Immunoreactivity of a Synthetic Pentadecapeptide Corresponding to the N-Terminal Region of the Scrapie Prion Protein

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SUMMARY

A pentadecapeptide with an amino acid sequence corresponding to the amino-terminal region of the scrapie prion protein was synthesized. Immunization of a rabbit with the peptide conjugated with ovalbumin induced specific antibodies. The antibodies reacted with all three of the major polypeptides in a proteinase K-treated fraction obtained from brains of mice infected with the Obihiro strain of scrapie agent. Some peptides in the proteinase-untreated fraction also shared antigenicity with the three major polypeptides. Specific polypeptides were also detected by the antiserum in a fraction prepared from spleens, but only two of the three major polypeptides were found and the amounts of the polypeptides were less than in brain.

The scrapie agent causes progressive subacute encephalopathy not only in its natural hosts, sheep and goats, but also in a variety of species inoculated experimentally (Gajdusek, 1977; Gibbs, 1980). An immune response to the agent has not been demonstrated in these hosts at any stage of infection.

Recently, unique rod-shaped structures, called scrapie-associated fibrils (SAF) or prion rods, have been found to co-purify with scrapie infectivity (Diringer et al., 1983a; Prusiner et al., 1982, 1983). This led to the assumption that SAF or the prion is the scrapie agent itself, or is at least one of the components of the agent (Barry et al., 1985; Bolton et al., 1982, 1984; Diringer et al., 1983b; Kascak et al., 1985; McKinley et al., 1983; Prusiner et al., 1982, 1983, 1984). Analysis by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that SAF or the prion consists of a single 26000 mol. wt. (26K) protein (Diringer et al., 1983b; Hilbert & Diringer, 1984; Multhaup et al., 1985) or a 27K to 30K polypeptide (Bolton et al., 1982, 1985; McKinley et al., 1983). More recently, however, it has been reported that SAF from some strains of scrapie agent consists of three polypeptides (Bode et al., 1985; Kascak et al., 1985).

Specific antibodies to SAF and to the polypeptide of the prion rod (prion protein) have been raised in rabbits (Barry et al., 1985; Bendheim et al., 1984; Diringer et al., 1984; Takahashi et al., 1986) and in mice (Bode et al., 1985). Immunological analysis using the antibodies revealed that a polypeptide encoded by a cellular gene, which is expressed in brain as well as various other tissues, is related antigenically to the prion protein (Oesch et al., 1985) and, moreover, that specific proteins associated with Creutzfeldt-Jakob disease are also related to the scrapie prion or SAF protein (Bendheim et al., 1985; Bode et al., 1985; Manuelidis et al., 1985). Recently, Prusiner et al. (1984) reported a sequence of 15 amino acid residues from the amino terminus (N-terminus) of the prion protein. It was found that a mRNA encoding this sequence of amino acids is present in both scrapie-infected and uninfected mouse and hamster brain (Chesebro et al., 1985).
We prepared an infectious fraction as described (Diringer et al., 1983b; Hilmert & Diringer, 1984) from mouse brains infected with the Obihiro strain of scrapie (Shinagawa et al., 1985). This fraction contained SAF-like structures and three polypeptides of apparent mol. wt. 24.5K, 21K and 17K were the major components of the fraction (Takahashi et al., 1986).

In this communication, we describe the synthesis of a pentadecapeptide corresponding to the N-terminal region of the prion protein reported by Prusiner et al. (1984), the raising of antibodies to the synthetic peptide, and the antigenic relationship between this peptide and the polypeptides found in our fractions prepared from scrapie mouse brains and spleens.

Mouse-passaged Obihiro strain of scrapie agent (Shinagawa et al., 1985) was inoculated intracerebrally into 4-week-old female ICR mice (specific pathogen-free, SPF) to obtain infected brains. Preparations of fraction P4, which is comparable to SAF (Diringer et al., 1983b; Merz et al., 1981), and of the anti-P4 serum have been described elsewhere (Takahashi et al., 1986). Fraction P4 was also prepared from spleens of infected mice.

Synthesis of the pentadecapeptide corresponding to the prion protein N-terminal sequence, Gly–Gln–Gly–Gly–Gly–Thr–His–Asn–Gln–Tyr–Asn–Lys–Pro–Ser–Lys (Prusiner et al., 1984), was carried out by Merrifield’s (1963) solid phase method starting from tert-butoxycarbonyl-lysine(2-chlorobenzyloxycarbonyl)phenylacetamidomethyl resin (Lys content: 0.16 mmol/g of resin) (Kaiser et al., 1970). The groups employed for protecting the secondary function of amino acids were 2-chlorobenzyloxycarbonyl for Lys, benzyl for Ser and Thr, and p-toluenesulphonyl for His. All coupling reactions were carried out by the dicyclohexylcarbodiimide/hydroxy-benztriazole method. The crude peptide obtained was purified by gel filtration or~a Sephadex G-15 column, and by reverse phase high performance liquid chromatography using a column of YMC-Pak S-343 I-15 (ODS-5, C18; 2.5 mm × 30 cm, Yamashina Kagaku Co., Tokyo, Japan) with gradient elution by CH3CN-H2O containing 0.1% trifluoroacetic acid.

To prepare an anti-peptide serum, 6 mg of the peptide was conjugated to an equal amount of ovalbumin with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Yanaihara et al., 1981) and the volume was adjusted to 2 ml. The conjugate (2 ml) was mixed with an equal volume of Freund’s complete adjuvant, and half of the mixture was injected intramuscularly at multiple sites on a JW-NIBS male rabbit. The rabbit received three additional injections of 0.5 ml of the mixture at 2 to 3 week intervals. Blood was taken 10 days after the last injection.

Detection of antibodies to the synthetic peptide was carried out by blot immunoassay. Biotinylated anti-rabbit IgG–donkey IgG and biotinylated horseradish peroxidase–streptavidin complex (Amersham) were used for the detection of immune reactions. The synthetic peptide conjugated with bovine serum albumin (BSA) was used as an antigen for assay of the anti-peptide serum.

Various amounts of the peptide antigen (0.78 to 100 ng protein) were blotted on to nitrocellulose membranes and reacted with preimmune serum and the anti-peptide serum at dilutions of 1:125 and 1:32000, respectively. As shown in Fig. 1 (a), positive signs of reaction could be detected down to 3.13 ng of the antigen, while the preimmune serum failed to react with any amount of antigen used. None of the sera reacted with the carrier protein, BSA. The titre of the anti-peptide serum was determined using 6.25 ng of the antigen blotted on nitrocellulose membranes. The serum reacted clearly up to 1:128000 and faintly up to 1:512000 (Fig. 1 b).

An experiment was performed to examine whether the anti-peptide serum reacted to fraction P4 prepared from scrapie-infected mouse brains. Various amounts of P4 (6.25 to 50 ng protein) which had been previously denatured by heating in the presence of 2% SDS and then diluted with phosphate-buffered saline, were blotted on nitrocellulose membranes. The membranes were reacted with the antiserum diluted 1:16000. A positive sign was detected at 12.5 ng or more of fraction P4 (Fig. 1 c). The antiserum reacted with 50 ng of fraction P4 at 1:32000 dilution (Fig. 1 d).

Since fraction P4 contains three major polypeptides and all of them react with the anti-P4 serum (Takahashi et al. 1986), we examined which of the major polypeptides reacts with the anti-peptide serum by Western blot analysis. The polypeptides of P4 were separated by SDS–PAGE, transferred to nitrocellulose membranes, and reacted with the anti-peptide serum diluted 1:3200. All three of the major polypeptides (Fig. 2a, lane 1) were immunologically
Fig. 1. Immunoreactivity of anti-peptide rabbit serum. (a) One of the duplicate nitrocellulose filters blotted with various amounts of the peptide antigen (conjugate of the peptide and BSA) using the Bio-Dot apparatus (Bio-Rad), was reacted with preimmune serum diluted 1:125 (row 1) and the other with the anti-peptide serum diluted 1:32000 (row 2). Numbers on the top indicate the amount (ng) of the peptide antigen blotted. (b) Filters containing 6.25 ng of the peptide antigen each were reacted with preimmune (row 1) and the anti-peptide serum (row 2) diluted variously as indicated on the top. (c) Various amounts of fraction P4 prepared from scrapie-infected mouse brains (Takahashi et al., 1986) blotted on nitrocellulose filters were reacted with the preimmune serum diluted 1:125 (row 1) and the anti-peptide serum diluted 1:16000. Numbers on the top indicate the amount (ng) of fraction P4 blotted. (d) Filters containing 50 ng of fraction P4 each were reacted with variously diluted preimmune (row 1) serum and the anti-peptide serum (row 2). Numbers on the top indicate dilution of the serum. Immunoreactivity was detected using biotinylated donkey anti-rabbit IgG (Amersham), biotin–streptavidin–horseradish peroxidase complex (Amersham), diaminobenzidine tetrahydrochloride and hydrogen peroxide by the procedures recommended by the supplier of the reagents.

stained (Fig. 2b, lane 1), being comparable to the result obtained by using the anti-P4 serum (Fig. 2c, lane 1). Some bands of higher mol. wt. were stained faintly; however, they were absent from other preparations of P4 (data not shown). When the polypeptides in a proteinase K-untreated fraction were separated and stained in the same way, in addition to the three major polypeptides, at least two polypeptides of mol. wt. about 30K and 34K were heavily stained (Fig. 2b, lane 2). The results were essentially the same as those obtained by using the anti-P4 serum (Fig. 2c, lane 2). No polypeptide in analogous fractions prepared from normal mouse brains was stained (Fig. 2b, c; lanes 3 and 4).

In order to examine whether the major polypeptides could be detected in spleens, fraction P4 prepared from spleens (about 200 mg) of two mice was analysed by Western blotting. As shown
Fig. 2. Western blot of fractions prepared from scrapie mouse brains. Samples consisting of fraction P4 prepared from scrapie mouse brains (lanes 1), an analogous fraction from normal mouse brains (lanes 3), a fraction which was the same as P4 except for proteinase K treatment (lanes 2), and an analogous fraction prepared from normal mouse brains (lanes 4) were prepared from the same amount of brains and suspended in the same vol. of distilled water. A triplicate set of the four samples was mixed with an equal vol. of double-concentration SDS electrophoresis sample buffer, heated at 100 °C for 3 min and electrophoresed on a 15% SDS–polyacrylamide gel according to the method of Laemmli (1970). One set of polypeptides was stained by silver (a) (Oakley et al., 1980). The remaining sets were electrophoretically transferred to nitrocellulose filters, and reacted with either the anti-peptide serum diluted 1:3200 (b) or the anti-P4 serum (Takahashi et al., 1986) diluted 1:800 (c). Detection of immunoreactivity was as in Fig. 1. Mol. wt. (× 10⁻³) of marker proteins (cytochrome c monomer and oligomers; Oriental Yeast, Tokyo, Japan) are shown in lane M.

in Fig. 3, two polypeptides of mol. wt. about 24K and 21K were faintly stained, indicating that the amount of the polypeptides in the spleen was small.

Immunization with the synthetic polypeptide induced production of a fairly high titre of antibodies. Since the synthetic peptide is pure and the region acting as the antigenic determinant is limited, the antiserum to the peptide can be used to analyse immunological relationships among the polypeptides related to scrapie.

Prion rods and SAF are prepared from scrapie-infected animal brains by different procedures (Bolton et al., 1982; Diringer et al., 1983b; Merz et al., 1981; Prusiner et al., 1982). The major component of prion rods and SAF is a single polypeptide of apparent mol. wt. 27K to 30K and 26K on SDS–PAGE, respectively. Recently, however, it was reported that SAF from some strains (ME7 and 139A) of scrapie agent contain three polypeptides and their proteinase K sensitivity varies (Kascsak et al., 1985). Our fraction P4 from brains of scrapie mouse infected with the Obihiro strain also contains three major polypeptides (Takahashi et al., 1986). The anti-peptide serum reacted with all three major polypeptides in fraction P4, indicating that these polypeptides share a common antigenicity with the N-terminal region of the prion protein. This strongly supports the suggestion that SAF and prion are synonyms for one and the same structure (Hilmert & Diringer, 1984). Bands at 30K and 34K in addition to the three major polypeptides were stained with the anti-peptide serum in the proteinase-untreated fraction, indicating that the proteinase-sensitive polypeptide also shares the same antigenicity. The proteinase-sensitive 30K and 34K polypeptides seem to be associated with scrapie since an analogous proteinase-untreated fraction prepared from uninfected mouse brains did not contain the corresponding polypeptide (Fig. 2b, c; lane 4). The relationship between these polypeptides sharing the same antigenic determinant(s) is unknown. It was assumed that the 29K polypeptide
detected in the proteinase K-treated fraction (of both prion and SAF) was a breakdown product(s) of the higher mol. wt. polypeptide(s) found in proteinase K-untreated fractions (Manuelidis et al., 1985). In the present study, since the three major polypeptides were present in both proteinase-treated and -untreated fractions, they can not be mere breakdown products of the 30K and 34K polypeptides. It is an attractive assumption that the proteinase-sensitive polypeptides are the precursors of the major polypeptides, i.e. the proteinase-sensitive polypeptides aggregate tightly to form rods which are resistant to proteinase treatment after or during processing in an unknown way. Two of the three specific polypeptides associated with scrapie could be detected in the fraction prepared from spleens, suggesting that the processing might be somewhat different in different organs.

The amount of the polypeptides in the spleen was less than in brain. This agrees with the infectivity titres of scrapie agent in those organs, i.e. the infectivity in the brain is about tenfold higher than in the spleen (Outram, 1976). We intended to examine the peripheral lymph nodes as well, but the lymph nodes in the SPF mice used were too poorly developed to be collected. The detection of specific polypeptides using highly specific antibodies in peripheral organs such as the lymph nodes may open the way to precise diagnosis of scrapie in living animals.

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REFERENCES


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