Characterization of Antisera against Scrapie-associated Fibrils (SAF) from Affected Hamster and Cross-reactivity with SAF from Scrapie-affected Mice and from Patients with Creutzfeldt-Jakob Disease

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SUMMARY

Antisera raised in rabbits and also for the first time in mice against scrapie-associated fibril (SAF) protein from hamster brain have been quantified by a modified ELISA technique (NC-ELISA) and used for a detailed analysis of SAF proteins obtained from hamster, mouse, and from patients who died of Creutzfeldt-Jakob disease. The antisera predominantly detected five bands in a Western blot analysis with apparent molecular weights of about 26000 (26K), 24K, 20K, 18K and 16K. By gel electrophoresis these antigens seem to be identical in mouse, hamster and man. The amount of material in the various bands, however, varies according to host or agent. In control materials from healthy brain SAF protein was found to be absent even when this material was used in a 50-fold excess compared to diseased brain.

INTRODUCTION

Unconventional slow viruses infecting their hosts produce always fatal progressive degenerative destruction of the central nervous system without any apparent immunological reaction. The use of the model system scrapie in hamsters (Kimberlin & Walker, 1977) resulted in the discovery of disease-specific fibrils (Merz et al., 1981) which were subsequently found in mouse scrapie as well as in the human Creutzfeldt-Jakob disease (CJD) (Merz et al., 1983, 1984). These scrapie-associated fibrils (SAF) were shown to contain a major protein component (SAF protein) (Diringer et al., 1983a; Prusiner et al., 1983) which had been discovered a year before as a disease-specific protein then designated prion protein (PrP) (Bolton et al., 1982). As a considerable amount of infectivity is associated with SAF, it was thought that they perhaps represent the infectious agent (Diringer et al., 1983a; Prusiner et al., 1983), a possibility raised also by their discoverers (Merz et al., 1983, 1984). However, SAF could also represent a pathological product caused by an infectious agent (Diringer et al., 1983a; Merz et al., 1983, 1984; Prusiner et al., 1983). We now believe that the latter is the case (Braig & Diringer, 1985).

With a purification method developed to yield large quantities of SAF protein from a few hamster brains (Hilmert & Diringer, 1984) we were able to raise antisera in rabbits (Diringer et al., 1984), as have others using a laborious method involving 1000 hamster brains (Bendheim et al., 1984). Our antisera have in the present study been characterized qualitatively and quantitatively and have been studied with respect to their cross-reactivity with SAF protein obtained from mice and CJD patients. Furthermore, we have compared the rabbit sera with three mouse sera prepared for the first time against hamster SAF protein using formic acid-denatured SAF protein.

METHODS

Scrapie strains and infection of animals. Hamsters of the inbred CLAC and the outbred AURA strains were infected intracerebrally (i.c.) with the 263K strain of hamster scrapie (Diringer et al., 1983b). Inbred STU mice were infected i.c. with the mouse scrapie strain 139A (Ehlers et al., 1984).
Clinical cases of CJD. Frozen autopsy and biopsy materials of CJD patients were collected and had been stored at −40 °C at the Institute of Neurology, Catholic University, Rome, since 1977. From this collection, frozen brains of five CJD patients (four females, one male, aged at death 49 to 70 years) were selected according to clinical, electroencephalogram (EEG) and neuropathological criteria. Detailed clinical and neuropathological descriptions of two of these cases (CJD-3 and CJD-2) have been reported (Rossini et al., 1979; Villa et al., 1982; Macchi et al., 1984). All patients had a history of rapid and progressive mental deterioration along with pyramidal and extrapyramidal signs, ataxia, myoclonus and a typical EEG of pseudoperiodic bi- or triphasic sharp waves generalized in both of the hemispheres. The duration of clinical disease was less than 6 months in four patients, but 18 months in the fifth (CJD-2). Neuropathological examination showed marked spongiosis of the grey matter with neuronal loss and proliferation of hypertrophic astrocytes. In addition, amyloid plaques and extensive white matter degeneration were observed in two cases (CJD-3 and -2). As controls, we used two frozen brains from patients who died without any neurological or infectious disease (N-6, aged at death 69 years, and N-1, age at death unknown).

Preparation of antigen. SAF proteins from various sources as well as control materials were prepared according to the method described recently (Multhaup et al., 1985). Brains from scrapie mice and hamsters were used at the terminal stage of the disease to ensure a high yield of SAF.

Preparation of rabbit and mouse antisera. The method for raising rabbit sera directed to hamster SAF protein has been described (Diringer et al., 1984). Mouse sera were raised by the same protocol, but immunogen used was formic acid-denatured SAF protein from hamster prepared by the method of Multhaup et al. (1985). Three injections per mouse each representing material obtained from three hamster brains were sufficient to raise antisera directed to SAF protein.

Immuno electron microscopy (IEM). IEM was performed using negative staining in the preadsorption mode of antigens under study as described in detail for an IEM study of bacterial pili (Gelderblom et al., 1985). Anti-rabbit IgG-gold complexes (gold 10 nm diam.) were purchased from Janssen Life Sciences Products (Bearse, Belgium) and were used at a 1:80 dilution. Anti-SAF serum was used at a 1:10 dilution.

Antibody detection methods.

NC-ELISA. The quantitative determination of antibodies to SAF protein in rabbit antisera was carried out by a modified ELISA procedure (NC-ELISA) which was developed recently (Beutin et al., 1984; Bode et al., 1984). The NC-ELISA is characterized by the use of nitrocellulose (NC) discs inside microtitre plates for efficient immobilization of antigen and a special histochemical staining procedure (which allows simple reading of the results by the naked eye).

The materials used as antigens were formic acid extracts prepared as described for the isolation of SAF protein (Multhaup et al., 1985). The dried formic acid extracts were dissolved in Tris (0.1 M)-buffered saline (TBS) pH 8.0 by short ultrasonic bursts (three times 3 s) with a sonicator cell disrupter (Ultrasonics, Inc.). Samples of 1 ml were spotted immediately onto nitrocellulose. Frozen samples were re-sonicated in an ultrasonic bath for 20 min before use. The solutions spotted contained the following concentrations of brain extracts per 100 ml: hamster, 0.5 to 2 brain equivalents, i.e. SAF material obtained from 0.45 to 1.8 brain; mouse, 2 to 4 brain equivalents, i.e. SAF material obtained from 0.9 to 1.8 brain; human, SAF material obtained from 10 g brain. All control samples from normal brains were used in the same concentrations as those from infected brains. The rest of the test procedure was carried out according to our previous description (Beutin et al., 1984; Bode et al., 1984); rabbit sera were used in serial dilutions starting from 1:1000 (in a few cases from 1:100) up to 1:64 000; alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago, Burlingame, Ca., U.S.A.) was diluted 1:2000 in all tests. The washing procedure was shortened to four changes instead of 10, with a 5 min interval after the second wash, by use of a 12-channel manual washer for microplates (Nunc), thus allowing the whole test to be performed within 4 to 5 h.

Western blot. Samples of SAF protein from hamster and mice brains and CJD-associated protein from human brains, respectively, extracted by the formic acid method (Multhaup et al., 1985), were prepared and separated by 12 to 17% SDS-PAGE according to standard procedures (Laemmli, 1970). Proteins were transferred from the gel to NC membranes (Schleicher & Schüll, pore size 0.45 μm) by overnight electrophoresis (30 V, Bio-Rad Trans-Blot Cell) using a Tris (25 mm)-glycine (192 mm) transfer buffer pH 8.3 containing 20% methanol, followed by a higher voltage transfer (60 V) for 1 h at room temperature.

The immunoassays of the Western blots were developed following a similar procedure as for NC-ELISA using the same reagents, times and temperatures for the subsequent incubation steps with bovine serum albumin blocking solution, serum, conjugate, and substrate. For each NC-blotted sample a volume of 10 ml of reagent was used. The NC strips were washed twice, each time for 10 min, gently rotating them in 50 ml per sample of NC-ELISA washing solution. The rabbit anti-SAF immune serum was diluted 1:1000 or 1:2000; conjugate and substrate dilutions were the same as for NC-ELISA.

RESULTS

Rabbit antisera to denatured SAF protein (Diringer et al., 1984) were first tested for their reactivity towards the intact SAF. For this purpose purified SAF (Diringer et al., 1983a)
Specificity of antisera against SAF

Fig. 1. Rabbit antibodies to hamster SAF protein reacting with intact SAF in IEM using anti-rabbit IgG-gold complexes (10 nm diam. particles). The specificity of this reaction is demonstrated by incubating a mixture of SAF and TMV with either anti-TMV serum (a) or anti-SAF serum (b) diluted 1:500 and 1:10, respectively.

Serum

(a) Rabbit
(b) Mouse
(c) Rabbit
(d) Rabbit

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Antigen

Hamster Hamster Mouse Man

Fig. 2. Quantitative determination of rabbit and mouse antibodies against hamster SAF protein by NC-ELISA showing reactions of one rabbit serum with hamster SAF protein from two different preparations (a), cross-reactions with mouse SAF protein (c), SAF-like protein isolated from brains of human CJD cases (d), and reaction of one mouse serum with hamster SAF protein (b). Preimmune serum (row 0) and twofold dilutions of immune serum (rows A to F) from (a, c, d) rabbit 536 and (b) mouse 3, starting at 1:1000, were tested with 1 μl of hamster SAF (columns 1, 3, 4) and normal hamster brain (columns 2, 5) (from 0.9 g brain/100 μl); with 1 μl of mouse SAF (column 6) and normal mouse brain (column 7) (from 0.9 g brain/100 μl), and with 1 μl of human CJD preparations (CJD-7, -3, -4, -2, -5 in columns 9, 10, 11, 13, 14) and normal human brain (N-6, -1 in columns 8, 12) (from 10 g brain/100 μl).
Table 1. Antibody titres of rabbit immune sera against hamster SAF protein determined by NC-ELISA using SAF protein from hamster and mouse brain and SAF-like protein from human CJD cases

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Reciprocal antibody titres with</th>
<th>Normal human brains (N patient no.)†</th>
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<tr>
<td></td>
<td>Scrapie hamster brain and normal hamster brain*</td>
<td>Scrapie mouse brain and normal mouse brain*</td>
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<td></td>
<td>(a) ≤ 32000 (2000)†</td>
<td>(b) &gt; 32000 (2000)</td>
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<tr>
<td>537</td>
<td>≥ 32000 (2000)</td>
<td>NT§</td>
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<tr>
<td>534</td>
<td>16000 (&lt;1000)</td>
<td>≥ 16000 (&lt;500)</td>
</tr>
<tr>
<td>535</td>
<td>NT</td>
<td>32000 (&lt;200)</td>
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* One µl samples derived from the following preparations were used. (a, b) Different lots of SAF protein from 0.45 g and 0.9 g hamster brain/100 µl, respectively; (c, d) different lots of SAF protein from 0.9 g and 0.3 g mouse brain/100 µl, respectively (normal animal brain used in same amounts).
† CJD and normal human brain isolates from 10 g brain/100 µl.
‡ NT, Not tested.
§ NT, Normal activity in parentheses.
‖ Not tested below 1000.
Specificity of antisera against SAF admixed with tobacco mosaic virus (TMV) were adsorbed to carbon-reinforced electron microscopy grids and reacted with either anti-SAF serum or anti-TMV serum. Rabbit antibody bound specifically to either SAF or TMV was revealed by the presence of electron-dense anti-IgG–gold complexes (Fig. 1). It can be seen that the serum raised against SAF protein reacted with the fibril, whereas the serum raised against TMV reacted with the virus only. The preimmune sera did not react with SAF (not shown).

We then quantified the amount of antibody in the four rabbit anti-SAF sera, by testing serial dilutions of the sera against standardized amounts of hamster brain SAF protein adsorbed to nitrocellulose by the NC-ELISA method (Bode et al., 1984). All sera reacted positively to SAF protein up to high dilutions, whereas control material obtained from healthy brains only reacted at low dilutions. No reaction was obtained with the preimmune sera. The three mouse sera raised against SAF from hamster brain as formic acid-denatured SAF protein (Multhaup et al., 1985) also reacted specifically in this test. Typical NC-ELISA results with one rabbit and one mouse serum are shown in Fig. 2(a, b).

The test was found to be positive using different SAF preparations from hamster brains. Cross-reactivity with SAF material isolated from mouse brain could also be demonstrated (Fig. 2a, c). One of the rabbit sera was used to determine whether it reacted specifically also with material from human brains of patients who had died of CJD (Fig. 2d). The CJD antigen reacted with the anti-hamster SAF serum in all five cases. Control material was negative in only one case (N-1), whereas the other human control showed a positive reaction comparable to the two weakly reacting CJDs (CJD-2 and -5) (Fig. 2d). Table 1 summarizes the quantification of antibodies in the rabbit sera and gives information about their reactivity with the various antigens. Sera 536 and 537 reacted with hamster SAF protein as well as with mouse SAF protein up to dilutions of about 1:32000 to 1:64000, whereas with normal material they reacted up to dilutions of about 1:1000 to 1:4000. Serum 534 was somewhat less potent, and the weakest reactivity was measured for rabbit 535. Against human CJD material, sera 536 and 537 reacted up to various antibody titres depending on the patient the material was derived from. With material from patient CJD-7, the sera were as potent as they were with the hamster-derived antigens.

Rabbit serum 536 was then used in Western blot analyses at a 1:2000 dilution in order to characterize SAF proteins obtained from the different sources, and also to investigate whether we could detect SAF protein in normal brain material. The latter seemed essential, because the tests described above revealed some non-specificity of our sera.

In Fig. 3, it can be seen that Western blot analyses were much more suitable than silver staining of SDS–polyacrylamide gels for differentiating between a variety of bands in scrapie brain material from hamster and mouse as well as in human CJD material. At least five different bands of antigen present at nearly identical positions could be differentiated in all diseased brain materials. The amount of material present in these bands varied, however, according to either the host species or the kind of agent. Whereas in hamster SAF protein band 1 with the highest molecular weight of about 26K was most prominent, in mouse material the antigen was almost equally distributed between bands 1 to 4 with a possibly slight excess of material in band 4 representing a molecular weight of about 18K.

No SAF protein was detected in normal hamster or mouse brain preparations, even if these materials were used up to the tested 10-, 20- or 50-fold excess (hamster) and to 5- or 10-fold excess (mouse). Mouse sera raised against hamster SAF when used in Western blots showed identical band patterns (data not shown).

In the two human CJD materials which were available in sufficient quantities for Western blotting the strongest reactions were observed with bands 2 and 4 representing molecular weights of about 24K and 18K, respectively. As well as the five bands also present in hamster and mouse SAF, the rabbit antiserum detected additional material at positions of about 12K and 10K. This material was also present in the preparation of human control brain N-6 which reacted positively in the NC-ELISA, but not in a twofold concentrated sample of control brain N-1 which showed no non-specificity in ELISA. Furthermore, in diseased brains from all sources, a high molecular weight band (about 40K) could be detected if large amounts of antigen were blotted.
Fig. 3. Characterization of SAF protein derived from different hosts (hamster, lanes 1 to 5; mouse, lanes 6 to 8; man, lanes 9 to 12) by Western blot using rabbit anti-hamster SAF antibodies (lanes 3 to 12) and comparison with hamster SAF protein detection by silver stain (lanes 1, 2) after separation by 12 to 17% SDS-PAGE. The immune serum used was from rabbit 536 which was tested against SAF protein from hamster and mouse brain (serum diluted 1:2000), and with SAF-like protein from fibrils isolated from human CJD cases (serum diluted 1:1000). The samples were: lane 1, 1/20 normal hamster brain; lane 2, 1/20 scrapie hamster brain; lane 3, 1/2 normal hamster brain; lane 4, 1/20 scrapie hamster brain; lane 5, 1/40 scrapie hamster brain; lane 6, 1 normal mouse brain; lane 7, 1 scrapie mouse brain; lane 8, 1/5 scrapie mouse brain; lane 9, 1 g normal human brain (N-6); lane 10, 1 g CJD human brain (CJD-2); lane 11, 0.5 g CJD human brain (CJD-7); lane 12, 2 g normal human brain (N-1). Molecular weights and band numbers are indicated.

DISCUSSION

Rabbit antisera raised against SAF protein from inbred CLAC hamsters infected with the 263K strain of scrapie detect the same antigen in CLAC as in the outbred AURA hamsters. These antibodies obtained with SDS-denatured SAF protein (Diringer et al., 1984) reacted with the intact fibrils prepared by a sucrose gradient technique (Diringer et al., 1983a). Antibodies prepared by others in a more laborious way using preparations from 1000 hamster brains (Bendheim et al., 1984) instead of only 15 also react with the intact fibril (Prusiner, 1984). In our experiments as well as in those published recently (Bendheim et al., 1984), in Western blots the antibodies detect various antigen-containing bands with apparent molecular weights of about 26K, 24K, 20K, 18K and 16K. Furthermore, we found additional reactive material at about 40K if high amounts of antigen were electrophoresed and blotted. As has been reported for two cases of CJD in man and for CJD in mice (Bockman et al., 1985; Bendheim et al., 1985) similar bands have been detected in our experiments with two more cases of the disease. Thus, because of the striking similarities in reactivity of our antibodies and those prepared by others with material from hamsters as well as from man isolated by similar but not identical procedures, we feel that our previous suggestion that SAF and prions are synonyms for one and the same structure (Hilmert & Diringer, 1984) is supported, a conclusion which so far has not been generally accepted (Bockman et al., 1985; Bendheim et al., 1985).

Our studies also show that antibodies against SAF react specifically with material from scrapie-affected mice. In addition we have been able to raise antisera to hamster SAF in mice although only with formic acid-denatured SAF protein and not with SDS-denatured material. The ability to raise anti-SAF sera in mice makes it possible to prepare monoclonal antibodies to SAF; these might be useful for increasing the specificity of the reaction and thus for searching in large-scale screening experiments for the disease-specific antigen in crude materials using the NC-ELISA test. The method should be able to detect around 50 ng or even less of SAF protein. This amount of antigen was present in the material we spotted as 1 µl of solution onto the nitrocellulose, representing 5 mg of hamster brain (Hilmert & Diringer, 1984).
SAF fractions used for immunization may have contained up to 10% of impurities. Thus, it is to be expected that antisera may also contain antibodies directed against host components. This seems indeed to have been the case, as shown by the NC-ELISA test where low dilutions of sera also reacted with normal brain material, whereas at higher dilutions the NC-ELISA and the Western blot specifically detected SAF antigen.

The quantitative differences in the amount of antigen in the five main bands detected by Western blotting could be caused either by the various agents used, i.e. the 139A and the 263K strains of scrapie and the CJD agent or by the different hosts, i.e. mouse, hamster and man, respectively. We suspect the host to be of more importance. It was shown recently with hamster scrapie in two different strains of animals that separation of the SAF antigen into various bands can be caused by a variation in glycosylation of a single peptide chain (Multhaup et al., 1985). Complete removal of the sugar side-chains resulted in a single peptide with a molecular weight of about 7000. Size, amino acid composition as well as sequencing data have shown that we are dealing with an amyloid-like peptide (Multhaup et al., 1985). The results chemically confirm earlier observations on intact SAF by electron microscopy (Merz et al., 1981; Diringer et al., 1983a; Prusiner et al., 1983) or Congo red staining (Prusiner et al., 1983). Kinetic data also indicate that SAF protein is an amyloid caused by a preceding virus replication (Braig & Diringer, 1985) rather than being a constituent of the scrapie agent itself (Bolton et al., 1984). Whether the amyloid in extracellular deposits seen in some scrapie agent/mouse strain combinations (Bruce et al., 1976) is chemically identical with SAF protein remains to be seen. From our data we would conclude that the SAF amyloid antigen must have been derived from a precursor glycoprotein structurally and functionally similar if not identical in all three hosts. This assumption is additionally supported by an impressive study by Oesch et al. (1985) demonstrating strong evidence that the 27K to 30K polypeptide designated PrP (Bolton et al., 1982), purified from scrapie hamster brain (and which is apparently identical with SAF protein) is encoded by a cellular gene. Confirmatory results have just been obtained independently by Chesebro et al. (1985) detecting PrP-specific mRNA in both normal and scrapie-infected mouse and hamster brain, thus suggesting that PrP is closely related to a normal protein of the brain of these species. Furthermore, Western blot analyses done by Oesch et al. (1985) showed some evidence that an antigenically PrP-related protein of 33K to 35K which could be detected only in crude normal and scrapie-infected brain homogenates without protease treatment might be a precursor from which the 27K to 30K protein is derived. However, a definite proof remains to be established.

From all these data it seems likely that the identification of the predicted SAF protein precursor would be valuable for further attempts to analyse the mechanisms involved in the pathogenicity of scrapie infection. The application of recent immunological techniques as presented in this study using antibodies to SAF protein may be useful for this purpose.

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