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Purification and Properties of an Intranuclear Virus-specific Antigen from Tissue Infected with Borna Disease Virus

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SUMMARY
A virus-specific antigen was extracted from brains of rats and from MDCK cells infected with Borna disease (BD) virus and purified to homogeneity by immunoaffinity chromatography and HPLC. The antigen consists of two components which are almost equal in size (38000 mol. wt.), and it forms aggregates in its native form. The virus specificity of the two antigenic entities was confirmed by immunoblots with convalescent serum and monoclonal antibodies. Immunofluorescent staining with monoclonal antibodies and a hyperimmune serum prepared against the purified antigen showed the intranuclear fluorescence typical for BD virus-infected cells.

INTRODUCTION
Borna disease (BD) is a rare and fatal encephalitis occurring naturally in horses and sheep. Rats and many other species can be infected, and it is clear that immunopathological effects determine the pathogenesis of the disease (Narayan et al., 1983). The virus has not been characterized as yet. However, a virus-specific soluble antigen can readily be demonstrated in brain extracts by classical serological procedures, such as complement fixation (von Sprockhoff, 1954) or immunodiffusion (Ludwig et al., 1973). By indirect immunofluorescence in infected cells an intranuclear antigen can be demonstrated, and this provides a very sensitive test for virus-specific antibodies (Wagner et al., 1968; Danner, 1976; Herzog & Rott, 1980).

It has been suspected that it might be possible to extract this intranuclear antigen from infected cells and that it represents at least the main component of the 'soluble antigen'. In view of the scarcity of information about the infectious virus a precise characterization of this viral component seemed to be an important step towards description of the virus and the immune reaction involved in the pathogenesis of BD. Efforts were undertaken, therefore, to purify this antigen and to determine some of its properties.

Promising results towards efficient purification of a virus-specific component had been obtained previously by use of antibody-mediated affinity chromatography (Ludwig & Becht, 1977). In the present study, this method has been used for isolation of the intranuclear antigen and to prepare monoclonal antibodies for more precise serological analysis of the purified antigen.

METHODS
Virus and preparation of cell extracts: Lewis rats were infected intracerebrally with BD virus of strain He/80 and killed 3 weeks later. A 10% suspension of their brains in TN buffer (0.02 M-Tris-HCl, 0.1 M-NaCl, pH 7.2) was homogenized and stirred for 1 h at room temperature after the addition of 1% Triton X-100 and 0.5% deoxycholate. The homogenate was centrifuged for 2 h at 30000 r.p.m. in a Beckman 45Ti rotor to remove particulate matter. The supernatant was diluted 1:5 in TN buffer and applied to the affinity column (see below).

MDCK cells persistently infected with BD virus (Herzog & Rott, 1980) were grown in Roux bottles. When the cells had reached confluency the medium was removed and the cells were scraped from the bottom of the bottles, suspended in TN buffer (1 ml buffer per bottle), homogenized and treated like the brain homogenates.
\textbf{BD virus-specific antisera.} Rabbits were infected intracerebrally and were exsanguinated 4 to 6 weeks later when clinical signs of BD had developed. Sera that had titres by the indirect immunofluorescence (IF) test of at least 1:1000 were used.

\textbf{Antibody-mediated affinity chromatography.} The procedure, which was based on proposals by Axen & Ernback (1971) and Porath & Sundberg (1972), has been described (Becht & Malole, 1975) for the isolation of 'soluble' nucleoprotein antigen from influenza virus-infected chorioallantoic membranes and was applied with minor modifications. Sepharose CL-6B was pretreated with phloroglucinol and epichlorhydrin, activated with cyanogen bromide dissolved in acetonitrile, and conjugated with the gamma globulin fraction of pooled sera from BD virus-infected rabbits at 4 °C overnight. About 300 mg of protein was used per 10 ml of packed, activated Sepharose. A column with the antibody-coated Sepharose was equilibrated with TN buffer containing 0:1% Triton X-100. After application of the tissue extracts it was washed with buffer plus detergent and finally with TN buffer alone. The material retained on the immunosorbent was eluted with alkaline buffer (0.15 M-ethanolamine hydrochloride, 0.1 M-NaCl, 10% glycerol), pH 11.2. The eluate was neutralized as it appeared from the column and was concentrated by pressure dialysis (Amicon, Membrane PM 10).

\textbf{High-pressure liquid chromatography (HPLC).} The concentrated eluate from the rabbit globulin affinity column was passed through a Biosil TSK 250 column in a buffer containing 0.05 M-NaH$_2$PO$_4$ and 0.02 M-Na$_2$SO$_4$, pH 6.8.

\textbf{Monoclonal antibodies.} BALB/c mice were immunized intraperitoneally with 100 μg of the crude antigen emulsified in Freund's incomplete adjuvant or adsorbed onto aluminium hydroxide (Alu-Gel-S; Serva, Heidelberg, F.R.G.). Immunization was repeated after 3 weeks and after a further 3 to 5 weeks, antigen plus 100 μg muramyl dipeptide (Institut Pasteur, Paris, France) was injected intravenously. Other BALB/c mice were injected intracerebrally at the age of 4 weeks with BD virus that had been passed twice in newborn mice, and the animals were killed 2 weeks later. For the fusion (Köhler & Milstein, 1975) of spleen cells with P3-X63-Ag8 653 plasmacytoma cells the procedure outlined by Fazekas de St. Groth & Scheidegger (1980) was followed. The supernatants of hybridomas were tested for BD virus-specific antibodies by the IF test in persistently infected MDCK cells. For the production of large amounts of monoclonal antibodies the hybridoma cells were grown in RPMI 1640 culture medium (Imperial Laboratories, U.K.) containing 10% horse serum in Roux bottles. Pure antibodies were recovered from the medium by affinity chromatography on Sepharose to which anti-mouse globulin had been conjugated by the method mentioned above. Anti-Ig was prepared in a hen immunized intramuscularly with the gamma globulin fraction of mouse serum emulsified in Freund's complete adjuvant. The gamma globulin was isolated from egg yolk by precipitation with dextran sulphate and sodium sulphate according to Jensenius et al. (1981). Antibodies from the hybridoma culture retained by the anti-globulin immunosorbent was eluted with 0.1 M-glycine–HCl, 0.1 M-NaCl, pH 2.8.

\textbf{Serological methods.} The medium used for immunodiffusion tests in agar gel (AGDT) consisted of 1% agarose, 3% polyethylene glycol, 0.5% KCl, 0.1% sodium azide in phosphate-buffered saline. Indirect immunofluorescence was carried out with persistently BD virus-infected MDCK cells grown in chamber slides as described (Herzog & Rott, 1980). For immunoblots the proteins separated by electrophoresis in 15% polyacrylamide gels (Laemmli, 1970) were transferred to nitrocellulose (Towbin et al., 1979) and treated with antibodies according to Talbot et al. (1984). Chicken anti-mouse or anti-rat globulin conjugated with peroxidase was used to stain the antibodies retained by the antigen bands.

\section*{RESULTS}

Extraction procedures and purification steps were monitored by AGDT with a potent convalescent serum from a BD virus-infected rabbit. The intensity of the precipitation lines showed that good extraction and solubilization of virus-specific antigen from BD virus-infected brains or MDCK cells was obtained by addition of detergents to the tissue homogenate.

The concentrated eluate from the affinity column formed a precipitation line when it diffused against a BD virus-specific serum and was negative with a normal serum. Additional proof that the eluate contained BD virus-specific antigen was obtained by injecting this material into a rabbit. With such a hyperimmune serum the typical brilliant nuclear fluorescence could be seen in infected MDCK cells. Since these indicator cells are derived from a species different from the rat, which served as a source of antigen, one can be certain that this fluorescence is virus-specific, besides the fact that uninfected cells did not show any fluorescence.

The eluate could also be used to prepare monoclonal antibodies. Hybridomas could be isolated which secreted antibodies that stained the intranuclear viral antigen in BD virus-infected cells by the usual IF procedure. There was the typical brilliant granular fluorescence that can be seen with whole convalescent serum (Wagner et al., 1968). When equal volumes of virus inoculum
and monoclonal antibodies were mixed before infection of rabbit glial cultures no reduction of virus titres could be noticed.

PAGE analysis of the affinity column eluates, whether derived from infected rat brains or from infected MDCK cells, always contained two closely adjacent bands with a molecular weight of about 38K (Fig. 1). Different preparations had additional bands which were stained at variable intensities by Coomassie Brilliant Blue. Examples of such variations are visible by comparing Fig. 1(b) with eluate in Fig. 3(c). These obvious contaminants could largely be eliminated by passing the crude eluate through a HPLC molecular sieve column. BD virus antigen demonstrable by AGDT appeared from the column at the same and at a slightly retarded position as the thyroglobulin marker (Fig. 2a). The size of the non-denatured antigen could therefore be estimated as being in the range of about 300K to 600K.

PAGE analysis showed that hardly any contaminating protein that had been attached nonspecifically to the affinity column remained in this fraction besides the 38K bands (Fig. 2b). The antigen content of the front fraction (I) was so low that it was only visible as two faint bands at 38K.

Immunoblots demonstrated unequivocally that these 38K components represent BD virus-specific antigens (Fig. 1). This became evident by staining of the two bands on nitrocellulose at
the 38K position of the polyacrylamide gel with a convalescent serum from a rat that was clinically ill and had a BD virus-specific antibody titre of 1:10000. This component could also be stained with monoclonal antibodies prepared in different ways. One type was from hybridomas that had been prepared by immunizing a mouse with the crude eluate from the affinity column (clones no. 18 and 47). Other hybridomas were obtained by fusing spleen cells from mice which had been infected with BD virus intracerebrally (clones no. 8 and 23). In all these cases two closely spaced bands were revealed as in gels stained with Coomassie Brilliant Blue (Fig. 1). With an immunoaffinity column of Sepharose conjugated with monoclonal antibody no. 47 the same two 38K proteins could be isolated from the tissue homogenates (Fig. 3b). In the eluates from the monoclonal antibody column, hardly any background contamination was visible in gels stained with Coomassie Brilliant Blue. With rat serum, either with serum from an infected animal or with normal serum, there was constantly a non-specific staining on the immunoblots.
that corresponded to two high molecular weight positions (Fig. 1). The same was true for rabbit serum but this could never be seen with mouse serum (not shown).

Staining of a gel with fluorescein isothiocyanate-conjugated lectins (wheat germ agglutinin and Lens culinaris agglutinin; Maher & Molday, 1977) did not reveal any signs of glycosylation in either of the two 38K bands.

**DISCUSSION**

Attempts have been made before to define the BD virus-specific antigens by PAGE analysis of immunoprecipitates (Ludwig & Becht, 1977). Since viral antigens represent only a minor fraction among labelled cellular proteins, the identification of the two presumptive virus-specific peaks of mol. wt. 40K and 22K was not unequivocal. Isolation of the antigen in purified form was therefore necessary for a definite characterization.

Immunoaffinity chromatography proved to be an efficient and decisive purification step. It became obvious that the eluate from the affinity column contained a virus-specific antigen, because there was a clear precipitation line when it diffused against a standard convalescent serum in an AGDT and there was no longer any precipitation when the flowthrough from the column was tested. An alkaline buffer system (Becht & Malole, 1975) was chosen for elution, because it is known from previous experience that many viral antigens have the tendency to precipitate at an acidic pH. It also became clear that the eluate contained the intranuclear BD virus antigen because a hyperimmune serum prepared against this material produced the typical brilliant intranuclear fluorescence in BD virus-infected MDCK cells while non-infected controls remained negative. In PAGE analysis of the eluate only a few bands were visible in a stained gel (Fig. 3); their number and intensities varied somewhat from one preparation to another. A constant finding, however, was two closely adjacent 38K bands. The virus specificity of these could be demonstrated in immunoblots stained with convalescent sera from infected rats or rabbits. Further support for the virus-specific nature of the two 38K bands came from immunoblots stained with monoclonal antibodies. Since nuclear fluorescence was the criterion used for the selection of hybridomas, one can be certain that the 38K component represents the intranuclear antigen. All monoclonal antibodies originating from mice immunized with the eluate or infected with BD virus stained both bands equally well (Fig. 3). It is highly probable, therefore, that they are structurally closely related. Since the 38K bands do not appear to be glycosylated the microheterogeneity of the two bands cannot be due to carbohydrate side-chains. A slightly faster migration rate of one type of polypeptide may result from a loss of a small number of amino acids by proteolysis, or a retarded migration of the other band may be due to polypeptides which were not fully denatured or reduced by the methods employed.

It is remarkable that in the upper region of the gels two components, most likely of cellular origin, bind to gamma globulins from normal rat and rabbit serum, but have no affinity for mouse serum, although mice are susceptible to BD virus infection without becoming clinically ill (Kao et al., 1984). This lack of non-specific adsorption of cellular material by mouse Ig was also found when a monoclonal antibody was used for the preparation of an immunoaffinity column. Besides the 38K double band, hardly any background contamination was visible in a PAGE analysis of an eluate from such a column (Fig. 3). The contaminating cellular proteins in the affinity chromatography eluate could virtually be eliminated by passing it through a HPLC gel filtration column. The only fraction which underwent a specific precipitation and contained the 38K double band in PAGE appeared with the thyroglobulin marker (670K). It can be roughly estimated, therefore, that the antigen exists in its native form as a polymer consisting of more than 10 monomers. The question as to whether the isolated antigen is a structural component of the virus remains open. This might be so, because the tendency of the 38K component to form aggregates could reflect a self-assembly phenomenon. Similarly to convalescent sera, none of the monoclonal antibodies had any neutralizing capacity, but it is not clear yet whether or not the 38K antigen is able to induce protective immunity. So far, only two rats have been immunized by intraperitoneal application of the purified antigen and challenged 1 week later intracerebrally with infectious BD virus. The animals developed virus-specific antibodies, but typical symptoms appeared and the disease ran its normal course.
Borna disease virus-specific nuclear antigen

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REFERENCES


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