Veterinary Immunology and Immunopathology, 31 (1992) 361–369
Elsevier Science Publishers B.V., Amsterdam

Borna disease virus-specific antigens. II. The soluble antigen is a protein complex

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(Accepted 14 March 1991)

ABSTRACT

Bause-Niedrig, I., Jackson, M., Schein, E., Ludwig, H. and Pauli, G., 1992. Borna disease virus-specific antigens. II. The soluble antigen is a protein complex. *Vet. Immunol. Immunopathol.*, 31: 361–369.

Borna disease virus-specific soluble antigen from persistently infected rat brains was purified to homogeneity using preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The soluble antigen is a complex of three proteins with apparent molecular weights of 35 kDa, 38 kDa and 24 kDa. The 35/38 kDa antigen double band was separated into its two components. The 24 kDa protein has no common epitopes with the 35/38 kDa protein.

ABBREVIATIONS

BD, Borna disease; FPLC, fast protein liquid chromatography; s-antigen, soluble antigen; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

INTRODUCTION

Borna disease (BD) naturally occurs in horses and sheep inducing a fatal encephalomyelitis. Experimentally a variety of other species can be infected, e.g. rats (Nitzschke, 1963; Hirano et al., 1983), mice (Kao et al., 1984) or rabbits (Zwick, 1939). It seems to be likely that the pathogenesis of the disease is determined by immunopathological effects (Narayan et al., 1983). Up to now the virion has not been characterized regarding structural proteins. Recent investigations describe the BD genome as a single-strand minus RNA

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of 8.5 kb with major transcripts of 2.1 and 0.8 kb (Lipkin et al, 1990; De La Torre et al., 1990). A virus-specific soluble antigen (s-antigen) was demonstrated as early as 1954 by Von Sprockhoff. Two different antigens with molecular weights of 22–24 kDa and approximately 40 kDa could be precipitated using sera of diseased animals (Ludwig and Becht, 1977; Ludwig et al., 1977). Further experiments demonstrated that the higher molecular weight antigen was a double band of 35/38 kDa (Haas et al., 1986; Bause-Niedrig et al., 1991).

With regard to the limited information we have about the virus, the purification and partial sequencing of the soluble antigen seem to be one possibility to characterize the virus. We describe the purification of the s-antigen and demonstrate that the 35/38 kDa double band obviously contains modifications of the same protein.

MATERIALS AND METHODS

s-Antigen preparation

Soluble antigen was prepared from BD virus infected rat brains (Hirano et al., 1983). Brains were ultrasonicated (10% in PBS, w/v) and insoluble materials removed by consecutive centrifugation steps (10 min, 1000×*g*; 10 min, 10 000×*g*; 60 min, 100 000×*g*; 4°C). The supernatant was submitted to fractionated ammonium sulfate precipitation. The majority of the antigen precipitated between 30% and 60% saturation. Desalting was done by gel filtration on PD 10 columns (Pharmacia). To further enrich the soluble antigen fast protein liquid chromatography (FPLC) was performed using an anion-exchange column (Mono Q, Pharmacia). As buffer A 20 mM Tris–HCl (pH 8.6), and as buffer B 20 mM Tris–HCl (pH 8.6), 1 M NaCl were used. The following gradient of buffer A/B was applied: after 4 ml buffer B reached 20%, after 34 ml 80% and after 36 ml 100%, respectively. Proteins eluting between 30% and 50% buffer B contained the 35/38 kDa as well as the 24 kDa antigen.

Preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The preparative SDS-PAGE was performed using a gel tube system with continuous elution (BRL 1100PG Unit, Giacobino et al., 1984). A 13% running gel was poured to a height of 7 cm overlaid by a 5 cm 6% stacking gel. As electrode buffer Tris–glycine buffer according to the Lämmli system was used (Lämmli, 1970). As elution buffer a three times concentrated electrode buffer was used. Three milliliters of the FPLC s-antigen preparation were mixed with 0.8 ml of 5× Lämmli sample buffer and applied to the gel. Protein concentra-

tion did not exceed a total of 2 mg. Separation and elution of proteins were done at 12 mA and 6 mA constant current, respectively. One milliliter fractions were collected at a flow rate of 10 ml h^{-1}.

Analytical SDS-PAGE and Western blotting

Purification of the antigens was followed by immunoelectrophoresis (Ludwig et al., 1977) and by PAGE analysis. For SDS-PAGE a 40 μl aliquot of each fraction was run on a vertical electrophoresis unit (LKB), and separated proteins were demonstrated by silver staining (Ochs et al., 1981). BD virus-specific antigens were detected by Western blot analysis (Burnette, 1981) using a sheep antiserum (S-5) directed against the antigen complex (for details see Bause-Niedrig et al., 1991). Gels to be submitted to silver staining and immunoblotting were run in parallel.

Preparation of monospecific antisera

Five goats were immunized with purified antigen obtained by preparative SDS-PAGE. Two goats (G-144, G-151) were immunized with fractions containing the s-antigen double band (35/38 kDa), one goat (G-146) was immunized with fractions containing the lower molecular weight band (35 kDa) and a fourth goat (G-149) received the higher molecular weight band (38 kDa), respectively. The fifth goat (G-142) was immunized with fractions containing the 24 kDa antigen. The first injection (2 ml total volume) was done subcutaneously (s.c.) using Freund's complete adjuvant. The first booster was done s.c. using Freund's incomplete adjuvant followed by two intravenous boosters (1 ml) of proteins being extensively dialysed against PBS. Each booster was performed with 20–40 μg protein. After pre-sera had been collected, injections were done every 3–4 weeks, and the animals bled 10–14 days after each immunization step. Sera were tested for anti-BD virus antibodies by immunoblotting.

Determination of the protein concentration

Protein concentrations were determined by a modified Bradford procedure (Bradford, 1976). Briefly: 100 μl of diluted sample were mixed with 30 μl of the Bradford reagent (Bio-Rad Protein Assay). After incubation for 10–30 min the suspension was transferred to round bottom microtiter plates (Nunc) and absorption was determined at a wavelength of 590 nm with a Dynatech ELISA reader. Bovine serum albumin served as the protein standard (1–10 μg 100 μl^{-1}).

RESULTS

Purification of BD-antigens

s-Antigen of BD virus-infected rat brains was enriched by fractionated ammonium sulfate precipitation. An equivalent of two brains (15 mg protein) was applied to the Mono Q column for each FPLC run. Fractions eluting between 30% and 50% of buffer B contained the BD virus-specific 35/38 kDa and 24 kDa antigens constituting approximately 50% of the total protein concentration (3–5 mg 11 ml^{-1}) (Fig. 1).

The three antigens (24 kDa, 35 kDa, 38 kDa) could be separated from each other by preparative SDS-PAGE. Reproducibility in consecutive runs depended mainly on the amount of protein applied to the gel and on the elution conditions, i.e. flow rate and current. The number of fractions containing the 35 kDa, the 38 kDa or both proteins depended on the run as well as whether results of the immuno- or silver staining were examined (Fig. 2a,b).

Fractions of the preparative SDS-PAGE containing either the 35 kDa or

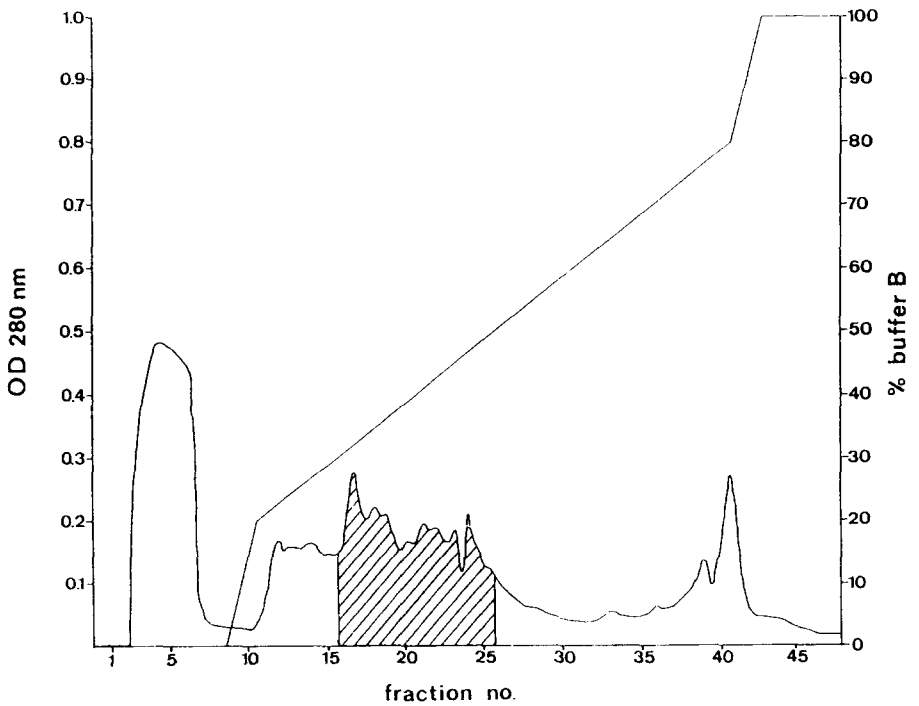

Fig. 1. Purification of the BD virus-specific s-antigen by FPLC. Preparations being enriched for the s-antigen of BD virus-infected rat brains were applied to an anion-exchange column for further purification. Fractions eluted between 30% and 50% buffer B (transversal lines) contained the BD virus-specific 35/38 kDa and 24 kDa proteins.

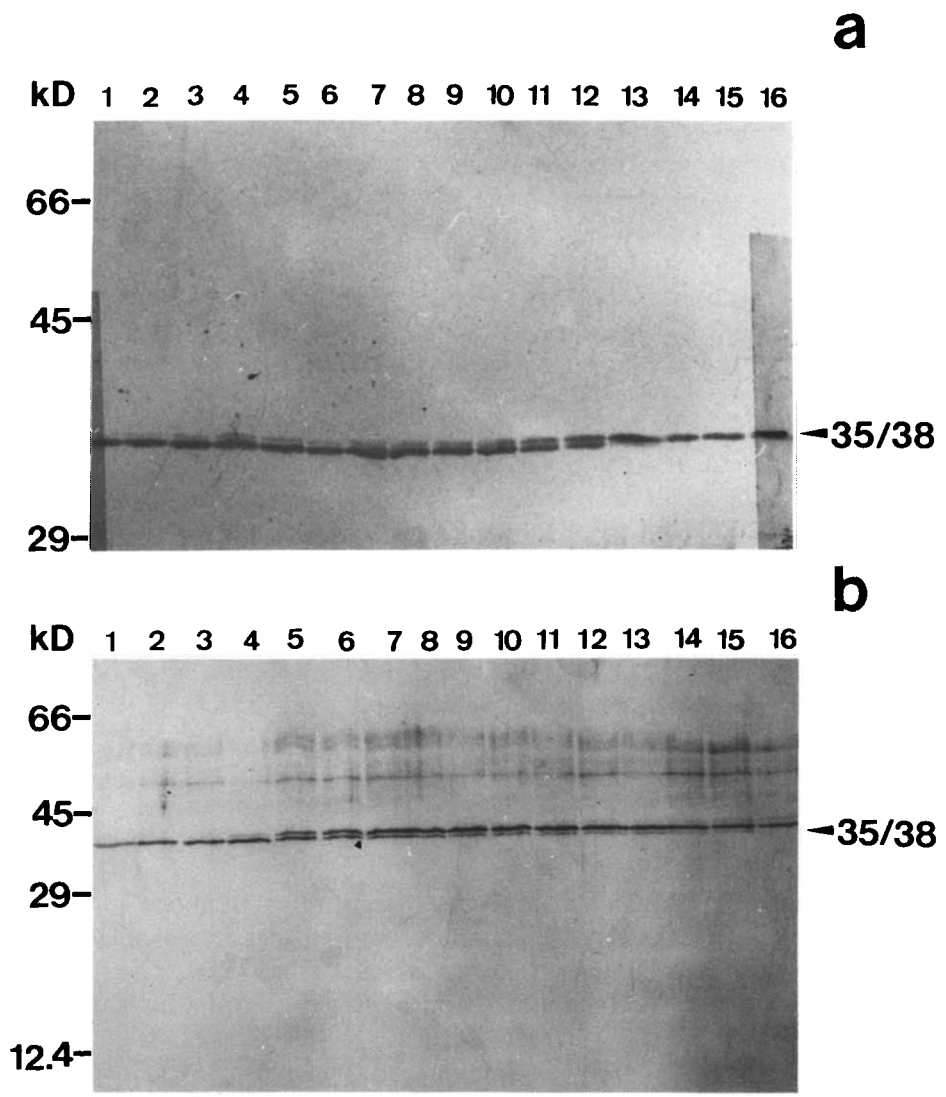

Fig. 2. Demonstration of the purity of BD virus-specific antigens. Fractions obtained by preparative SDS-PAGE were analysed by Western blotting (S-5 sheep serum) (a) and silver staining of analytical SDS-PAGE (b), respectively. Lanes 1–16 show a representative run of sequentially collected fractions. Examining the Western blot, (a) lane 1 contained the 35 kDa protein, lanes 2–13 the 35/38 kDa double band, and lanes 14–16 the 38 kDa protein. Examining the silver staining, (b) lanes 1–3 contained the 35 kDa protein, lanes 4–15 the 35/38 kDa double band, and lane 16 the 38 kDa protein.

the 38 kDa protein as monitored by immunoblotting and silver staining, were used for immunization (Fig. 2a, lanes 1 and 16).

In the silver stained gel one further band of a slightly higher than 38 kDa

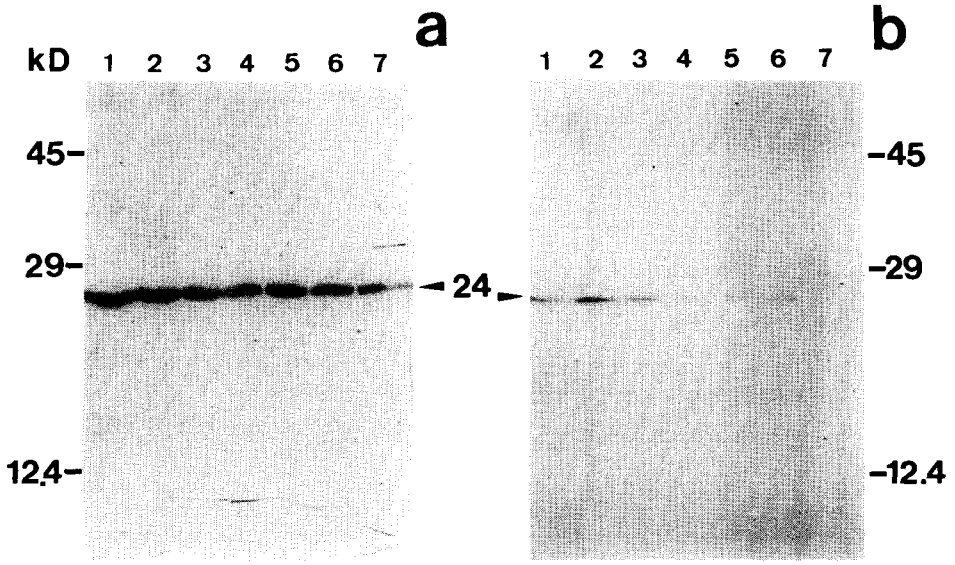

Fig. 3. Purification of the 24 kDa protein. Fractions obtained by preparative SDS-PAGE were analysed by immuno- (a) and silver staining (b), respectively. Lanes 1–7 show a representative run of sequentially collected fractions containing the 24 kDa protein. It was always more difficult to demonstrate the 24 kDa protein by silver staining (b).

molecular weight was visible (Fig. 2b, lane 16). This protein was not recognized by the BD virus-specific antiserum S-5 (Fig. 2a, lane 16), nor did it induce antibodies reactive in the Western blot analysis of the BD virus-specific antigens (see Fig. 4).

Weak bands occurring in the molecular weight range of about 50–60 kDa throughout the whole gel constituted non-protein unspecific staining. The 24 kDa antigen was eluted in three to nine fractions (Fig. 3a,b). The first fraction of the 35 kDa antigen was eluted approximately 50 ml after the 24 kDa antigen had come off.

Serological differentiation of the antigens

Due to the low amount of protein used for each immunization, BD virus-specific antibodies were produced after only four injections. All goats which had received the 35/38 kDa antigen (G-144, G-151), the 35 kDa (G-146) or the 38 kDa (G-149) protein induced antibodies reacting with the 35/38 kDa double band in immunoblotting. The goat (G-150) which had been immunized against the 24 kDa protein developed antibodies reactive with this protein (Fig. 4).

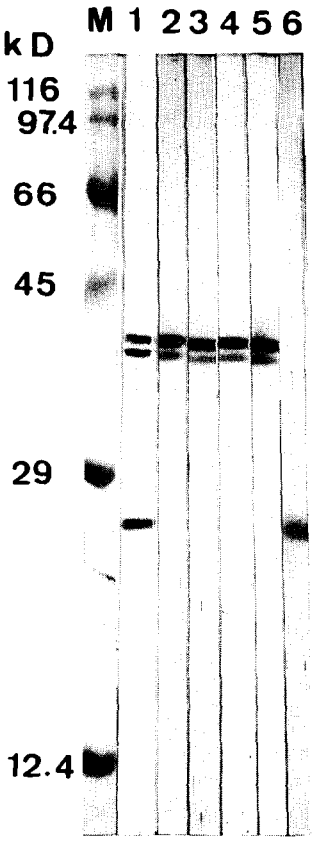

Fig. 4. Western blot analysis of the hyperimmune goat sera. s-Antigen preparation containing partially purified 24 kDa as well as 35/38 kDa proteins (FPLC) was separated on SDS-PAGE and transferred to NC-sheets. Lane 1: positive control, i.e. the S-5 sheep serum reacting with the 24 kDa and the 35/38 kDa antigens. Lanes 2 and 3: staining pattern of the goat sera G-144 and G-151, both immunized with the 35/38 kDa double band. Lane 4: staining pattern of the goat serum G-146 immunized with the 35 kDa protein. Lane 5: staining pattern of the goat serum G-149 immunized with the 38 kDa protein. Lane 6: staining pattern of the goat serum G-150 immunized with the 24 kDa protein. Lane M: molecular weight marker proteins run on the same gel.

DISCUSSION

Attempts to biochemically define the BD virus-specific soluble antigen have been unsuccessful. Immunoprecipitation of radioactively labeled proteins with sera of diseased animals revealed that two antigens with molecular weights of approximately 40 kDa and 22 kDa were precipitated (Ludwig and Becht, 1977; Ludwig et al., 1977).

The purification of the s-antigen was achieved by immunoaffinity chro-

matography and HPLC (Haas et al., 1986). These preparations contained proteins running in SDS-PAGE as a double band with a molecular weight of approximately 38 kDa. With these purification techniques, however, it was not possible to separate the two proteins. Under these conditions the 24 kDa protein was not detectable (Haas et al., 1986). If this was due to separation of the 24 kDa antigen from the 35/38 kDa antigen during purification or to the lack of antibodies directed against the lower molecular weight antigen is unclear.

We showed that the soluble BD virus-specific antigen eluted from the FPLC column as a protein complex consisted of three BD virus-specific antigens with molecular weights of 24, 35 and 38 kDa. The 35/38 kDa double band as well as the 24 kDa antigens could be separated from each other after denaturation and were purified to homogeneity by preparative SDS-PAGE. Immunization with the purified proteins revealed that the 35 and the 38 kDa antigen harbor common antigenic epitopes and therefore seem to be modifications of the same protein. In agreement with Haas et al. (1986) we found that the proteins were not glycosylated as demonstrated by radioactive labeling and lectin binding studies. Experiments to demonstrate phosphorylation gave equivocal results (N. Klein et al., personal communication, 1989). Preliminary results of protease digestion studies of the 35 and 38 kDa protein showed identical peptide patterns with one modification (N. Klein et al., personal communication, 1989) confirming the above described resu'ts.

The animal immunized with the 24 kDa protein developed only antibodies against this protein showing no cross-reaction to the 35/38 kDa antigen. Vice versa, the sera of the animals immunized with the 35/38 kDa protein or the single components showed no reaction with the 24 kDa protein.

These data confirm and extend results obtained with monoclonal antibodies (Bause-Niedrig et al., 1991) which demonstrated common epitopes on the 35 and 38 kDa antigens and which were not shared with the 24 kDa protein. Furthermore, the monoclonal antibody directed against the 24 kDa antigen was not reactive with the 35/38 kDa protein.

At present the purified proteins are further investigated by aminoacid sequence analysis. These proteins are, however, very labile and thus easily lost. Furthermore, the amino terminus seems to be blocked which hampers the sequencing of these proteins.

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