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Scrapie: concept of a virus-induced amyloidosis of the brain

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After an intraperitoneal infection disease-specific incorporation of [^{3}H]leucine into protein and [^{3}H]uridine into RNA in the brain precede clinical scrapie in hamsters. Onset of both incorporations are the earliest measurable events in the disease. Infectivity and subsequent clinical symptoms appear only after this biochemical activity has ceased. The disease-specific [^{3}H]protein co-purifies with scrapie-associated fibrils (SAF) and infectivity during differential centrifugation and buffer extraction. SDS-PAGE shows that the [^{3}H]protein is not SAF protein but a protein with an apparently higher mol. wt. The [^{3}H]RNA is metabolically stable and separates from SAF and the main portion of infectivity in the last step of the purification. The appearance of SAF-protein is a late event and correlates with severe clinical symptoms. SAF seems to be derived from a brain protein turning over slowly. Our data are consistent with early pre-clinical virus replication. In this case treatment aimed at suppressing virus replication in the clinical phase of the human Creutzfeldt-Jakob disease is unlikely to produce any beneficial effect.

Key words: scrapie/hamster/SAF/amyloidosis/*in vivo* kinetics

Introduction

The clinical progressive phase of subacute spongiform virus encephalopathies (SSVE) in man and animals is preceded by an exceptionally long incubation period without any apparent symptoms. The clinical phase in general terminates in death within a few months. Scrapie 263K in hamsters (Kimberlin and Walker, 1977) has become an important model system for SSVE. The discovery of disease-specific fibrils, i.e., scrapie-associated fibrils (SAF) (Merz *et al.*, 1981) was followed by the discovery of a disease-specific, proteinase K-resistant protein (Bolton *et al.*, 1982), and the recognition that this protein is the major constituent of SAF, which is closely associated with infectivity (Diringer *et al.*, 1983a). This protein was designated prion protein (Prusiner *et al.*, 1983). Using this term, however, implies agreement with the prion hypothesis (Prusiner, 1982) according to which the scrapie agent is a new kind of infectious particle. The small size of the scrapie agent is claimed to distinguish it from conventional viruses, and 'the monomeric form of the scrapie agent may be considerably smaller than that of a viroid' (Prusiner, 1983). Our experiments lead us to disagree with this hypothesis (Diringer and Kimberlin, 1983), we prefer therefore to use the term SAF protein rather than prion to indicate that this protein is a major constituent of SAF.

SAF could either be the scrapie agent itself (Diringer *et al.*, 1983a; Merz *et al.*, 1983,1984; Prusiner *et al.*, 1983; Hilmert and Diringer, 1984) or a pathological proteinaceous structure resembling amyloid arising as a consequence of virus infection (Merz *et al.*, 1981,1983; Diringer *et al.*, 1983a; Prusiner *et al.*, 1983). Thus, knowledge of the kinetics of the biosynthesis of SAF is necessary to understand the pathogenesis of SSVE. A rapid and efficient method to concentrate and enrich SAF, SAF protein and infectivity together in a single fraction (Hilmert and Diringer, 1984) using just one hamster brain has enabled us to perform *in vivo* labelling studies with radioactive precursors of protein, DNA and RNA synthesis. Here we report pulse labelling experiments strongly suggesting virus replication during the clinically inconspicuous early phase of the scrapie 263K in hamsters.

Results

To study the sequence of biochemical events in the brain during the development of a SSVE, we infected inbred CLAC hamsters i.p. with 100 μl of a 10^{-2} suspension of scrapie brain to avoid any unnecessary damage to the brain of the recipient animals. Controls received normal brain homogenate. At various times throughout the incubation period as well as in the clinical phase an animal was given 1 mCi [^{3}H]leucine i.p. Leucine is a constituent of SAF protein (Multhaup *et al.*, 1985). All fractions from the purification procedure were analyzed for tritium content, but differences between controls and scrapie-infected animals were detected only in the P_E fraction. This fraction contained only 0.02 – 0.05% of the 5×10^6 c.p.m. incorporated into the total, acid-precipitable protein of the brain homogenate.

Figure 1a shows transiently increased incorporation of [^{3}H]leucine into P_E isolated from scrapie-infected animals between infection and clinical manifestation of the disease. After 50 days disease-specific incorporation began, reached a peak around 60 days, and disappeared 70 days after infection. The labelled material was sensitive to treatment with 25 μg proteinase K/ml for 2 h at 37°C. This labelled protein differs from SAF protein: whereas the latter migrates into a 12 – 17% SDS-polyacrylamide gel, behaving like a 25-kd protein, the [^{3}H]protein did not enter the gel (data not shown). SAF protein was not labelled at any time during the experiment.

When [^{3}H]uridine was used for labelling, we only detected differences between scrapie and normal brain when the labelling period following a single injection of 1 mCi of [^{3}H]uridine (40 Ci/mmol) was extended over 10 – 30 days. These differences were found in various fractions, being most prominent, however, in fraction E_3 which contained ~1% of the total brain RNA. The results with this fraction are given in Figure 1b. Disease-specific, increased labelling was first detected 35 days after infection and declined at about the same time as in the incorporation of [^{3}H]leucine, i.e., ~70 days after infection. The [^{3}H]uridine-labelled fraction was sensitive to RNase digestion.

If [^{3}H]thymidine (110 Ci/mmol) was used as a precursor, we were unable to detect differences between scrapie and control animals in any fraction prepared and at any time during the experiment. For fraction E_3 this is shown in Figure 1c.

We scored the animals for clinical symptoms during the course

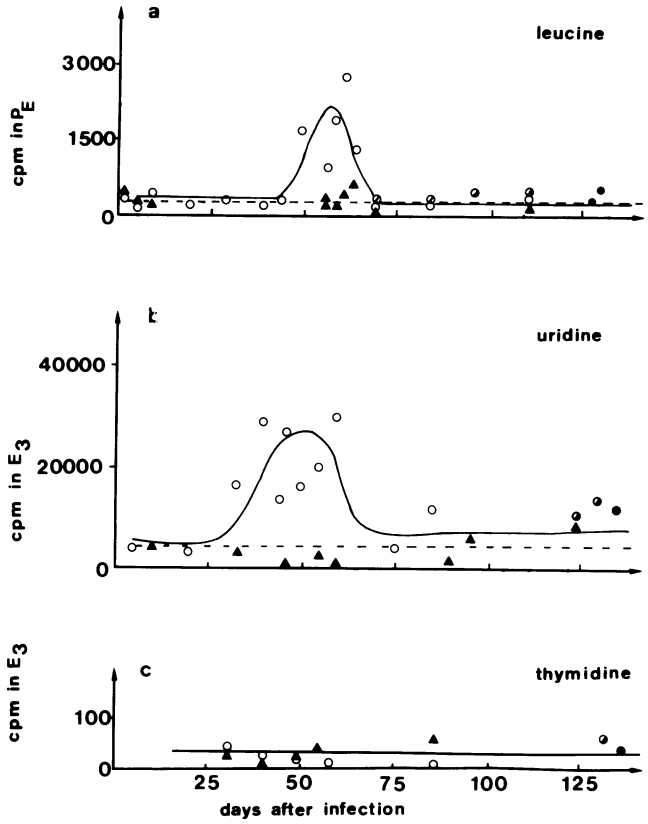

Fig. 1. Incorporation of radioactive leucine **(a)**, uridine **(b)** and thymidine **(c)** during the development of disease into the P_E fraction **(a)** and the E_3 fractions **(b–c)**, respectively, prepared from control (▲), or scrapie (○) hamster brains after i.p. infection. Each point represents a single animal. P_E is a fraction obtained from a brain homogenate as a pellet at 215 000 *g* after differential centrifugation which subsequently was extracted three times with buffers. E_3 is the last of these extracts. Symbols of diagnosis: ○ no symptoms: ∅ beginning of symptoms (nervousness and sensitivity to noise, fear); ◕ advanced symptoms (beginning of head bobbing and uncoordinated movements); ● severe scrapie (inability to turn or to stand upright).

Table I. Time dependence of clinical symptoms in hamsters i.p. infected with scrapie.

Days after infection	60	75	85	95	115	125
Diagnosed animals						
Σ	19	17	15	14	12	10
○	19	15	11	6	2	–
∅	–	2	3	7	8	5
◕	–	–	1	1	1	2
●	–	–	–	–	1	3

Animals used in the [^{3}H]leucine experiment presented in Figure 1a, which were left at indicated times, were scored. For symbols of diagnosis see Figure 1. (Σ) total number of animals left.

of the experiment using various diagnostic criteria (see legend to Figure 1). The results, given in Table I, show that even earliest symptoms of disease were detected only after the biochemical synthesis of [^{3}H]protein and [^{3}H]RNA had decreased. Seventy days after infection only two out of 17 remaining animals, which had not yet been used for pulse-labelling studies, showed early signs of disease. Advanced symptoms in the first animal were scored at day 85 and the first full case of scrapie was recorded

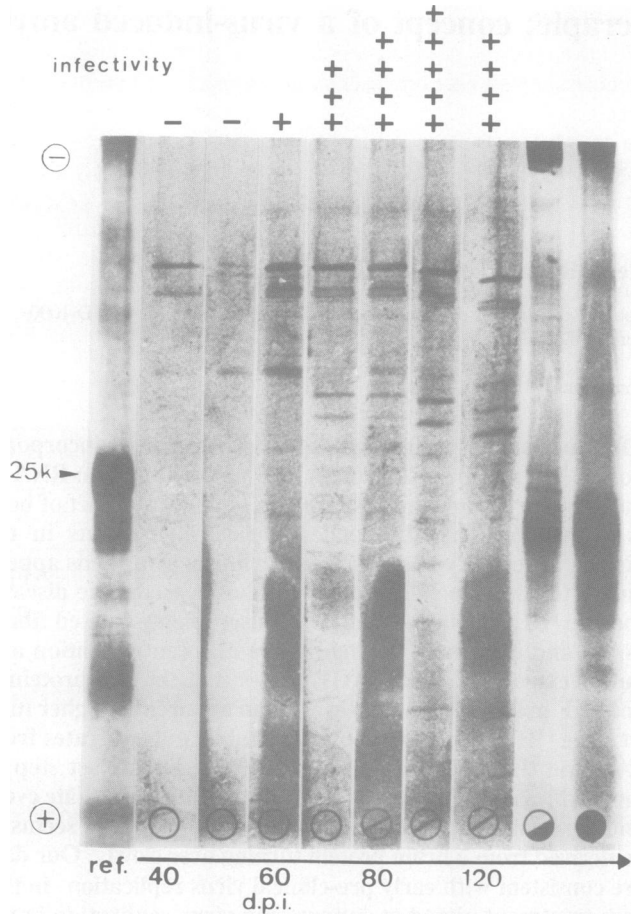

Fig. 2. Increase of infectivity in brains of individual hamsters after an i.p. infection and appearance of SAF protein in the P_E fraction of the same animals during the development of disease. Infectivity was estimated by measurement of the incubation period. Relative titers: no infectivity detected (−), an ~10-fold difference is represented by (+). Symbols of diagnosis are as in Figure 1. A 20, 10 or 5 μl sample taken from 100 μl of a sonicated suspension of P_E, prepared from the brain of a scrapie-infected hamster, was run at each time. This represents 0.2 (no symptoms and beginning of symptoms), 0.1 (advanced symptoms), and 0.05 (severe scrapie) brain equivalent, respectively. Left lane: a control preparation of SAF protein representing 0.05 brain equivalent of a terminal case of hamster scrapie.

at day 115. At this time all but two animals showed clear signs of disease. A specific incorporation of tritiated precursor was never observed during the clinical phase.

To relate the appearance of SAF protein to infectivity and clinical symptoms, P_E was isolated from brains of i.p.-infected animals without prior addition of 'carrier brain' and without labelling. We homogenized brains of individual hamsters and injected intracerebrally 50 μl of a 10^{-2} dilution of these homogenates into recipient animals to estimate infectivity (Diringer *et al.*, 1983b); then we prepared P_E from these homogenates and scored for SAF protein by SDS-PAGE, a method allowing easy detection of this protein in P_E, if one-twentieth of a brain equivalent is applied to the gel (Hilmert and Diringer, 1984). SAF protein could not be detected during the first 100 days after infection, not even in P_E fractions obtained from animals showing early clinical symptoms (Figure 2). The presence of SAF protein in the last two lanes of Figure 2 demonstrates that the appearance of large amounts of SAF is an event, predominantly occurring during the late, clinically manifested phase.

Discussion

We have reported here four observations. (i) After intraperitoneal incubation of scrapie brain, there is very early, transient disease-specific synthesis of protein co-purifying with infectivity and SAF up to the P_E stage of purification. It does not, however, co-migrate with SAF protein in gel electrophoresis. (ii) Disease specific RNA can be labelled and separated from SAF and most of the infectivity. Both these events occur in the brain during the clinically inapparent phase of the disease. The beginning of RNA synthesis seems to precede the beginning of protein synthesis. (iii) Infectivity of brain extracts increases before clinical symptoms. (iv) SAF protein appears late in the course of the disease. Its formation seems to be restricted to the period of clinical symptoms, in particular the very late period and it is not associated with [^{3}H]leucine labelling. The kinetics of the hitherto undescribed incorporation of radioactive precursors first into RNA and subsequently into protein as well as the occurrence of these biochemical events prior to an increase of infectivity and the appearance of clinical symptoms strongly suggest virus replication. These biochemical changes are the earliest which have ever been measured in scrapie. Low levels of infectivity have been detected in the brains of hamsters only 5 – 10 days after an i.p. infection without further increase during the next 30 days (Diringer, 1984). Thus, the biosynthetic activities we measured between 35 and 65 days cannot be a consequence of preceding virus replication.

To decide whether some of the labelled RNA and protein are constituents of the scrapie virus, or whether we have measured the expression of a cellular reaction towards ongoing replication, several additional experiments will have to be performed: (i) RNA and protein will have to be characterized; (ii) in hybridization experiments we will have to investigate whether or not all of the labelled RNA is of cellular origin. The appearance of infectivity in hamster scrapie will have to be related to the biochemical synthesis in a more detailed study. Here we have shown that infectivity in individual hamsters increases dramatically before the onset of symptoms.

Our infectivity data in hamsters are supported by an earlier, very extensive investigation into the pathogenesis of scrapie in mice after an s.c. infection (Eklund *et al.*, 1967). These authors reported maximum titers of infectivity before the onset of clinical symptoms and concluded that 'the long continued presence of large amounts of virus in the CNS seems to be necessary to produce clinical disease and lethal damage to this structure'. Also, in hamsters inoculated intracerebrally, the titers of infectivity increased before definite pathologic changes were recognized and before clinical symptoms appeared (Baringer *et al.*, 1983). If replication of virus in scrapie, and possibly SSVE in general, begins and terminates before the onset of clinical symptoms, then it is highly unlikely that treatment of SSVE with substances interfering with virus replication would have any beneficial effect for the host, if given at a time when symptoms have already appeared. Indeed, trials to treat patients suffering from Creutzfeldt-Jakob disease with antiviral drugs (Brown, 1984; Newman, 1984; David *et al.*, 1984) have failed completely.

Although SAF also co-purify with infectivity and are strongly associated with it (Diringer *et al.*, 1983a; Prusiner *et al.*, 1984; Hilmert and Diringer, 1984), the late appearance of the main amount of SAF protein and our failure to label the protein (containing leucine and constituting 0.01% of the total protein in a scrapie brain) favour the possibility that the amyloid-like SAF are formed from a pre-existing brain protein turning over very slowly. This concept is in agreement with the currently well established hypothesis that amyloidoses are diseases resulting from abnormal protein degradation (Glenner, 1980). Experiments in search of a possible precursor protein with antibodies directed against SAF protein (Bendheim *et al.*, 1984; Diringer *et al.*, 1984), and comparative protein sequencing analyses of this putative precursor and SAF protein will show whether indeed SAF are formed by such mechanisms.

Removal of carbohydrate from SAF protein (which is a glycoprotein) clearly shows that the major constituent of SAF has a mol. wt. of 7 kd and, as a highly glycosylated protein, can hardly be considered as part of the detergent-insensitive scrapie virus (Multhaup *et al.*, 1985).

Ever since the discovery of amyloid plaques in the brain of scrapie-infected mice (Fraser and Bruce, 1973; Wisniewski *et al.*, 1975) this disease has been considered as a possible model for Alzheimer disease (Dickinson *et al.*, 1983). Most of the amyloid plaques in scrapie appear during the late clinical phase (Bruce, 1981). This is similar to our observation of the accumulation of large amounts of SAF protein. However, amyloid plaque formation in scrapie is restricted to certain virus and mouse strain combinations (Bruce *et al.*, 1976) whereas the formation of the amyloid-like SAF is a general observation in model systems of SSVE (Merz *et al.*, 1981,1983,1984) as well as in naturally occurring SSVE of man (Merz *et al.*, 1983,1984). Therefore, the more general concept of scrapie and other SSVE being virus-induced amyloidoses of the central nervous system seems warranted.

Materials and methods

Purification procedure for SAF and infectivity

The inbred CLAC strain of hamster (Diringer *et al.*, 1983b) was used. An animal was anaesthetized with ether, killed, bled, and the brain transferred into 10 ml of Tris-buffered saline (pH 7.4) (TBS). 10 ml (in the case of uridine-labelling 5 ml) of a large pool of a 10^{-1} homogenate of unlabelled scrapie hamster brain in TBS were added, serving as SAF carrier material. After homogenization, a differential centrifugation procedure was performed as described (Multhaup *et al.*, 1985). The final pellet, P_E, was enriched in SAF, SAF protein and infectivity (Multhaup *et al.*, 1985). Aliquots of the various fractions were analyzed for tritium content in a liquid scintillation counter.

SDS-PAGE

Samples for SDS-PAGE were mixed with an equal volume of Laemmli buffer and heated (Diringer *et al.*, 1983). Gel electrophoresis of the P_E fraction was performed on round and slab gels, and the latter were stained as described (Diringer *et al.*, 1983). For determination of radioactivity, the round gels were frozen at −10°C and cut into 2 mm slices. These were incubated in 0.3 ml Soluene (Packard Instruments, Frankfurt/Main, FRG) at 50°C for 2 h, and were counted in 2 ml of a toluene-based scintillation solution.

[4,5-^{3}H]L-leucine (110 – 120 Ci/mmol), [5,6-^{3}H]uridine (35 Ci/mmol), and [methyl,1′,2′-^{3}H]thymidine (110 Ci/mmol) were obtained from Amersham-Buchler (Braunschweig, FRG).

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References

Baringer,J.R., Bowman,K.A. and Prusiner,S.B. (1983) *J. Neuropathol. Exp. Neurol.*, **42**, 539-547.

Bendheim,P.E., Barry,R.A., De Armond,S.J., Stites,D.P. and Prusiner,S.B. (1984) *Nature*, **310**, 418-421.

Bolton,D.C., McKinley,M.P. and Prusiner,S.B. (1982) *Science (Wash.)*, **218**, 1309-1311.

Brown,P. (1984) in de Clercq,E. and Walker,R.T. (eds.), *Targets for the Design of Antiviral Agents*, Plenum, NY, pp. 131-157.

Bruce,M.E. (1981) *J. Comp. Pathol.*, **91**, 589-597.

Bruce,M.E., Dickinson,A.G. and Fraser,H. (1976) *Neuropathol. Appl. Neurobiol.*, **2**, 471-478.
David,A.S., Grant,R. and Ballantyne,J.P. (1984) *Lancet*, **i**, 512-513.
Dickinson,A.G., Bruce,M.E. and Scott,J.R. (1983) in Katzman,R. (ed.), *Banbury Report 15. Biological Aspects of Alzheimer's Disease*, Cold Spring Harbor Laboratory Press, NY, 387-398.
Diringer,H. (1984) *Arch. Virol.*, **82**, 105-109.
Diringer,H. and Kimerlin,R.H. (1983) *Biosci. Rep.*, **3**, 563-568.
Diringer,H., Rahn,H.C. and Bode,L. (1984) *Lancet*, **ii**, 345.
Diringer,H., Gelderblom,H., Hilmert,H., Özel,M., Edelbluth,C. and Kimberlin,R.H. (1983a) *Nature*, **306**, 476-478.
Diringer,H., Hilmert,H., Simon,D., Werner,E. and Ehlers,B. (1983b) *Eur. J. Biochem.*, **134**, 555-560.
Eklund,C.M., Kennedy,R.C. and Hadlow,W.J. (1967) *J. Infect. Dis.*, **117**, 15-22.
Fraser,H. and Bruce,M.E. (1973) *Lancet*, **i**, 617-618.
Glenner,G.G. (1980) *N. Engl. J. Med.*, **302**, 1283-1292, 1333-1343.
Hilmert,H. and Diringer,H. (1984) *Biosci. Rep.*, **4**, 165-170.
Kimberlin,R.H. and Walker,C.A. (1977) *J. Gen. Virol.*, **34**, 295-304.
Merz,P.A., Somerville,R.A., Carp,R.J. and Wisniewski,H.M. (1981) *Acta Neuropathol. (Berl.)*, **54**, 63-74.
Merz,P.A., Somerville,R.A., Wisniewski,H.M., Manuelidis,L. and Manuelidis, E.E. (1983) *Nature*, **306**, 474-476.
Merz,P.A., Rohwer,R.G., Kascsak,R., Wisniewski,H.M., Somerville,R.A., Gibbs,C.J.,Jr. and Gajdusek,D.C. (1984) *Science (Wash.)*, **225**, 437-440.
Multhaup,G., Diringer,H., Hilmert,H., Prinz,H., Heukeshoven,J. and Beyreuther,K. (1985) *EMBO J.*, in press.
Newman,P.K. (1984) *Lancet*, **i**, 793.
Prusiner,S.B. (1982) *Science (Wash.)*, **216**, 136-144.
Prusiner,S.B. (1983) in Court,L.A. (ed.), *Virus non Conventionnels et Affections du Système Nerveux Central*, Masson, Paris, France, pp. 57-83.
Prusiner,S.B., McKinley,M.P., Bowman,K.A., Bolton,D.C., Bendheim,P.E., Groth,D.F. and Glenner,G.G. (1983) *Cell*, **35**, 349-358.
Wisniewski,H.M., Bruce,M.E. and Fraser,H. (1975) *Science (Wash.)*, **190**, 1108-1110.

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