Isolation and Characterization of a 14500 Molecular Weight Protein from Brains and Tissue Cultures Persistently Infected with Borna Disease Virus

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SUMMARY

A protein with an apparent molecular weight of 14500 (14.5K) was extractable from homogenates of Borna disease virus-infected brains and tissue cultures using high concentrations of detergent and salt and by differential centrifugation procedures. The protein, present in an aggregated form, was remarkably resistant to proteinase K. Specific antibodies prepared in the homologous system (rat) recognized the 14.5K protein from various sources (infected brain of rat, mouse or chicken, and tissue cultures), but did not neutralize infectivity nor stain Borna disease virus-specific antigens from in vitro or in vivo preparations. Post-infection immune sera from different animal species did not detect the protein. This 14.5K protein was infection-specific but not disease-specific, and is inferred to be part of an internal virion component.

Borna disease (BD) is caused by an unclassified conventional, enveloped virus (Ludwig & Becht, 1977; Danner & Mayr, 1979). Besides the poorly characterized soluble (s-) antigen extractable from brains or cell cultures, which most probably correlates with a 42K protein (Ludwig et al., 1977), no defined virus structures or disease-associated proteins have been characterized. Since BD virus appears to be strongly bound to cellular membranes (Pauli & Ludwig, 1985) we adapted a procedure using detergent and salt extraction which had led to the analysis of scrapie-associated fibrils (Hilmert & Diringer, 1984; Multhaup et al., 1985) and report here on a protein found in BD virus-infected brain tissues and cell cultures.

In comparative analyses, suspensions of detergent-dissolved brains harvested from 3-week-old persistently or mock-infected Wistar rats (Hirano et al., 1983) or BALB/c mice (Kao et al., 1984) were processed as outlined in the flow chart (Fig. 1a, i to v). No obvious differences were detectable when either the total homogenates (Fig. 1b, i) or their supernatants cleared by centrifugation (Fig. 1b, ii) were analysed by SDS-PAGE. However, an infection-specific protein band with an apparent molecular weight of 14.5K appeared in the pellet fraction obtained by high-speed centrifugation (Fig. 1b, iii). After this pellet was dissolved in 10% salt and centrifuged, the 14.5K protein was present only in preparations from infected brains (Fig. 1b, iv). It remained detectable in the pellet (PE), even after several buffer extractions (Fig. 1b, v). Since we have been able to adapt BD virus to the mouse and observed that, with passage, infectivity titres rose (Kao et al., 1984), brain suspensions of passages 1 or 6 were assayed. The 14.5K protein was detected only in the PE fraction (Fig. 2a) of passage 6. Some of this protein was solubilized by extraction, which holds true for rat brain material as well (Fig. 2b). Under the same conditions, no such protein was visible after PAGE and silver staining of brain material from persistently infected chickens which had been inoculated after hatching with $4 \times 10^4$ f.f.u. of rat-adapted virus and assayed 3 weeks later (Table 1).

Staining of this new protein was not observed in brain preparations derived from diseased animals, i.e. from naturally infected horses (animals NAS or KÜNZ; Gosztonyi & Ludwig, 1984) and rabbits experimentally infected with strain V. All animals showed the classical neuropathological lesions of BD. The failure to detect the 14.5K protein could be related to the fact that virus titres in these materials were considerably lower than in rat and mouse brains (Table 1).
Short communication

(a) 1:10 homogenate (i) + 10% detergent (N-lauroylsarcosine sodium salt); + 10 mM-NaH$_2$PO$_4$; 22000 g for 30 min

S$_{22}$ (ii)

215000 g for 2 h

P discard

S discard

P$_{215}$ (iii)

+ 1% detergent + 10% NaCl; + 20 mM-Tris-HCl, sonicate; 215000 g for 2.5 h

S discard

P$_{215S}$ (iv)

+ 1% detergent, 10% NaCl, sonicate; stir at 37 °C overnight; 5500 g for 10 min

S$_1$ store

P$_{55}$ + distilled water; stir at 37 °C for 1 h; 5500 g for 10 min

S$_2$ store

P$_E$ store (v)

(b) M C I

(ii) C I

(iii) C I

(iv) C I

(v) C I M

45

29

13
Table 1. *Borna disease virus*-infected tissues used for extraction and their infectivity titres

<table>
<thead>
<tr>
<th>Material</th>
<th>Pathology/disease*</th>
<th>Virus titre (f.f.u.)/g material</th>
<th>Material applied to gel (f.f.u. before extraction)</th>
<th>14.5K protein detectable on†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain</td>
<td>−/−</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^6$</td>
<td>Gel/Blot</td>
</tr>
<tr>
<td>Mouse brain</td>
<td>−/−</td>
<td>$5 \times 10^6$</td>
<td>$5 \times 10^5$</td>
<td>Gel/Blot</td>
</tr>
<tr>
<td>Chicken brain</td>
<td>−/−</td>
<td>$1 \times 10^6$</td>
<td>$2 \times 10^5$</td>
<td>−/Blot</td>
</tr>
<tr>
<td>Rabbit brain</td>
<td>+/+</td>
<td>$1 \times 10^5$</td>
<td>$2 \times 10^4$</td>
<td>−/−</td>
</tr>
<tr>
<td>Horse brain</td>
<td>+/+</td>
<td>$1 \times 10^3$</td>
<td>$2 \times 10^2$</td>
<td>−/−</td>
</tr>
<tr>
<td>ED/TL‡</td>
<td></td>
<td>$1 \times 10^8$</td>
<td>$2 \times 10^7$</td>
<td>?/Blot</td>
</tr>
<tr>
<td>Oligo/TL‡</td>
<td></td>
<td>$2 \times 10^7$</td>
<td>$4 \times 10^6$</td>
<td>Gel/Blot</td>
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<tr>
<td>JKG/TL‡</td>
<td></td>
<td>$3 \times 10^5$</td>
<td>$1 \times 10^4$</td>
<td>−/−</td>
</tr>
</tbody>
</table>

* +, Typical clinical symptoms and pathological central nervous system lesions present; −, not present.
† Gel, protein visualized after silver staining of SDS–PAGE slabs; blot, protein visualized after protein blot and immunostaining. ?, infected and control cell preparations show several proteins in the same mol. wt. range.
‡ ED/TL, equine dermal cell line persistently infected with rat-adapted BD virus. Cells were grown in Eagle’s medium, Dulbecco’s modification (EDM) supplemented with 5% foetal calf serum. Oligo/TL, human embryonic oligodendroglial cell line persistently infected with rabbit-adapted BD virus. Cells were grown in EDM with 5% newborn calf serum (NCS). JKG/TL, infected newborn rabbit brain cells; primary brain cells were passaged once and 24 h later infected with $10^5$ f.f.u. of rat-adapted BD virus per 5 cm Petri dish and then grown in EDM with 5% NCS. For further details on cell growth and virus titration, see Hirano et al., 1983; Kao et al., 1984.
To clarify whether the BD virus-specific protein was associated only with infected brains, persistently infected tissue cultures of neural (embryonic human oligodendrocytes: Oligo/TL) as well as non-neural origin (equine dermal cells: ED/TL) were subjected to the same extraction procedures. The 14.5K protein was present reproducibly after SDS–PAGE and silver staining in Oligo/TL cells, but could not be detected unequivocally in ED/TL cells (Table 1).

The differential centrifugation procedure used indicated that the PE fraction contained the 14.5K protein in an aggregated form. This conclusion was supported by the observation that this material remained on top of the slab gel after PAGE in the absence of SDS (data not shown) and that it withstood extensive proteinase K digestion (40 μg/ml at 37 °C; Fig. 3).

Newborn rats, which are known to be very sensitive to BD virus infection, were inoculated intracranially with the PE fraction to check for infectivity. In repeated experiments no infectious virus could be demonstrated under the conditions described earlier (Hirano et al., 1983).

The S₂ fractions (see Fig. 1a) from infected rat brains always contained fewer impurities than the PE fractions (compare Fig. 3a, lane 1 with c, lane 1) and were therefore used to raise antibodies in a homologous animal. The antisera bound specifically to the 14.5K protein in an aggregated form. This conclusion was supported by the observation that this material remained on top of the slab gel after PAGE in the absence of SDS (data not shown) and that it withstood extensive proteinase K digestion (40 μg/ml at 37 °C; Fig. 3).

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The brain extracts from diseased animals (horse, rabbit) which had not shown the 14.5K protein by conventional staining also remained negative by immunostaining using the 14.5K-specific sera. Rat hyperimmune sera prepared against the 14.5K protein did not neutralize infectivity. This is not surprising since the large amount of detergent used in the extraction procedure would destroy the lipid-containing envelope of BD virus, and it is assumed that the new protein is part of an internal virion component. The antisera, furthermore, did not stain infected tissue culture cells or brain sections, using indirect immunofluorescence, ELISA or

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**Fig. 3. PAGE analysis of the fractions (a) PE and (c) S₂ containing the 14.5K protein or (b) S₂ from control brains, after treatment with proteinase K for 1 h at 37 °C. Mol. wt. (× 10⁻³) is shown (M). Electrophoresis and protein staining were carried out as described in Fig. 1(b). Proteinase K concentrations used were 0 (lanes 1), 4 (lanes 2), 10 (lanes 3), 20 (lanes 4) and 40 (lanes 5) μg/ml.**
Fig. 4. Immunostaining of protein blots. (a) Extracts from BD virus-infected rat brains (R) and aliquots of these, treated with 40 μg/ml proteinase K (1 h, 37 °C) (Rp) were electrophoresed (Fig. 1 b) and transferred to nitrocellulose as outlined by Burnette (1981). PE fraction in R is in left strip, in Rp in the left and middle strips; S2 fraction in R and Rp right strips. Antisera against the 14.5K protein were raised in adult Wistar rats by intradermal inoculation (0.5 ml) of a homogenate consisting of equal amounts of S2 from infected rats (Fig. 1 a) and Freund's complete adjuvant. Animals were boosted with S2 (now using Freund's incomplete adjuvant) and bled at intervals of 20 days. Binding of the antisera to the electrophoresed 14.5K protein was demonstrated by a substrate reaction with 3-amino-9-ethylcarbazole (Sigma) after incubation first with biotin-coupled species-specific anti-IgG (Vector Laboratories, Burlingame, Ca., U.S.A.) and then with the peroxidase-coupled streptavidin–biotin complex (Amersham) according to the manufacturers’ instructions. Mol. wt. (× 10⁻³) is shown. (b) PE fractions from BD virus-infected (Ch) and mock-infected chicken brains (C) and ED/TL cells (E) were electrophoresed and treated as described in (a). Application of post-infection immune sera gave results typified by lane C.

peroxidase–antiperoxidase techniques (Hirano et al., 1983; Pauli et al., 1984; Gosztonyi & Ludwig, 1984). This indicates that antibodies were raised against antigenic structures exposed only in a denatured form of the protein. The finding of the new protein in different animal species initiated immunoblotting experiments with post-infection immune or reference sera from different animal species. Sets of sera from an infected horse, infected rabbits, rats or mice, some of which had high neutralization and/or s-antigen titres (for details, see Hirano et al., 1983), did not react with 14.5K protein blotted to nitrocellulose. The results were similar to those with control filters (compare Fig. 4 b).

BD virus infection is known to cause a broad spectrum of effects in different animal species. This enabled us to test the brains of clinically diseased animals with characteristic histopathological lesions, persistently infected animals without clinical symptoms or apparent
alterations in the brain, and a variety of tissue culture cells for the presence of the 14.5K protein. The data summarized in Table 1 indicate that detection of the protein does not depend upon infection itself but on the presence of high levels of infectious virus. From this it seems likely that the protein is associated with the virion rather than being a product of cell damage.

The possibility that we are dealing with a cellular or modified cellular protein is unlikely since only antibodies directed against the 14.5K protein were induced under these immunization conditions, although our preparations were not absolutely pure.

BD virus and the scrapie agent are indigenous in sheep, the natural host of both agents. The infectivity of these conventional and unconventional viruses which infect the central nervous system is closely associated with cellular membranes. Besides these common features the above extraction procedure enabled infectivity- and disease-associated products to be distinguished in scrapie (H. R. Braig & H. Diringer, unpublished), but resulted in the isolation of a different, infection-specific protein in BD. Here a correlation with virus structures is obvious and the accumulation of a pathological product seems to be less likely, although the function of the protein has to be further elucidated.

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REFERENCES


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