Antisera to Scrapie-Associated Fibril Protein and Prion Protein Decorate Scrapie-Associated Fibrils

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Scrapie-associated fibrils (SAF) are an infection-specific structure observed in the unconventional-agent diseases. Polyclonal antisera raised to scrapie proteins were used to test the antigenic relationship between purified fibrils and SAF isolated from non-protease-treated synaptosomal-mitochondrial preparations. The experimental design utilized fibrils from scrapie strain 263K-infected hamsters, scrapie strain 139A-infected mice, and scrapie strain ME7-infected mice. Preparations were examined by negative-stain immune electron microscopy and Western blot analysis of the polypeptides. Fibrils and polypeptides from each preparation reacted with a rabbit antiserum raised to each of the following: hamster 263K prion protein (PrP 27-30), hamster 263K SAF protein, and mouse ME7 SAF protein. Immune electron microscopy and Western blot analysis revealed similar antigenic relationships among the three scrapie antisera. Thus, fibrils and polypeptides can be considered to be the same in each preparation. No reactivity of the fibrils was observed with antisera raised to Alzheimer neurofibrillary tangles or a synthetic peptide of cerebrovascular amyloid. Thus, the fibrils observed in purified preparations share structural and antigenic similarities plus biochemically related peptides with SAF present in non-protease-treated preparations.

Scrapie-associated fibrils (SAF) were seen first by electron microscopy in detergent-treated synaptosomal-mitochondrial (S-M) fractions from scrapie-infected brains (24). Subsequently, SAF were visualized in similar preparations from Creutzfeldt-Jakob disease, kuru, chronic wasting disease of mule deer, and natural scrapie in sheep. They have not been found in brain extracts from normal individuals or patients with other neurological conditions and as such are an infection-specific marker for the unconventional-agent diseases (23). Several laboratories have isolated fibrils in association with both specific proteins and infectivity in hamsters infected with scrapie strain 263K (10, 30) and C57BL/6J mice infected with scrapie strain ME7 (17). It has been asserted that SAF as originally described are different in structure from the purified fibrils, prion rods (10, 30). The development of antisera to prion protein and SAF protein in several laboratories has provided a means to resolve this question (2, 5, 11, 16, 31). We refer to the prion protein or SAF protein as a *protease-resistant protein* (PrP). In the work described below, we used three antisera raised to PrPs of SAF or prion rods to compare the immunoreactivity of isolated fibrils and their constituent polypeptides prepared by a purification procedure which uses protease treatment and by a nonpurification procedure, originally used to identify SAF, which does not use enzyme treatment.

MATERIALS AND METHODS

Animals and scrapie agents. Hamster scrapie strain 263K and mouse scrapie strain 139A were kindly provided by Richard H. Kimberlin (ARC & MRC Neuropathogenesis Unit, Edinburgh, Scotland), and mouse scrapie strain ME7

was kindly provided by Alan G. Dickinson (ARC & MRC Neuropathogenesis Unit). Scrapie strains ME7 and 139A were inoculated intracerebrally into female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine), and scrapie strain 263K was inoculated intracerebrally into female LVG/LAK hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Normal C57BL/6J mice and LVG/LAK hamsters were inoculated with normal brain suspensions. Inoculum preparation, injection, scoring, and sacrificing of animals were done as previously described (9). All scrapie-infected animals used in this study were at the clinical stage of disease, and the animals inoculated with normal brain suspensions were age matched to the scrapie-infected animals.

Tissue preparation. Purified fibrils were first prepared from scrapie-infected brains (mice were used for scrapie strains ME7 and 139A and hamsters were used for scrapie strain 263K) as previously described (17). The final gradient fraction contained 10^9 50%-lethal-dose units per ml for the 263K strain and 3×10^7 50%-lethal-dose units per ml for the ME7 and 139A strains. Infectivity was determined by intracerebral inoculation of serial 10-fold dilutions of the gradient fractions for endpoint titration in either weanling LVG/LAK hamsters for scrapie strain 263K or weanling C57BL/6J mice for scrapie strains ME7 and 139A. Evaluation of aliquots of these preparations by both negative-stain electron microscopy and polyacrylamide gel electrophoresis confirmed the presence of SAF and SAF polypeptides (17).

S-M extracts of normal and scrapie-infected brain material were prepared as previously described (24).

Immune electron microscopy. For immune electron microscopy, aliquots of samples were placed on freshly glow-discharged, carbon-coated, 400-mesh nickel grids for 1 min and washed with 4 drops of distilled water. Nonspecific protein-binding sites were blocked with 0.1% bovine serum

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albumin in 0.04 M phosphate-buffered saline (PBS) (pH 7.2) for 10 min. Samples were incubated for 1 h with the primary antibody (1:500 or 1:1,000 dilution in 0.1% bovine serum albumin in PBS), washed with 4 drops of PBS for a total of 8 min, and exposed for 1 h to colloidal gold-conjugated goat anti-rabbit immunoglobulin (Polaron Instruments, Inc., or Janssen Life Sciences Products) diluted 1:25 with 0.1% bovine serum albumin in PBS. The grids were washed with 4 drops of PBS for a total of 8 min and with 2 drops of distilled water, and the material was stained with 3% sodium phosphotungstate (pH 7.2) or 2% uranyl acetate and air dried. The specimens were examined for the presence of colloidal gold in a Philips EM 420 at magnifications of $\times 10,000$ to $\times 60,000$. Those specimens negatively stained and exhibiting a low background of colloidal gold were further evaluated for the number of SAF labeled with colloidal gold. In each experiment, a total of 50 fibrils for each scrapie strain were counted. The deposition of 2 to 10 colloidal gold particles along a fibril was regarded as positive immunobinding.

For the S-M preparations, samples were diluted 1:20 or 1:50 with 0.32 M sucrose in 1 mM NaHCO₃ for immune electron microscopy. Proteinase K (Boehringer Mannheim Biochemicals) treatment consisted of incubating aliquots of 1:20 or 1:50 dilutions of normal or scrapie (139A, ME7, or 263K)-infected samples with 50 μ g of proteinase per ml for 30 min at room temperature. Samples were processed for immune electron microscopy as described below.

Antisera. All antisera raised to isolated scrapie proteins are prefixed by R-263K or R-ME7 for consistency. R-ME7-M-PrP antisera were prepared by using SAF protein isolated from scrapie strain ME7-infected mice by the procedure of Hilmert and Diring (15) and further purified by gel elution of protein from the 20- to 30-kilodalton (kDa) region (31). R-263K-SAF (R-263K-PrP-2) antisera were prepared by using SAF protein isolated from scrapie strain 263K-infected hamsters by the same procedure but without additional gel purification (5); they were a gift of Heino Diring of the Robert Koch Institute, West Berlin, Federal Republic of Germany. R-263K-PrP (R-263K-PrP-1) antisera were from the fourth bleed and were prepared by using denatured prion protein isolated from scrapie strain 263K-infected hamsters by the procedure of Prusiner and co-workers (2, 30); they were a gift of Paul Bendheim of this institute. R-263K-PrP-1 reacts with prion proteins isolated from hamsters infected with scrapie strain 263K and from mice and humans infected with scrapie strain CJD (2-4). R-ME7-M-PrP and R-263K-PrP-2 likewise react with SAF proteins isolated from hamsters infected with 263K, mice infected with ME7 or 139A, hamsters infected with CJD, and human brains infected with CJD (5, 12, 20). R-263K-PrP-1 has been shown to immunodecorate prion rods isolated from scrapie strain 263K-infected hamsters (1). R-263K-PrP-2 likewise immunodecorates SAF isolated from scrapie strain 263K-infected hamsters (5). Rabbit antisera raised to Alzheimer neurofibrillary tangles (R-ANT) was a gift of Inge Grundke-Iqbal of this institute (14). Rabbit antisera raised to a synthetic peptide (21, 25) representing amino acids 1 to 24 of a partial amino acid sequence of cerebrovascular amyloid (13) was a gift of David Miller of this institute, and these antisera are known as rabbit antisera raised to synthetic cerebrovascular amyloid peptide (R-SCVAP).

Western blot analysis. Proteins were solubilized in 2% sodium dodecyl sulfate-0.5% β -mercaptoethanol, electrophoresed on 12% Laemmli gels, and electrophoretically transferred to nitrocellulose as previously described (34).

For the S-M preparations, 0.03 brain equivalents of an S-M fraction was added per lane (see Fig. 4). The S-M preparation was also treated with proteinase K (50 μ g/ml) for 15 min at 37°C prior to solubilization and electrophoresis. Approximately 200 ng of purified 263K SAF was added to each lane (see Fig. 4). Western blot staining was carried out as previously described (16, 31) with secondary antibody conjugated to alkaline phosphatase and a Nitro Blue Tetrazolium-based substrate.

RESULTS

All three antisera (at dilutions of 1:500 and 1:1,000) decorated purified fibrils isolated from brains infected with scrapie strains 263K, ME7, and 139A (Fig. 1). These antisera had differing reactivities with the purified fibrils both in the number of gold particles bound per fibril and in the percentage of fibrils that were decorated (Table 1). In all cases, the R-ME7-M-PrP antisera were most reactive with the purified fibrils, with 70 to 100% of the fibrils being stained. The next most reactive antisera were R-263K-PrP-2, with 40 to 70% of the fibrils being stained. The least reactive antisera were R-263K-PrP-1, which stained only 20 to 40% of the fibrils. It is not surprising that R-ME7-M-PrP and R-263K-PrP-2 antisera showed the greatest reactivity with these SAF preparations, since these antisera were prepared by using SAF proteins isolated by a similar procedure, while R-263K-PrP-1 antisera were raised to denatured proteins isolated by a different procedure. All three antisera reacted with one or more epitopes present on SAF isolated from mice infected with scrapie strain 139A and ME7 and from hamsters infected with scrapie strain 263K. The only consistent decoration pattern observed was that the greatest number of gold particles tended to be present at the ends of the fibrils (Fig. 1b). This result is not surprising, since all three antisera were raised to solubilized polypeptides, and thus the epitopes located on the surfaces of the fibrils could be fewer in number than those located at the ends of the fibrils. The major cause of high background staining was found to be inefficient washing or very high concentrations of the first or second antibody or both (Fig. 1b; see also Fig. 2c). In these situations, ferritin tended to be labeled (Fig. 1b).

Since other abnormal fibrils deposited *in situ* in Alzheimer's disease (amyloid and paired helical filaments) have shown a tendency to nonspecifically bind immunoglobulins (34a, 35) and since there has been speculation that the amyloid observed in Alzheimer's disease is composed of prions (28), we tested antisera raised to these abnormal fibrils for reactivity with isolated SAF. R-ANT and R-SCVAP both reacted in negative-stain immune electron microscopy with the homologous structures to which they had been raised (25). These antisera (1:500 and 1:1,000 dilutions) and normal rabbit sera (1:500 and 1:1,000 dilutions) did not react with SAF from either ME7- or 139A-infected mice or 263K-infected hamsters (Table 1). Thus, SAF derived from ME7-, 139A-, and 263K-infected animals do not share surface antigens with other abnormal fibrils, and there was no nonspecific binding of the colloidal gold to the fibrils under the labeling conditions used. R-ME7-M-PrP antisera do not react with Alzheimer's disease abnormal fibrils in tissue sections (25) or with isolated Alzheimer's amyloid or paired helical filaments.

The above-mentioned data showed that the three scrapie antisera reacted specifically with purified scrapie fibrils. We then examined the ability of SAF in the nonpurified S-M fractions from ME7- and 139A-infected C57BL/6J mouse

FIG. 1. Electron micrographs of purified SAF from (a) scrapie strain 139A reacted with R-ME7-M-PrP antisera (1:500 dilution) (note the deposition of colloidal gold); (b) scrapie strain ME7 reacted with R-263K-PrP-1 antisera (1:1,000 dilution) (note the deposition of colloidal gold); and (c) scrapie strain ME7 reacted with R-ANT antisera (1:1,000 dilution) (note the absence of colloidal gold deposition). The preparations were stained with phosphotungstate (a) and uranyl acetate (b and c). Magnification, $\times 143,000$.

brains and 263K-infected hamster brains to bind the three antisera. The original observation of SAF in scrapie-infected mice was made by negatively staining a sample of a similar S-M fraction (24). In addition, we examined the ability of the scrapie antisera to react with SAF which had not been treated with proteinase K. Proteinase K was used in the purification procedures (15, 30, 31) employed to prepare the immunogen for all three antisera. Since the nonpurified S-M fractions contained trapped soluble proteins and Fc receptors, colloidal gold-conjugated protein A and colloidal gold-conjugated goat anti-rabbit immunoglobulin were evaluated for specific binding to SAF in the absence of any primary antisera. These controls did not react with SAF. SAF in the non-protease-treated preparations were immunolabeled by the scrapie antisera, yielding patterns similar to those shown for the purified fibrils (Fig. 2). SAF were not immunolabeled by protein A, goat anti-rabbit-immunoglobulin, R-ANT, or R-SCVAP. Immune electron microscopy of these samples was repeated after proteinase K treatment (50 $\mu\text{g}/\text{ml}$ for 30 min at room temperature). All three scrapie antisera immunolabeled SAF following such treatment (Fig. 3). Decoration of SAF with these three antisera was not dependent upon exposure of SAF to proteinase K or changed by such exposure.

S-M preparations from scrapie strain 263K-infected hamsters and fibrils purified from scrapie strain 263K-infected hamsters were examined by Western blot analysis (34) for reactivity with the three antisera. All antisera reacted with a broad protein band at 33 to 35 kDa and with a 26- to 28-kDa protein band in the S-M preparations (Fig. 4, lane 1). As previously noted (17), the staining intensity of SAF protein bands varied with the antisera used in Western blot analysis. R-263K-PrP-1 reacted most strongly with the S-M preparations, intensely staining protein bands at 33 to 35 kDa, 26 to 28 kDa, 23 to 24 kDa, and 19 to 20 kDa. R-263K-PrP-2 reacted less well, revealing protein bands at 33 to 35 kDa and 26 to 28 kDa. This difference in the Western blot profiles reflects the intensity of antibody reactivity and the lower protein concentration of the lower-molecular-weight proteins. R-ME7-M-PrP reacted least strongly but stained proteins at 33 to 35 kDa and 26 to 28 kDa similar to those stained by R-263K-PrP-2 and R-263K-PrP-1. This weaker reactivity may be related to species differences in SAF proteins (17). Upon treatment of the S-M preparations with proteinase K (50 $\mu\text{g}/\text{ml}$ for 15 min at 37°C) prior to electrophoresis, there was a change in the Western blot profiles (27). Immunoreactive bands at 26 to 28 kDa, 23 to 24 kDa, and 19 to 20 kDa were now present in the Western blot profiles with R-263K-PrP-1 and R-263K-PrP-2 antisera (lane 2), with a loss of the band at 33 to 35 kDa, and these bands appeared to be identical to those present in the purified SAF preparations (lane 3). The reactivity of R-ME7-M-PrP was again less intense, with only the bands containing higher concentrations of protein (26 to 28 kDa and 23 to 24 kDa) being stained. Size-heterogeneous protein bands in the range of 43 to 56 kDa were often present at low concentrations in PrP preparations (7) and may reflect dimer or polymer aggregation of different-molecular-weight forms of PrP. The variation in the staining patterns of proteins in this size range seen in these Western blots with different antisera (i.e., lane 3) indicated both the variability of this aggregation and differences in antibody reactivity to them. The Western blot studies correlated with staining (immunodecorating) in that all three antisera reacted with SAF and SAF protein prior to and following proteinase K treatment. However, in terms of reactivity, R-263K-PrP-1 exhibited the greatest reactivity in

FIG. 2. Electron micrographs of SAF in S-M preparations from (a) scrapie strain ME7 reacted with R-SCVAP antisera (1:500 dilution) (note the absence of colloidal gold deposition); (b) scrapie strain 263K reacted with R-263K-PrP-2 antisera (1:500 dilution) (note the deposition of colloidal gold); and (c) scrapie strain ME7 reacted with R-ME7-PrP-2 antisera (1:250 dilution) (note the deposition of colloidal gold). All preparations were stained with phosphotungstate. Magnification, $\times 143,000$.

Western blots and the least reactivity with isolated fibrils, while both R-263K-PrP-2 and R-ME7-PrP reacted more intensely with isolated fibrils than in Western blots.

Since SAF were originally detected by electron microscopy upon the addition of detergent to membrane fractions and since electron microscopy revealed a close association of SAF with membranes (19, 24), an additional experiment was performed in which S-M preparations from hamsters

infected with 263K or from normal animals were reacted with the various scrapie antisera prior to the addition of detergent. SAF were not detected in the S-M preparations, but specific focal areas of some membranes in the scrapie S-M preparations were immunolabeled with the three scrapie antisera. S-M preparations from normal animals did not exhibit this staining pattern (Fig. 5). Mitochondria were not immunolabeled in either the normal or the scrapie

FIG. 3. Electron micrographs of SAF in S-M preparations, following treatment with 50 μ g of proteinase K per ml at room temperature for 30 min, from (a) scrapie strain ME7 reacted with R-263K-PrP-1 antisera (1:1,000 dilution) (note the deposition of colloidal gold); (b) scrapie strain 139A reacted with R-ME7-M-PrP antisera (1:500 dilution) (note the deposition of colloidal gold); and (c) scrapie strain ME7 reacted with normal rabbit sera (1:500 dilution) (note the absence of colloidal gold deposition). All preparations were stained with uranyl acetate. Magnification, $\times 143,000$.

material. The staining pattern further suggests the presence of SAF protein in membranes.

DISCUSSION

The three scrapie antisera immunodecorated SAF under a variety of isolation conditions. Each antiserum reacted the

best against the homologous fibril from which the component polypeptides had been used as an antigen. The decoration pattern was not dependent on exposure of the preparations to proteinase K. The pattern of reactivity was surprising, in that the whole fibril was not heavily decorated with the colloidal gold. In fact, most of the published decoration patterns of SAF or prion rods could be interpreted as light (1,

TABLE 1. Immunolabeling of SAF

Prepn tested	Scrapie agent	Immunolabeling ^a of SAF by indicated antisera:				
		R-ME7-M-PrP	R-263K-PrP-1	R-263K-PrP-2	R-ANT	R-SCVAP
Purified	139A	75.0 (3-10)	22.0 (1-3)	45.0 (3-6)	1.0 (0-1)	0.5 (0-1)
	ME7	93.0 (3-20)	31.5 (2-4)	56.0 (4-7)	0.5 (0-2)	1.0 (0-1)
	263K	65.5 (3-5)	43.5 (4-6)	73.0 (4-9)	1.0 (0-1)	0.5 (0-2)
S-M	139A	71.0 (3-10)	25.0 (1-4)	46.0 (4-8)	0.5 (0-1)	0.5 (0-2)
	ME7	97.0 (2-21)	29.0 (1-5)	61.5 (4-6)	0.5 (0-1)	1.0 (0-1)
	263K	62.5 (3-10)	47.5 (4-9)	65.0 (1-10)	0.5 (0-2)	1.0 (0-1)
S-M treated with proteinase K ^b	139A	78.0 (2-9)	25.0 (1-5)	42.0 (3-7)	1.0 (0-1)	0.5 (0-1)
	ME7	95.0 (3-22)	30.0 (1-4)	58.5 (4-6)	1.0 (0-1)	1.0 (0-1)
	263K	68.5 (1-5)	46.5 (3-8)	75.5 (3-12)	0.5 (0-1)	1.0 (0-1)

^a Data represent the percentage of fibrils on which two or more colloidal gold particles were deposited (number of gold particles present on an individual fibril).

^b The S-M preparations (after detergent treatment) were incubated with 50 µg of proteinase K per ml for 30 min at room temperature.

5), while those reported here could be interpreted as heavy. This result indicates that the polyclonal antibodies raised to the solubilized proteins may only react with a few conformational epitopes present on the surface of the fibril. Antisera or monoclonal antibodies raised to the intact fibril may yield a heavier repetitive decoration pattern which could be used for epitope mapping of the fibril. This use would aid in understanding how the fibril is constructed. The consistent lack of gold particles decorating the SAF when other, nonrelated antisera were used was a good control for the observed positive staining with the three scrapie antisera. Considering that the immunoglobulin conjugated to the colloidal gold is 10 nm in length and the antibody binding to SAF is also 10 nm in length, it is not surprising that a 5- to 10-nm space was observed at times in the binding of the colloidal gold to the exterior of SAF. The colloidal gold could be seen mainly alongside and not on top of the fibril. Our interpretation of this phenomenon is that the weight of the colloidal gold in contrast to the length of the reacting antibody causes the colloidal gold particle to fall over alongside the fibril. SAF rest 10 to 15 nm above the supporting carbon coat, and neither SAF nor reacting antibodies are chemically fixed, so the antibodies can be considered flexible and therefore capable of falling to the side during processing

of the grid. When embedded thin sections are used for postimmunodecorating, this is not a problem, since support is present across the entire thin section.

These studies showed that both SAF and prion rods observed in purified preparations shared structural epitopes and antigenically related component polypeptides with SAF originally observed in nonpurified preparations. Both SAF and prion rods copurify with infectivity (10, 17, 30, 33) and biochemically and antigenically related PrPs in a variety of procedures (8, 10, 17, 20, 30, 31). SAF in purified and nonpurified preparations were immunodecorated with R-263K-PrP-1 antisera, raised to the protein component of prion rods. Likewise, SAF in purified and nonpurified preparations were immunodecorated with R-ME7-M-PrP and R-263K-PrP-2 antisera, raised to the protein components of ME7 and 263K SAF, respectively. The reactivities of all three antisera used in this study were very similar in both immunodecoration and Western blots. At this time, it is reasonable to conclude that the fibrils observed under different isolation conditions from brains infected with scrapie agents are the same.

Immunocytochemistry performed on Formalin-fixed or frozen brain sections from infected animals with the scrapie antisera has been disappointing in that the only specific structures consistently immunolabeled have been amyloid plaques in certain scrapie agent-host combinations (2, 22a). In the present study, antisera directed against the scrapie peptides were observed to react with non-detergent-treated membranes. Antibody reactivity appeared as small focal areas of staining on only certain portions of the membranes. The topography of antigen sites detected by this technique might ultimately indicate where SAF antigens and possibly the agent are located within the membrane (18, 24, 26, 32).

At the time of the original observations of the fibrillar structures, the term SAF was coined as a descriptive term which encompassed both their disease of origin and their appearance (24). Similar structures subsequently described in partially purified preparations were referred to as rods (29) and then prion rods (30). The present work strongly indicates that the structures originally described and those in partially purified preparations do not differ in structure, protein composition, or antigenicity. The proteins associated with scrapie infectivity have received several designations: PrP (6), unique protein (29), prion protein (22), and SAF protein (10). PrP has been used as an abbreviation with two different meanings, prion protein (22) and protease-resistant protein (6). Part of this confusion can be eliminated by use of terms in the order of their chronological appearance until a common consensus is agreed upon. All fibrils would then be

FIG. 4. Western blot analysis of nonpurified and purified SAF proteins. SAF protein in S-M preparations (lanes 1 and 2) or purified SAF protein (lane 3) was electrophoresed and transferred to nitrocellulose paper (28). S-M preparations in lane 2 were treated with proteinase K at 50 µg/ml for 30 min at 37°C prior to electrophoresis. Antisera were used at the following concentrations: R-ME7-M-PrP (A), 1:5,000; R-263K-PrP-1 (B) and R-263K-PrP-2 (C), 1:10,000. Blots were developed by using alkaline phosphatase-conjugated secondary antibody and a Nitro Blue Tetrazolium-based substrate (16).

FIG. 5. Electron micrographs of non-detergent-treated S-M membranes from (a) normal hamster brains reacted with R-263K-PrP-2 antisera (1:1,000 dilution) (note the absence of colloidal gold deposition); and (b) scrapie strain 263K-infected hamster brains reacted with R-263K-PrP-2 antisera (1:1,000 dilution) (note the deposition of colloidal gold). All preparation were stained with uranyl acetate. Magnifications, $\times 56,000$ (a) and $\times 102,000$ (b).

referred to as scrapie-associated fibrils, SAF (24). Their component polypeptides would be referred to as protease-resistant-proteins, PrPs, as denoted by the first identification of this protein (6). Commonality of terms would foster easier communication and understanding among laboratories inside and outside the scrapie field.

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