major proteins by chromatography on a wheat germ agglutinin–agarose column. We were unable to detect sugar specific binding of either Gp34 or of infectivity to the matrix of the column using a variety of infectious fractions. With the more purified p215 salt fraction, Gp34, p26, and the bulk of infectivity were recovered in the void volume ('flow through', Figure 6B). In contrast, all other detectable minor sialoglycoproteins were specifically bound to the matrix. Although the binding of various

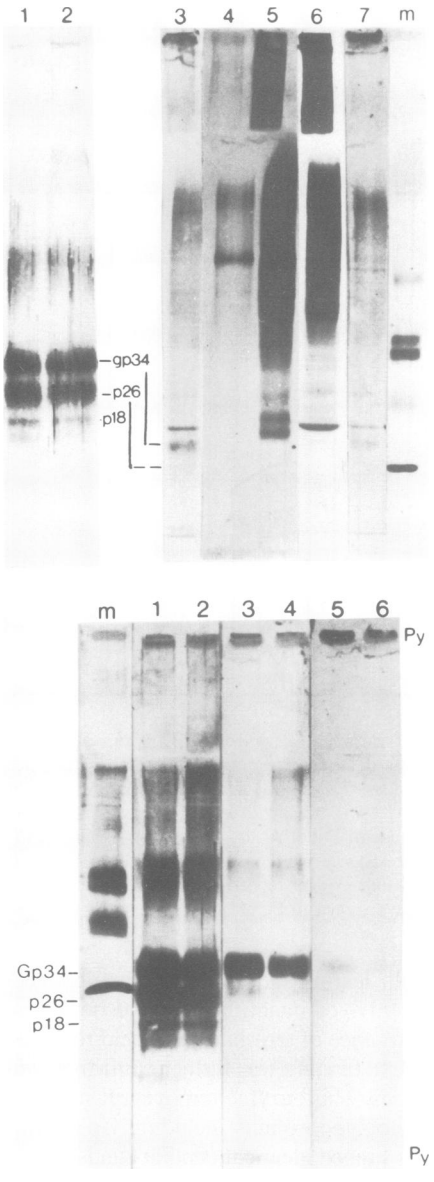

Fig. 5. Lectin (WGA-agarose) chromatography of the inoculated p215 fractions. **Top panel, lanes 1** and **2** are the flow through and flocculent residue fractions stained with anti PrP antibodies, and show comparable amounts of Gp34, p26 and p18 in each of these fractions after lyophilization. **Lanes 3–7** show all the chromatography fractions using ricin for detection. **Lane 3** is the flow through. **Lane 4** shows proteins eluted with sugar. **Lane 5** shows the same sugar-eluted proteins treated with neuraminidase (after elution) and reveals many minor sialic-acid-bearing peptides had bound to the matrix. **Lane 6** shows salt-eluted peptides (i.e. those binding to the matrix via ionic interactions), and **lane 7** are peptides from the flocculent residue fraction. In a repeat fractionation and chromatography experiment (**bottom panel**) antibodies (**lanes 1** and **2**) and WGA (**lanes 3** and **4**) again delineated roughly equal amounts of sedimentable Gp34 and p26 in the flow through and residue fractions. As in the first chromatography slightly less of the 34-kd ricin-binding peptide is present in the residue fraction (**lanes 5** versus **6**) but this minor difference of 60:40% could not account for large differences in assayed infectivity in these fractions. Aggregates of the major peptides are at 60 kd (e.g. **lanes 1** and **2**). Lanes marked m are mol. wt markers (29 kd, 43 kd, 52 kd and 94 kd) run and blotted from the same gel, stained with Indian ink. Py is pyronin Y used in gel for subsequent alignment of lanes.

sialoglycoproteins to WGA columns is highly dependent on the exact nature of the matrix (Furukawa *et al.*, 1986), it is more likely in our experiments that the insoluble nature of Gp34 aggregates in this salt-precipitated preparation (requiring >0.5% SDS for solubilization) prevented its interaction with the matrix. Therefore a more soluble, but less pure 215 000 *g* pellet prepared in the absence of salt (p215) was analyzed. This fraction also contains Gp34 and p26 only when prepared from infected brains whereas in control preparations these peptides remain in the supernatant.

This less pure preparation contained a large variety of sialoglycoproteins which bound to the matrix and were specifically eluted by β-*N*-acetylglucosamine. The majority of these sialoglycoproteins were shown to react with ricin only after removal of sialic acid with neuraminidase (Figure 5A, lanes 4 and 5). In addition, this p215 fraction reproducibly gave rise to a flocculent residue which failed to enter the matrix of the column. When the various column fractions were assayed for infectivity, 65% of the applied infectivity was recovered in the void volume (Figure 6A). This represents practical quantitative recovery of agent. The sugar and salt elutable fractions contained <2% of applied infectivity and the residue at the top of the column had less than 1%.

Gp34 and p26 were the major peptide species in the void volume (Figure 5, lane 1) and failed to interact with the matrix of the column either specifically or non-specifically. Surprisingly the flocculent material at the top of the column, which contained negligible infectivity, contained ~50% of the applied Gp34 and p26, and both residue and flow through fractions were identical whether concentrated by lyophilization or by sedimentation (Figure 5). Identical chromatography results were obtained with three separate p215 brain fractionations. Thus under mild conditions, where infectivity is quantitatively preserved, 50% of Gp34 is recovered in a fraction which is 1.8 logs (1.5%) less infectious than the remaining fraction with Gp34.

It is unlikely that these two fractions contain structurally distinct variants of Gp34. Their migration on gels and reactivity with lectins was identical. Both populations yielded identical 27-kd PrP bands following limited proteinase K treatment (not shown), and both had the sedimentation properties of the putative 'infectious' form. More significantly, neither fraction runs true on rechromatography. When the void volume is reapplied to the column, it again separates into two fractions, one which does not enter the column matrix and one which is recovered in the void volume. Similarly resuspension of the flocculent material at the surface of the column divides into two equivalent fractions on rechromatography. Electron microscope examination of both Gp34 containing fractions showed comparable abundant fluffy material characteristic of non-proteolyzed Gp34. The material which failed to enter the column in addition contained a web of structurally intact collagen fibers. The most likely explanation of the chromatographic behavior of Gp34 is that it is an inherently sticky molecule that variably aggregates with itself and other protein species, and is not an intrinsic component of the CJD agent.

Discussion

Despite the many unusual hypotheses concerning the nature of the infectious agents of CJD and scrapie, we know little about the molecules that constitute these agents. Recent attempts to purify infectivity have led several investigators to suggest that the major proteins detected in infectious fractions represent the infectious agent (cf. Introduction). The above studies were designed to find if any of these major peptide species are integral agent components that contain the necessary information to code for

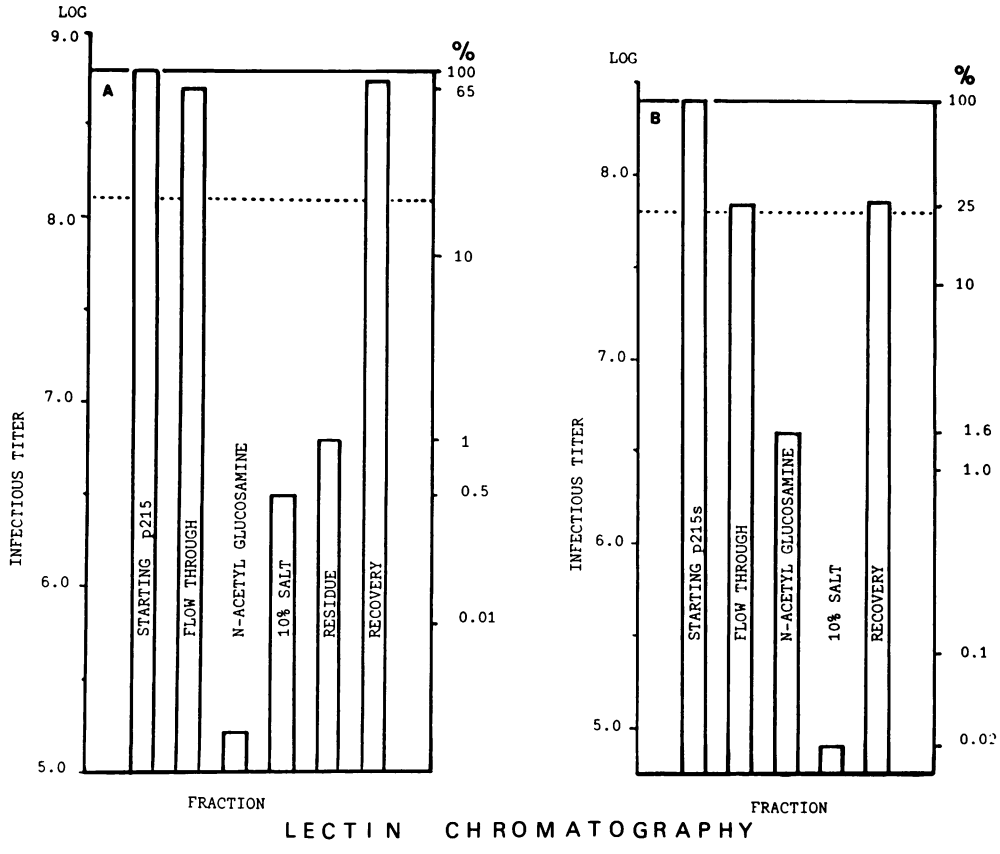

Fig. 6. Titration of infectivity in the p215 **(panel A)** and p215 salt **(panel B)** chromatography fractions. In **panel A**, the p215 fraction applied to the column was derived from three brains (~3 g); in **panel B** the p215 salt fraction (derived from 1 g of brain) was applied to the column (i.e. the relative titers in **panel B** are lower). Significant infectivity was only present in the flow through (void volume) fraction. The sugar-eluted, salt-eluted and residue fraction contained 2% of the applied infectivity. Recoveries of agent in the flow through were reasonably quantitative within assay limits (65 and 25%, respectively, of the starting material in these two experiments).

their replication in susceptible hosts. Several conclusions can be drawn.

In CJD the major 34- and 26-kd peptides can clearly be separated from infectivity under mild non-denaturing conditions where infectivity is quantitatively preserved and are neither, as previously suggested 'inseparable from infectivity' (Oesch *et al.*, 1985) nor stoichiometrically related to infectious titer (McKinley *et al.*, 1983). We have found no evidence for distinct 'infectious' and 'non-infectious' forms of Gp34. It has been speculated that post-translational modification of this highly conserved membrane protein could be responsible for infectivity. However, removal of sugar chains from Gp34, its only known modification, had no effect on the titer of agent in various infectious fractions which contain this protein. Therefore it is unlikely that Gp34 (or its derivative, PrP) represents the 'informational' and replicating component of the CJD agent.

Gp34 prepared from brains and infected animals appears to readily aggregate both with itself and with other proteins. Ultrastructural examination of preparations highly enriched in intact Gp34 reveal fluffy material (Manuelidis and Manuelidis, 1986). Notably, virtually no extracellular 'scrapie associated fibrils' (SAF) (amyloid-like) plaques are seen microscopically in the brains of this CJD rodent model. SAF appear only in CJD preparations treated with proteinase K which contain the cleaved 27-kd PrP form of the molecule. It is possible therefore that Gp34 can become associated with the CJD agent during the typical global disruption of membranes in diseased brains, and that this aggregation is enhanced by proteolytic cleavage of isolated fractions. It is also well known that highly purified conventional viruses frequently contain inseparably associated normal host membrane proteins including HLA antigens and receptor molecules such as the T4 antigen (Maddon *et al.*, 1986). Indeed, CJD infectivity may never be completely separable from the Gp34 membrane glycoprotein in brain fractions derived from later stages of disease. In accord with this, the sedimentable form of PrP is detected in brain only subsequent to large increases of infectious titer (Czub *et al.*, 1986). However, the aggregation of Gp34 in infected brains may have a role in organ specific pathologic effects and the deposition of plaques.

CJD infectivity is variably sensitive to treatment with proteinase K. In the triton-treated preparations described above it is exquisitely sensitive. In the less soluble p215 salt preparation, infectivity is less rapidly degraded (unpublished data), although infectivity is still clearly reduced by treatments which convert the bulk of Gp34 to the presumably infectious 27-kd PrP form (Manuelidis *et al.*, 1985). This suggests that there are CJD-agent-associated proteins which play a role in infectivity. It remains to be determined whether these proteins are specific species, either host or agent encoded. Regardless, the titer of infectivity suggests that an agent-specific protein would be considerably less abundant than Gp34 (Manuelidis and Manuelidis, 1986), and the purification schemes applied to date do not sufficiently enrich the agent to allow identification of potential agent specific proteins. The insensitivity of infectivity to glycosidase treatments suggests that putative agent specific proteins are either not sialoglycoproteins (that can be cleaved by neuraminidase and Endo

H), or that glycoprotein sugar residues play essentially no role in the process of infection.

Previous studies in CJD would suggest that a single-stranded DNA or an RNA would be the most likely candidate for the 'informational' component of the agent (Manuelidis and Manuelidis, 1981). We suspect that there must be a nucleic acid intermediary in these unconventional infections because brain cells from virtually all CJD animals are permanently transformed (Manuelidis and Manuelidis, 1983b; Manuelidis *et al.*, 1986), and direct application of CJD-infectious material to 3T3 cells also causes permanent cell transformation (Oleszak *et al.*, 1986). One possible route for such transformation could be via a reverse transcription of an agent-specific RNA.

The lack of a demonstrable effect of RNase A on infectivity shown above does not contradict this hypothesis because there is clearly protection of the agent by associated proteins, the extent of which may vary with the type of preparation. Lack of a specific RNA in SDS-purified PrP preparations is meaningless since these preparations are not infectious (Prusiner *et al.*, 1984). Our working hypothesis is that these agents may be incompletely assembled or defective retroviral-like elements. It should be emphasized that no conventional virus-like structures have been consistently observed in infectious CJD or scrapie preparations. However, it is well known that some defective retroviruses can have variant morphologies that preclude their recognition as viral particles (e.g. Rohrschneider *et al.*, 1976). These incompletely assembled retroviral particles are found intimately associated with membranes, the same subcellular fraction that is most enriched in CJD and scrapie infectivity. An intimate association of this postulated retroviral-like agent with membranes may preclude their recognition by the immune system. Defective neurotropic retroviruses can produce pathologic membrane changes without an inflammatory response (Zachary *et al.*, 1986) and thus show similarities to CJD and scrapie. In summary, there is no evidence or reason to support the existence of a novel intrinsically infectious protein in CJD or scrapie, and we suggest that the 'prion' hypothesis is unwarranted at the present time.

Materials and methods

Proteinase K experiments

Fresh brains from passaged hamster CJD (Manuelidis *et al.*, 1978) were taken from clinically ill animals and homogenized in 10 vol buffer as previously described (Manuelidis and Manuelidis, 1983a) to yield a crude pellet and a first synaptosome extract (SM 1). The 400 *g* (15 min) crude pellet was vortexed in 6 vol buffer and lysed at room temperature by addition of Triton-X 100 (1% final concentration). The lysate was cleared by centrifugation at 1500 *g* for 15 min at 4°C. Clarified supernatants (1 ml) were aliquoted into two tubes, self-digested proteinase K was added to one tube (100 μg/ml final), both tubes were incubated at 37°C for 1 h, and immediately diluted 1:3000 for intracerebral inoculation (six hamsters each for control and proteinase-K-treated lysates). All other fractions from this same preparation were similarly diluted and inoculated. Incubation times were calibrated against a standard dilution curve of serial 10-fold dilutions of homogenate (Figure 1). The membrane-rich fraction (crude pellet plus SM 1) contained 65% of the starting infectivity, and the solubilized material (soluble crude pellet) was used for proteinase K and control incubations (Figure 2).

Glycosidase and RNase experiments

Highly infectious 215 000 *g* pellets (p215 and p215 salt) were made from two to three frozen brains as previously described in detail using 10% sarkosyl and protease inhibitors (Manuelidis *et al.*, 1985; Sklaviadis *et al.*, 1986). These fractions were treated with glycosidases as described (Sklaviadis *et al.*, 1986); RNase A was used at 285 μg/ml for 1 h at 37°C. Western blots, lectin and antibody binding were performed as previously described except that a more sensitive strepavidin alkaline phosphatase complex (Bethesda Research Laboratories) was used to detect the binding of biotinylated lectins. Immediately after enzymatic treatment, cleaved and control samples were identically diluted for infectivity assays (i.e. a 1:1000 of each 500 μl sample aliquot made from the same initial preparation and run in parallel incubations).

Lectin chromatography

Infectious p215 or p215 salt pellets derived from 1–3 g of brain were resuspended in 8–10 vol of column buffer (25 mM Tris–Cl, pH 7.5, 0.1% w/v Triton-X 100). This suspension was applied twice to a 1 ml wheat germ agglutinin–agarose column (Vector) made in a 1-cc syringe, using nylon mesh as a support; 8–10 ml of flow through (void volume) was pooled for inoculation. After column washes (10 ml), 5 ml of 0.5 M *N*-acetylglucosamine was used to elute sialic-acid-bearing proteins. This was followed by a wash with 10% NaCl in column buffer (5 ml) to elute proteins that bound to the column via ionic interactions. Visible flocculent material at the top of the column (residue) was removed mechanically by gently stirring the top of the gel, and these aggregates then passed through the column after the addition of initial column buffer (8–10 ml). The four pooled fractions (flow through, sugar eluted, salt eluted and residue) were comparably diluted after collection for infectivity assays. After inoculation, these fractions were dialyzed against 10 mM Tris–Cl, pH 7.5, and sample volumes were reduced by lyophilization for standard gel analysis (Manuelidis *et al.*, 1985). The 34/26-kd peptides in the flow through and residue fractions could also be easily and quantitatively precipitated by centrifugation at 13 000 *g* for 15 min (see Results, Figure 5B). Peptides eluted from the column with either sugar or high salt could not be similarly precipitated, i.e. were completely soluble and did not aggregate. For quantitative comparison of peptides in each chromatography sample, lyophilized or pelleted material was resuspended in identical final volumes of buffer; equal aliquots of each sample were loaded on 12% acrylamide gels for Western blot analysis.

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