Detection of Scrapie-associated Fibril (SAF) Proteins Using Anti-SAF Antibody in Non-purified Tissue Preparations

By RICHARD RUBENSTEIN,* RICHARD J. KASCSAK, PATRICIA A. MERZ, MICHAEL C. PAPINI, RICHARD I. CARP, NIKOLAOS K. ROBAKIS AND HENRYK M. WISNIEWSKI

New York State Office of Mental Retardation and Developmental Disabilities, Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York 10314, U.S.A.

(Accepted 9 January 1986)

SUMMARY

Antisera raised to scrapie-associated fibril (SAF) proteins were used to detect scrapie-specific polypeptides in three different non-purified brain preparations: a synaptosomal-mitochondrial fraction, 20% brain homogenate and 20% brain homogenate extracted with Sarkosyl. The concentration of SAF proteins in the preparations was greater than the quantity of SAF as detected by negative stain electron microscopy. This suggests that not all of the protein exists in the form of SAF. An immunologically reactive 33K to 35K protein was detected in both normal and scrapie brain preparations. This protein was susceptible to complete proteinase K (PK) digestion in normal brain preparations and it is suggested that scrapie infection is responsible for post-translational modifications which confer PK resistance in scrapie preparations. These modifications may also play a role in the antigenic differences seen in a variety of scrapie agents. SAF-specific proteins were also detected in the spinal cords and spleens from scrapie-affected animals. Detergent extraction of material followed by PK treatment and Western blot analysis is a highly specific and sensitive method for the detection of SAF proteins. This procedure could be applied to human neurological diseases of unknown aetiology.

INTRODUCTION

Scrapie-associated fibrils (SAF) are abnormal filamentous structures (Merz et al., 1981) observed only in the unconventional slow virus diseases (Merz et al., 1981, 1983b, 1984; McKinley et al., 1983). SAF were first observed by negative stain electron microscopy (EM) in detergent-treated synaptosomal-mitochondrial fractions from scrapie-affected mouse brain (Merz et al., 1981). SAF and infectivity co-purify (Diringer et al., 1983a, b; Kascsak et al., 1985), suggesting that SAF are closely associated with infectivity. SAF from different animal strain-scrapie agent combinations are distinguishable by such characteristics as polypeptide profile and sensitivity to proteinase K (PK) (Kascsak et al., 1985).

Silver staining of polyacrylamide gels has demonstrated that purified 263K agent SAF consist of a 26000 to 28000 (26K to 28K) mol. wt. protein whereas SAF obtained from mice affected with ME7 or 139A scrapie agents are composed of three polypeptides: 26K to 28K, 23K to 24K and 21K to 22K (Kascsak et al., 1985). It has not been possible to detect SAF-specific proteins in the original synaptosomal-mitochondrial preparations by silver staining because of the high background of contaminating host proteins. Recently, antisera has been generated to protein components of the purified ME7 SAF. This antiserum has been shown to react specifically with the SAF proteins by Western blot analysis (Gibbs et al., 1985; Manuelidis et al., 1985; R. J. Kascsak et al., unpublished). We have used this antiserum to monitor the presence of SAF-specific proteins in non-purified scrapie preparations. Since the SAF purification procedures...
involve the use of PK, it is uncertain what effect this enzyme treatment has on the SAF proteins during purification of these structures. Immunological analysis of synaptosomal–mitochondrial and other non-purified preparations, employing anti-SAF antisera, were also used to ascertain the effects of enzyme treatment on the SAF proteins.

**METHODS**

**Animals and scrapie agents.** The sources of animals and the scrapie agents ME7, 263K and 139A have been described previously (Kascak et al., 1985). The scrapie agent 22L was kindly provided by Dr A. G. Dickinson (AFRC & MRC Neuropathogenesis Unit, Edinburgh, U.K.). The scrapie agents ME7, 139A and 22L were inoculated into C57BL/6J mice. The scrapie agent 263K was inoculated into LVG/LAK hamsters. The inoculum preparation, intracerebral injections, scoring and sacrificing of animals were performed as previously described (Carp & Callahan, 1981). Control animals were injected with normal brain homogenate. Unless otherwise indicated the tissue for the preparations was obtained from animals sacrificed after showing clinical signs of scrapie disease for 3 consecutive weeks.

**Tissue preparation.** Synaptosomal–mitochondrial extracts of normal and scrapie brain material were prepared as previously described (Merz et al., 1981, 1983b, 1984). Subcellular fractionation of spleen material followed the procedure described by Merz et al. (1983b).

A 20% brain or spinal cord homogenate was prepared by Dounce homogenization in 1 mM-NaHCO3, 1 mM-MgCl2·6H2O and 0.32 M-sucrose. The homogenate was clarified at 2000 g for 15 min and the supernatant used. Detergent extracts were prepared in the same manner except that homogenization was performed in one of the following detergents: 1% Sarkosyl, 1% octyl glucoside, 1% sulphobetaine (SB) 3-14, or 2% SDS. The above 20% homogenates, prepared either in the absence or presence of detergents, are referred to as crude tissue preparations.

**Electron microscopy.** A 5 μl aliquot of a 1:10 to 1:50 dilution of a semi-purified preparation was applied for 1 min to freshly glow-discharged 400-mesh carbon-coated grids. Excess fluid was drained with filter paper and the sample was washed with distilled water and stained for 1 min with sodium phosphotungstate pH 7.2. The grid was air-dried and examined in a Philips EM300 or EM420 microscope at 80 kV or ×10000 to ×50000.

**Antisera.** Antisera to SAF protein, isolated from brains of C57BL/6J mice clinically affected with the ME7 scrapie agent, was prepared by immunization of a New Zealand white rabbit. SAF was purified as described elsewhere (Kascak et al., 1985), solubilized in 2% SDS and electrophoresed in 15% SDS–polyacrylamide gels (PAGE) (Laemmli, 1970). Areas containing the three ME7 SAF proteins (21K to 28K) were cut from the gel, eluted by H2O extraction and concentrated by lyophilization. Primary immunization consisted of inoculating 40 μg of purified eluted antigen in complete Freund's adjuvant (Difco) into the footpads and intramuscularly in an area near the hind leg lymph nodes. Each subsequent immunization was given in the same manner but in the presence of incomplete Freund’s adjuvant. The rabbit received four inoculations of gel-eluted antigen at approximately 2 week intervals. The antigen used for the fifth and final immunization was purified SAF. Titres of the antisera following the fourth and fifth immunizations were identical.

**Western blot analysis.** Proteins were electrophoresed in 15% polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose using a Trans-Blot Apparatus (Bio-Rad) at 40 V for 18 h according to Towbin et al. (1979). The nitrocellulose paper was blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 2% normal goat serum (NGS) for 45 min at room temperature. The paper was washed in PBS containing 0.2% Tween 20 (PBST). Primary antiserum (1:5000) in PBST containing 1% NGS was applied for 2 h at 37 °C. Following washing in PBST, secondary antibody [1:1000 goat anti-rabbit IgG conjugated with alkaline phosphatase (Tago, Burlingame, Ca., U.S.A.)] was applied for 1.5 h at 37 °C. The paper was washed with PBST and substrate (0.33 mg/ml nitro blue tetrazolium (Sigma) and 0.17 mg/ml 4-bromo-4-chloro-3-indolyl phosphate (Sigma) in 0.1 M-Tris, 0.1 M-NaCl, 0.05 M-MgCl2, pH 9.5) was added. The paper was incubated at room temperature until a noticeable colour change occurred. The reaction was stopped by washing in H2O.

**RESULTS**

SAF from a variety of scrapie agents could be observed by negative stain EM when prepared by the synaptosomal–mitochondrial procedure from the brains of scrapie-affected animals (Fig. 1). These structures were absent from normal material. The SAF observed in these fractions were structurally indistinguishable from those purified more extensively (Kascak et al., 1985) except for their greater length. The shorter lengths of the purified SAF were due to the frequent sonication steps in the purification procedure. Western blot analysis using anti-SAF antisera has previously indicated that purified ME7 SAF contained three immunologically reactive bands: 26K to 28K, 23K to 24K and 21K to 22K (R. J. Kascak et al., unpublished). The SAF from
Fig. 1. Electron micrograph of SAF obtained from detergent-treated synaptosomal–mitochondrial brain preparations of scrapie (a) 139A-affected mice, (b) 263K-affected hamsters, (c) 22L-affected mice and (d) ME7-affected mice. All samples were stained with 3% sodium phosphotungstate pH 7.2.

263K agent infection were also shown to be composed primarily of three stained bands: 26K to 28K, 23K to 24K and 19K to 20K (Bendheim et al., 1984; R. J. Kacsak et al., unpublished). Since purified SAF are exposed to PK during the isolation procedure, synaptosomal–mitochondrial preparations were examined with and without enzyme treatment. Synaptosomal–
Fig. 2 Western blot analysis of synaptosomal–mitochondrial fractions from brain of normal mouse (NM), normal hamster (NH), scrapie-affected brain of mouse (scrapie agent ME7) and hamster (scrapie agent 263K). Aliquots of fractions were incubated in the absence (lanes 1) or presence (lanes 2) of PK (50 μg/ml for 30 min at 37 °C) prior to electrophoresis and immunoblotting.

mitochondrial fractions from ME7-affected mice, which contained ME7 SAF according to negative stain EM, revealed three polypeptides by Western blot analysis, 33K to 35K, 26K to 28K and 23K to 24K (Fig. 2). Upon treatment with PK prior to electrophoresis there was a shift in the staining pattern to bands at 26K to 28K, 23K to 24K and 21K to 22K (Fig. 2). This pattern was identical to that seen in purified SAF prepared by procedures using PK. Synaptosomal–mitochondrial preparations shown to contain 263K SAF by negative stain EM revealed one major band in Western blots with anti-SAF antisera at 33K to 35K. As seen above for ME7, treatment of the 263K preparations with PK produced a change in the polypeptide profile, to molecular weights of 26K to 28K and 23K to 24K. This pattern is similar to that seen for purified 263K SAF. A lightly stained band at 19K to 20K was observed occasionally. It is apparent from these studies that PK treatment altered the banding profile of SAF proteins, resulting in lower molecular weight forms. It is also evident that SAF observed in the original synaptosomal–mitochondrial preparations (Merz et al., 1981, 1983b, 1984) contain SAF polypeptides that are antigenically identical to those isolated in highly purified preparations (Kascak et al., 1985). SAF antisera also stained a normal protein in the 33K to 35K region prior to PK treatment but failed to react with any proteins in uninfected preparations after enzyme treatment (Fig. 2).

Since SAF proteins were easily detected in synaptosomal–mitochondrial preparations by Western blot analysis, the possibility of detecting these proteins in less purified preparations was investigated. Twenty-% brain homogenates were prepared from mouse and hamster brains that had been inoculated either with normal brain material or with the scrapie agents ME7 and 263K, respectively. All extracts (see Methods) were examined with and without PK treatment prior to electrophoresis and blotting. SAF-specific proteins could be observed in homogenates with and without detergent following staining of blots with anti-SAF antisera (Fig. 3). The pattern was similar to that observed in Fig. 2, with bands at 33K to 35K, 26K to 28K and 23K to 24K from the brains of scrapie-positive mice and proteins at 33K to 35K and 26K to 28K from scrapie-positive hamsters. Following PK treatment there was again a shift to bands at 26K to
Antibody detection of scrapie proteins

Fig. 3. Western blot analysis of brain extracts prepared in the absence (a) or presence (b) of the detergent Sarkosyl as described in Methods. Aliquots of these samples were treated with PK as described in Fig. 2 prior to Western blotting.

Fig. 4. Appearance of SAF proteins in scrapie-infected brain homogenates. Mice infected with the ME7 scrapie agent were sacrificed at the indicated times (days) post-inoculation. A Sarkosyl-treated 20% brain homogenate was prepared as described in Methods. Aliquots were treated with PK as described in Fig. 2 and analysed on Western blots.

28K, 23K to 24K and 21K to 22K for scrapie-affected mice and 26K to 28K and 23K to 24K for 263K-infected hamsters. Sarkosyl treatment was more efficient in releasing SAF protein than homogenization without detergent as shown by the darker antibody staining. Using Sarkosyl
Fig. 5. An electron micrograph of SAF observed in scrapie strain ME7-affected spleen preparations. The sample was stained with 3% sodium phosphotungstate pH 7.2.

extraction, SAF proteins could be detected in 0.001 brain equivalents of material. A variety of other detergents (SB 3-14, octyl glucoside and SDS) were found to be equally effective in releasing the SAF proteins from brain material (data not shown). Sarkosyl extracts of normal brain material also showed a band staining in the 33K to 35K region. Staining of this band was lost following PK treatment. This band was observed both with and without detergent treatment but was more prominent in the latter preparations.

Since Western blot analysis of PK-treated crude brain homogenates appeared to be an easy
Antibody detection of scrapie proteins

and efficient method of detecting SAF proteins, we used it to determine the earliest appearance of scrapie-specific proteins in infected animals. As can be seen in Fig. 4, PK-resistant SAF proteins were first demonstrable at approximately 86 days post-inoculation. This is prior to the appearance of clinical symptoms which occurred between 130 and 145 days post-inoculation. The quantity of SAF protein continued to increase throughout the rest of the incubation period. This increase coincides with the rise in both infectivity and quantity of SAF (Bolton et al., 1982; Merz et al., 1983a; Diringer et al., 1983a, b; Hilmert & Diringer, 1984).

Spleens from infected animals have previously been shown to contain SAF (Merz et al., 1983b, 1984) and relatively high (compared to all tissues except brain) amounts of infectivity in the absence of any pathological changes (Carp et al., 1985). The spleens of ME7 scrapie agent-affected mice were examined for the presence of SAF-specific proteins. Negative stain EM of a subcellular fraction of spleen material (Fig. 5) shows SAF indistinguishable from that found in brain. Similar spleen preparations were examined by Western blot analysis before and after PK treatment using the anti-SAF antiserum (Fig. 6). Prior to PK treatment, light, diffuse staining was seen throughout the gel in both the normal mouse and ME7 scrapie agent spleen preparations. A high molecular weight band (55K) was seen consistently in non-PK-treated spleen material from scrapie-infected animals and occasionally in preparations of normal mouse spleen. Staining of the 55K band was lost after PK treatment, with the concomitant appearance of three polypeptides only in the scrapie preparations. The polypeptides had molecular weights similar to those of the SAF-specific proteins found in brain material.

The PK resistance of the scrapie proteins was used in a search for their presence in spinal cord. As can be seen in Fig. 7, Sarkosyl-treated spinal cord homogenates from ME7 and 263K scrapie-affected animals showed a staining pattern similar to that of scrapie proteins from brain preparations. Similar studies were also performed on crude homogenates obtained from a
variety of other organs (liver, kidney, lungs, heart and salivary glands), but so far we have been unable to detect SAF proteins in crude preparations of these organs. If SAF and the scrapie-specific proteins are present in these organs, it may prove necessary to concentrate the material prior to Western blot analysis.

We next extended our studies to mice affected with two other scrapie agents, 139A and 22L. Western blot analysis of PK-treated synaptosomal–mitochondrial fractions showed a polypeptide profile consisting of three bands: 26K to 28K, 23K to 24K and 21K to 22K. In the case of 139A the staining intensities of these bands increased as the molecular weights decreased, while for 22L all bands stained equally (Fig. 8). This profile was identical to Western blots of highly purified preparations. Western blot analysis was performed on PK-treated Sarkosyl extracts of crude brain and spinal cord homogenates prepared from mice affected with 139A and 22L (Fig. 8). The polypeptide profiles obtained from the brain and spinal cord crude material from mice affected by either agent were similar to those obtained in both cases from more purified preparations. Other detergents (SB 3-14, octyl glucoside or SDS) yielded identical results (data not shown).

**DISCUSSION**

Using anti-SAF antisera in Western blot analysis, SAF proteins were readily detected in three types of semi-purified or non-purified preparations, i.e. a synaptosomal–mitochondrial fraction, 20% brain homogenate and a 20% brain homogenate extracted with detergent. Using detergent treatment in semi-purified and non-purified preparations, SAF proteins have also been detected in spleens and spinal cord material, respectively. This is not surprising since SAF and infectivity have been detected in both the spleen and the central nervous system. Infectivity titres in the spleen have been found to be lower than in the central nervous system. This is reflected by the necessity to concentrate the spleen material in order to detect the SAF proteins. To detect the proteins in other organs of comparable infectivity titres, such as the salivary glands, it may also be necessary to concentrate the material prior to Western blot analysis. The immunostaining of
Antibody detection of scrapie proteins

scrapie-specific polypeptides in the SAF-enriched synaptosomal–mitochondrial preparations was observed with an antiserum raised to proteins from purified SAF. These results together with the structural similarities of the fibrils strongly suggest that SAF seen originally in synaptosomal–mitochondrial preparations by negative stain EM (Merz et al., 1981) are the same as those which co-purify with the infectivity following partial purification (Diringer et al., 1983a, b). SAF proteins in PK-treated non-purified preparations were measured by endpoint dilution and compared with the quantity found in purified preparations of known protein concentration. The quantity of SAF proteins obtained from the synaptosomal–mitochondrial and crude preparations was approximately 2 μg/brain equivalent. This represents a far larger amount than is obtained in SAF purification procedures (approximately 0.5 μg/brain equivalent) (R. Rubenstein et al., unpublished). However, comparison of the quantity of SAF in both purified and non-purified preparations, as determined by negative stain EM, does not reflect this difference. This either means that considerable SAF is lost in the purification scheme or that there exists a large pool of SAF proteins which are not assembled into SAF. The SAF structure has rarely been visualized in scrapie-affected brain preparations prior to detergent treatment (Somerville et al., 1985). This indicates that either SAF is so tightly bound to membrane material that detergent extraction is necessary, or that detergent itself plays a role in assembly of the SAF structure.

Western blots on crude extracts prior to and following PK treatment suggest that there is a breakdown of protein during the purification procedures which employ PK. Similar results have previously been reported by Oesch et al. (1985). All of our data suggest the presence of a precursor protein (33K to 35K in the central nervous system and possibly a 55K or some other undetected protein in the spleen) that is present prior to PK treatment. It has recently been reported (Bendheim & Bolton, 1985) that a 54K normal brain protein may be the precursor of the scrapie-specific proteins. The lower molecular weight bands that are seen routinely by Western blot analysis in this and other studies after using PK for purification of SAF (Bendheim et al., 1984; Kascak et al., 1985; Manuelidis et al., 1985) are probably the result of an effect of PK on this precursor protein or its modified form (see below). Definitive proof will be obtained once these proteins are sequenced.

The finding of a normal host protein in brain material with reactivity to anti-SAF antisera raises questions concerning the relationship of SAF and host proteins. Normal proteins are susceptible to complete PK digestion whereas SAF proteins are not. Staining of the 33K to 35K protein in normal material indicates the sharing of one or more antigenic sites with SAF proteins. The possibility that the normal protein has an unrelated site with reactivity to an antibody that is a minor component of the polyclonal antiserum seems unlikely since three different scrapie antisera preparations, all raised to purified SAF proteins, reacted with the 33K to 35K normal polypeptide (Manuelidis et al., 1985; Oesch et al., 1985; R. Rubenstein et al., unpublished). It has previously been reported that no differences were found in the mRNA from normal and scrapie brain material (Chesebro et al., 1985; Oesch et al., 1985). These results and those reported in this paper indicate that post-translational modifications together with structural changes of the host protein confer PK resistance. The SAF protein is known to be glycosylated (Bolton et al., 1985; Manuelidis et al., 1985; Multhaup et al., 1985; R. J. Kascak et al., unpublished). Structural or other chemical modifications may also occur. The different characteristics of the SAF polypeptides associated with different scrapie strains, such as antigen and lectin binding (R. J. Kascak et al., unpublished) shows that these post-translational modifications are not the same for each scrapie agent. In addition, competition studies between lectin and antibody binding indicated that lectin bound to the SAF proteins was not able to inhibit anti-SAF antibody binding (R. J. Kascak et al., unpublished). This suggests that the antibody is not directed against glycosylated sites on the SAF proteins.

Extraction of brain in 10% Sarkosyl followed by PK digestion prior to Western blot analysis was shown to be a highly specific and sensitive method for detection of SAF proteins. The detection of these proteins could be used to diagnose specimens of suspected cases of Creutzfeldt-Jakob disease or Gerstmann-Straussler syndrome. It has previously been reported (Bockman et al., 1985; Manuelidis et al., 1985) that proteins from the brains of patients who
were diagnosed as having Creutzfeldt-Jakob disease were immunologically reactive with an antibody raised in rabbits against scrapie-specific proteins. In addition, it has recently been reported that patients receiving human growth hormone therapy may have become infected with Creutzfeldt-Jakob disease from contaminated human pituitary glands (Brown et al., 1985). The technique reported in this paper may be useful for analysing samples from these patients. This procedure can be performed in 1 day and therefore offers great advantages over the long and expensive infectivity studies that are usually used for the diagnosis of these diseases.

We are grateful to C. Scalici, S. Callahan and A. Sanchez for their excellent technical assistance, R. Weed for photographic assistance and A. Monaco for typing the manuscript. This research was supported in part by NIH grants NS21349 and AG04220.

REFERENCES


Antibody detection of scrapie proteins


(Received 30 October 1985)