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PURIFICATION OF THE GROUP-SPECIFIC ANTIGEN OF BLUETONGUE VIRUS BY CHROMATOFOCUSING*

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A method for the purification of the precipitating antigen of bluetongue virus (BTV) is described. The results obtained indicate that the precipitating antigen is identical with a core protein of BTV having a molecular weight of 39,000 (P7). So far all bluetongue virus serotypes have been shown to possess this protein. It is evident therefore that a group-specific reaction could be based on the presence of antibodies against this core protein. The purification of this protein by chromatofocusing proved relatively easy to perform and immunodiffusion tests revealed a group-specific reaction.

bluetongue purification group-specific antigen immunodiffusion

INTRODUCTION

The double immunodiffusion test (ID) as a means of detecting group-specific precipitating antibodies against bluetongue virus (BTV) was first described by Klontz et al. (1962). While these authors used an extract of infectious mouse brain, Jochim et al. (1969) demonstrated the presence of a group-specific antigen in the supernatant of BTV infected cell cultures. A first attempt to isolate this antigen met with questionable results. Wang et al. (1972) used ion-exchange chromatography and other techniques of gel-chromatography to purify the antigen. Yet the molecular weight calculated for the protein was of a size which could not be accounted for in the bluetongue virus genome. It would therefore appear that this preparation was heavily contaminated with nonviral material. On the other hand Huismans (1979) showed that a protein (P7) of BTV is produced in excess in infected cell cultures and released in the supernatant. The group-specific character of this virus protein was also demonstrated by immunoprecipitation studies (Huismans et al., 1982). More recently, Gumm and Newman (1982) have shown that P7 can be isolated by isoelectric focusing. This lead us to attempt the isolation of

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that core antigen (P7) from infectious supernatant, since the isolation of a relatively pure precipitating antigen would greatly facilitate immunodiffusion tests. Experience has indicated that antigen obtained purely by concentration of the supernatant leads more often than not to the development of several precipitation lines, of which only one is BTV specific.

We now report the application of chromatofocusing to the isolation of the group-specific core protein of BTV and its application to the immunodiffusion test.

MATERIALS AND METHODS

Virus and cells

Bluetongue virus strain 8 – serotype 10 – was purchased from the Denver Serum Company, Denver, CO, U.S.A. The virus was plaque-cloned three times prior to infection of roller bottles seeded with BHK_{21} cells. Usually a multiplicity of infection (MOI) of 0.1 was chosen for infection. Before the infection, growth medium was drained off and the cultures were washed twice with phosphate-buffered saline (PBS) to remove serum globulins. Eagle's medium (Glasgow modification) enriched with 10% calf serum was used for cell propagation, whereas for virus growth the serum was omitted. As a rule infected cultures showed a complete cytopathic effect (CPE) within 48 h of infection.

Preparation of infectious supernatant

Cells and supernatant fluids from infected cultures with complete CPE, were harvested and centrifuged for 120 min in Rotor R19 at 18,000 rpm. The pellet contained most of the virus, which was used for other purposes. The resulting supernatant was either concentrated with an Amicon PDIH concentration unit or was precipitated with an equal volume of saturated $(NH_4)_2\ SO_4$, pH 7.4, solution. Concentration or precipitation was performed at room temperature. With both methods a 100-fold concentration of the supernatant containing soluble antigen was obtained. This protein solution was diluted to contain 30–40 mg protein/ml.

Gel filtration

Separation of the constituents of the concentrated supernatant was attempted by gel filtration on Sephacryl S-200. 2 ml of the concentrate were passed through a 2.5 × 100 cm column in 0.05 M Tris with 0.5 M NaCl at pH 8.0. Elution was carried out at room temperature and fractions containing peaks were precipitated with $(NH_4)_2\ SO_4$ and dialysed against PBS.

Chromatofocusing

Chromatofocusing was essentially as described by the manufacturer (Deutsche Pharmacia, Freiburg). Polyexchanger PBE 94 was dissolved in appropriate amounts in 0.025 M imidazol buffer, pH 7.4. Five ml of the concentrate, which had been previously dialyzed against 0.025 M imidazol buffer pH 7.4, were passed through a 0.9 × 30 cm column

filled with PBE 94. Care was taken that the sample applied did not exceed 300 mg protein. Before applying the test sample 5 ml polybuffer, pH 4.0, were applied to the column. Elution was performed with polybuffer, pH 4.0, and individual peaks were sampled and precipitated twice with 90% sat. $(NH_4)_2\ SO_4$ solution and finally dialysed against PBS.

Protein determination

The determination of the protein concentration was carried out with the aid of a commercially available protein assay kit (Bio-Rad).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method as described by Laemmli (1970) was used. Running conditions were 40 V for 14 h. The gel slab was fixed in methanol and stained with Coomassie brilliant blue and destained in 4% acetic acid.

Immunodiffusion

A 1% solution of Litex HSC agarose was prepared in veronal buffer, pH 8.4 (ionic strength 0.025). Microscope slides were overlaid with 3 ml of the agarose solution. A template consisting of 6 equidistant wells from a centre well was used. Each well was filled with 15 µl of reagent. Plates were kept in a moist chamber at room temperature. Inspection for precipitation lines was done after 24 h.

Sera

Bluetongue reference sera prepared in guinea pigs were obtained from Dr. J. Erasmus (Veterinary Research Institute, Onderstepoort, South Africa). Sheep and cattle sera were collected from experimental cases which had undergone a BTV infection. Normal BTV(-) sera were collected from animals having no contact with BTV. Rabbit anti-BHK serum was a gift from Dr. J. Cox from this Institute.

RESULTS

Concentration of crude precipitating antigen

Both concentration methods, namely pressure dialysis and salt precipitation, proved satisfactory with regard to the precipitation lines obtained with test serum. The relatively fast concentration achieved with the pressure dialysis procedure appeared to favor this method. On the other hand, if infectious supernatant, which had not been clarified in the ultracentrifuge was used this led to a prolonged concentration time probably due to clogging of the multifibre.

Isolation of the precipitating antigen by gel chromatography

Figure 1 shows the elution profile of the concentrate when passed through a Sephacryl S-200 column. Though some separation was achieved, the profile indicated overlapping

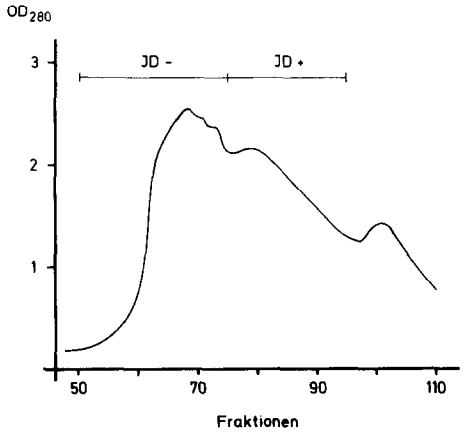

Fig. 1. Fractionation of crude antigen in a Sephacryl S-200 column. Sample 2 ml, fractions 3.3 ml, elution buffer 0.05 M Tris + 0.5 M NaCl, pH 8.0.

of a number of different proteins. Nevertheless the second peak coincided approximately with a molecular weight range smaller than 50,000 as judged from the elution profile with marker proteins. SDS-PAGE (Fig. 2) confirmed this, as there was a definite shift to lower molecular weight proteins from peak 1 to peak 2. As shown in Figure 3 it is evident that peak 2 carried the precipitating antigen, yet a considerable amount of cellular material was still present as seen in its reaction with anti-BHK serum.

Isolation of the precipitating antigen by chromatofocusing

A much clearer separation of individual protein fractions was achieved with chromatofocusing of the concentrated supernatant. Figure 4 illustrates that rather distinct protein fractions were eluted when polybuffer pH 4.0 was used. Obviously the majority of cellular substances present in the concentrate were not bound on the chromatofocusing column used. This is demonstrated by the size of the first peak fraction. In addition, a considerable amount of soluble material present in the concentrate was not eluted with the chosen buffer conditions. This, although not illustrated in Figure 4, was regularly observed after elution of the column with 1 M NaCl. Nevertheless, of the remaining protein-containing fractions only one reacted positively in the ID test. This fraction obtained at a pH value in the vicinity of pH 5.9 always elicited a clear precipitation line with BTV positive reference serum. The relative purity of the ID positive fraction was further demonstrated by the absence of any precipitating line with anti-BHK serum. In contrast, both the ID positive fraction obtained from gel chromatography as well as the crude antigen precipitation, led to the formation of several precipitating lines with anti-BHK serum (Fig. 3). Similarly, if BTV reference serum was used in the ID test (Fig. 5) chromatofocusing purified antigen yielded only one line whereas the crude antigen resulted in the formation of several precipitation lines. The antigenically active fraction

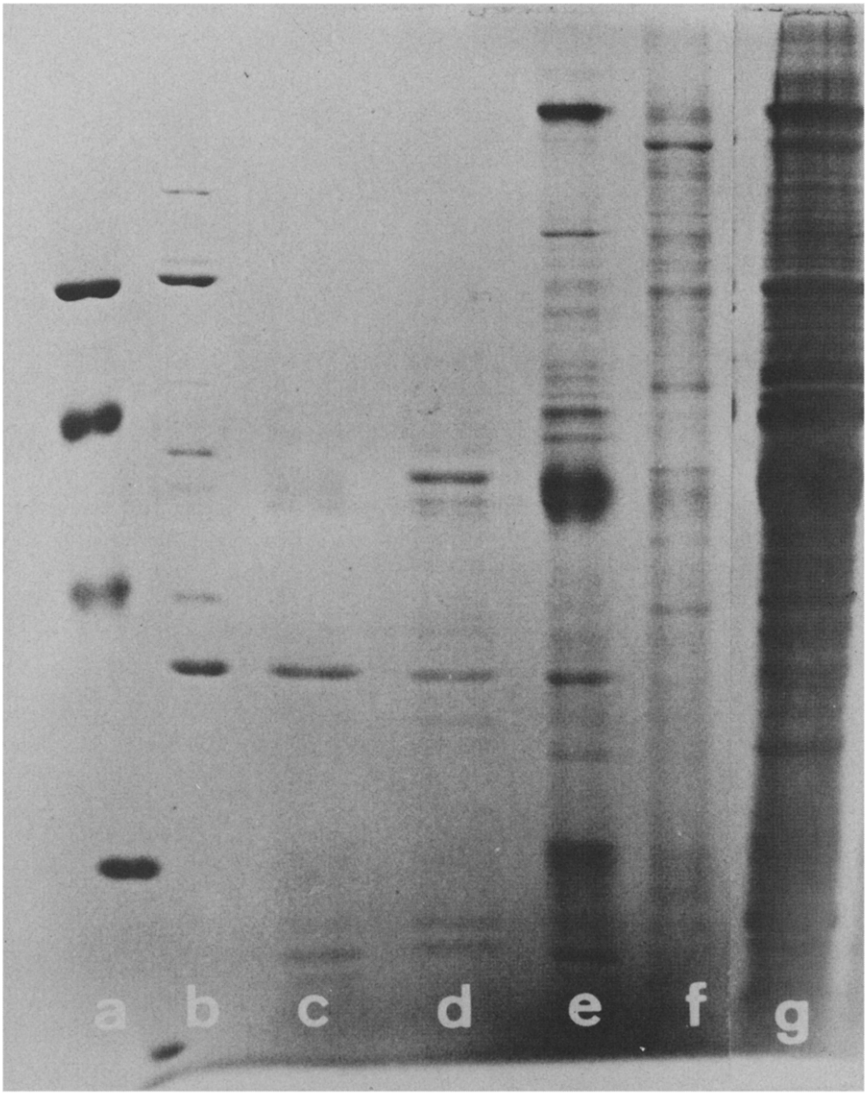

Fig. 2. SDS-PAGE of antigen samples. (a) Marker proteins, (b) BTV 10A, (c) chromatofocusing ID (+) peak, (d) as (c), faster elution, (e) S-200 ID (+) fraction, (f) S-200 D (-) fraction, (g) crude antigen preparation as applied to columns.

was further shown on SDS-PAGE (Fig. 2, lanes c and d) to consist of a protein with a molecular weight of 39,000. In addition this protein coincides with the protein fraction, P7, of BTV (Fig. 2, lane 6). These ID positive fractions were found regularly in the vicinity of pH 5.9 when concentrated material was passed through a chromatofocusing column with the above conditions. However, less purification was obtained if the elution was performed at a faster rate than 35 ml/h (Fig. 2, lane d).

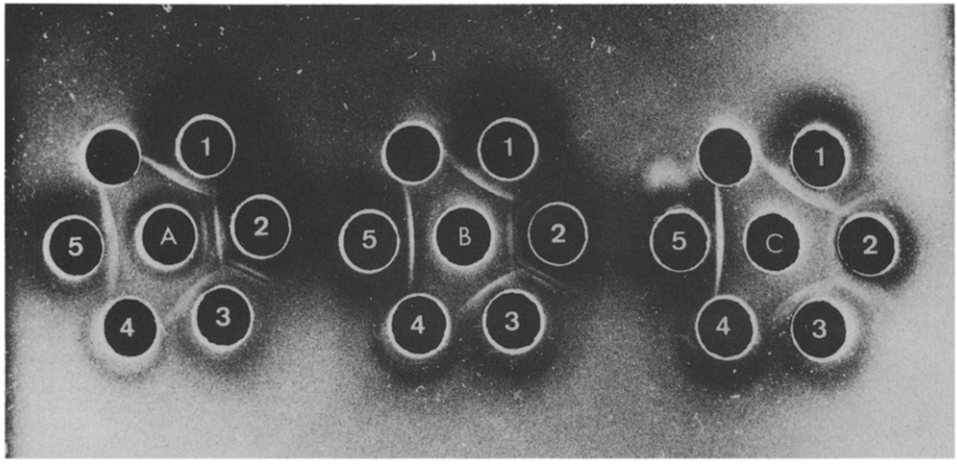

Fig. 3. Immunodiffusion of BTV: (A) crude antigen, (B) S-200 ID (+) fraction, (C) chromatofocusing ID (+) fraction. (1) sheep serum BTV (+), (2) rabbit-anti-BHK serum (3) bovine serum BTV (+), (4) normal bovine serum, (5) reference serum BTV (+).

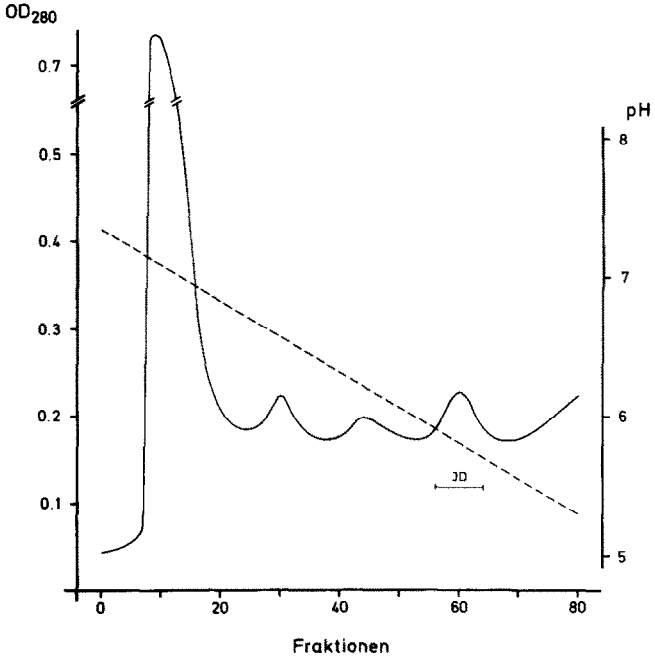

Fig. 4. Fractionation of crude antigen in a chromatofocusing column. Sample 5 ml, fractions 3.3 ml; elution buffer polybuffer pH 4.0.

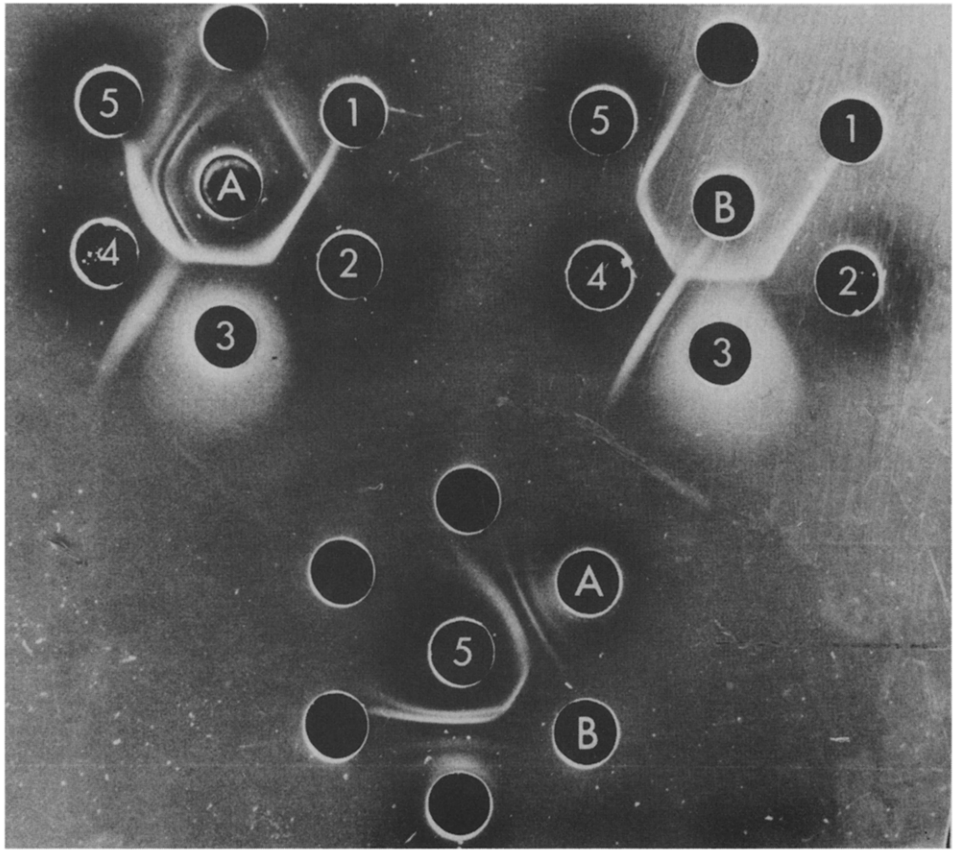

Fig. 5. Immunodiffusion of BTV: (A) crude antigen, (B) chromatofocusing ID (+) fraction. (1) rabbit-anti-BHK serum, (2) rabbit hyperimmune serum BTV T10, (3) BTV + sheep serum, (4) reference serum T17, (5) reference serum T13.

DISCUSSION

There are at least 20 different serotypes of bluetongue virus. Serodiagnosis based on type-specificity is therefore extremely difficult. A serological test based on a common antigen shared by all serotypes is indispensable. The first attempt to find a group-specific antigen was made by Kipps (1956) who demonstrated a group reaction by using a mouse brain derived soluble antigen in the complement fixation test. Other workers have proved since then the value of the double immunodiffusion test (Klontz et al., 1963; Jochim et al., 1969), the fluorescent antibody test (Ruckerbauer et al., 1967), the ELISA (Hübschle et al., 1981) and the haemolysis-in-gel test (Jochim et al., 1981) for the detection of group-specific antibodies. However, little attention was paid to the molecular basis of this group reaction. Verwoerd et al. (1969) stipulated that the two outer proteins surrounding the BTV core are responsible for serodifferentiation and possibly for its virulen-

ce. The five remaining proteins consequently are assembled in the virus core. From an evolutionary point of view, it appears more likely that the common antigen should be contained in the core. In support of this, Huismans et al. (1981) using an immunoprecipitation assay showed that protein 7 (P7) which forms part of the core, reacts with all serotype-specific sera tested. The same author (Huismans, 1979) indicated that P7 is produced in excess during virus replication. The isolation of this protein from infectious culture fluid appeared to be the next step for the clarification of an intergroup relationship. An attempt to isolate the group-reacting soluble antigen with ion-exchange chromatography (Wang et al., 1972) was met with a retrospectively dubious result. With the advance of new protein separation techniques (chromatofocusing) a relatively easy method for the isolation of protein P7 and its application in immunodiffusion tests was at hand. The results obtained from antigens isolated with chromatofocusing clearly demonstrate that P7 is present in considerable amount in the infectious supernatant and support the results of Huismans allocating group-specific reactivity to P7. Judged from the relative ease of performance chromatofocusing is therefore ideally suited for the preparation of a group-specific BTV antigen.

Because only one precipitation line is formed, the use of P7 in routine diagnosis will give unequivocal results. Such an antigen will certainly improve the understanding of the relationship between the various subgroups existing in the orbivirus group.

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