Correlation of Serotype Specificity and Protein Structure of the Five U.S. Serotypes of Bluetongue Virus

By JAMES O. MECHAM,* VICTORIA C. DEAN AND MICHAEL M. JOCHIM

Agricultural Research Service, Arthropod-Borne Animal Diseases Research Laboratory, P.O. Box 3965, University Station, Laramie, Wyoming 82071-3965, U.S.A.

(Accepted 15 August 1986)

SUMMARY

The relationship between serotype specificity and protein structure was studied by polyacrylamide gel electrophoresis, peptide mapping and radioimmune precipitation (RIP) of structural and non-structural proteins of the five U.S. serotypes of bluetongue virus (BTV). The surface proteins, VP2 and VP5, showed the most variation in size among the serotypes. Peptide mapping of the proteins showed that VP2 is unique for each of the U.S. serotypes. The nucleocapsid and non-structural proteins showed a high degree of conservation, whereas the other surface protein, VP5, showed intermediate conservation among the serotypes. Monospecific neutralizing antiserum produced in rabbits against each serotype was used in cross-RIP against cytoplasmic extracts prepared from cells infected with each BTV serotype. There were extensive cross-reactions among those proteins which showed a high degree of structural conservation, whereas VP2 was immunoprecipitated best in the homologous RIP system. Thus, a correlation between serotype specificity and protein structure was shown among the five U.S. serotypes of BTV.

INTRODUCTION

Bluetongue virus (BTV) is a member of the orbivirus group. At least 24 serotypes have been identified worldwide and five are found in the U.S.; these are designated BTV-2, BTV-10, BTV-11, BTV-13 and BTV-17 (Barber, 1979; Gibbs et al., 1983). BTV causes disease in sheep, cattle and wild ruminants that may result in severe economic loss to the livestock industry (Mulhern, 1985).

The genome of BTV is composed of 10 segments of double-stranded RNA (Verwoerd, 1969; Verwoerd et al., 1970) which code for at least seven structural and two or three non-structural proteins (Verwoerd et al., 1972; Huismans, 1979; Mertens et al., 1984). The different BTV serotypes possess both shared and unique antigenic determinants as determined by serological methods (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983).

Standard virological and serological techniques sometimes fail to identify BTV-infected animals and germ-plasm or confuse serological diagnosis due to cross-reactions with other orbiviruses (Della-Porta et al., 1985; Jochim, 1985). Attenuated vaccines against bluetongue disease often give incomplete protection or may provide an environment for reassortment of viral genetic material and for the emergence of new strains (Osburn et al., 1983; Campbell & Grubman, 1985). Subunit diagnostic probes and vaccines offer alternatives that may overcome some of these problems. However, to produce these probes it is necessary to characterize those group-specific and serotype-specific antigenic determinants which are of biological interest.

We have initiated the characterization of antigenic determinants on the proteins of BTV by analysis of structural and non-structural proteins of the five U.S. serotypes of BTV with polyacrylamide gel electrophoresis (PAGE), peptide mapping and radioimmune precipitation (RIP). Our results have allowed us to correlate some structural and serological features for the proteins of the different serotypes.
METHODS

Viruses and cell cultures. The viruses used for this study were BTV-2 (strain Ona A), BTV-10 (strain BT-8), BTV-11 (Station strain), BTV-13 (strain 67-41B) and BTV-17 (strain 63-66B). Each serotype had been plaque-purified by limiting dilution in cell culture.

Baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection and used to propagate virus for these studies. Cells were grown in Eagle's MEM supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml) and 10% bovine foetal serum.

Discontinuous SDS-PAGE. Protein samples were suspended in PAGE sample buffer (2.5% 2-mercaptoethanol, 1.25% SDS, 0.38 M-glycine, 0.05 M-Tris base, 10% glycerol) and electrophoresed in 1.5 mm thick slab gels similarly to the method of Laemmli (1970) as previously described (Mecham & Trent, 1983). Gels for peptide mapping contained 15% acrylamide, whereas all other gels contained 10% acrylamide. The following 14C-labelled proteins were used as molecular weight standards: phosphorylase B (97000), bovine serum albumin (69000), ovalbumin (46000) and carbonic anhydrase (30000) (New England Nuclear). Autoradiography was accomplished by impregnating the gels with either En3Hance or PPO-DMSO autoradiography enhancer (New England Nuclear) and by exposure of Kodak X-omat AR film for appropriate lengths of time.

Growth and purification of virus. Confluent monolayers of BHK-21 cells were infected at a multiplicity of 0.1 p.f.u./cell in MEM (without methionine) containing 2% bovine foetal serum and 5 μCi/ml [35S]methionine (New England Nuclear). The infected cells were incubated at 37 °C until 100% of the cells showed cytopathic changes. The cells were shaken off the culture flask and pelleted at 1500 g for 30 min. The pellet was resuspended in 0.002 M-Tris–HCl buffer pH 8.8 containing 1% Triton X-100 (TX-100) and stirred at 4 °C for 1 h. This suspension was then centrifuged at 1500 g for 30 min and the supernatant fluid was saved. The supernatant fluid was centrifuged through a 25% sucrose cushion at 25000 r.p.m. in a Beckman SW28 rotor at 5 °C for 2 h. The pellet was resuspended in Tris–HCl buffer and extracted with 1/3 vol. of Genosolv-D (Allied Chemical Corp., Morristown, N.J., U.S.A.) with vortex-mixing and centrifugation at 1500 g. The aqueous phase was then pelleted through a 25% sucrose cushion at 25000 r.p.m. for 2 h at 5 °C in the SW28 rotor. The pellet was resuspended in Tris–HCl buffer and centrifuged through a 25 to 50% sucrose gradient at 30000 r.p.m. for 2 to 4 h in a Beckman SW40Ti rotor. The virus bands were located with light, collected, pelleted through a 25% sucrose cushion and resuspended in PAGE sample buffer for analysis of viral proteins by SDS–PAGE and autoradiography.

Radioimmune precipitation. BHK-21 cells were infected with virus at 1 p.f.u./cell and at 18 to 24 h after infection were labelled for 1 h with 50 μCi/ml [35S]methionine (New England Nuclear) in MEM (without methionine) containing 2% bovine foetal serum. Cells were lysed with RIP buffer (Kessler, 1975) containing 1% TX-100 and 0.1% aprotinin (Sigma) and centrifuged at 15000 g for 5 min. The cell lysates were immunoprecipitated by using antiserum produced in rabbits against each of the five U.S. BTV serotypes. Staphylococcus aureus Protein A (Pansorbin, Calbiochem Behring) was used as the solid phase in the reaction. The immunoprecipitated viral proteins were eluted from the Pansorbin by boiling in PAGE sample buffer. These samples were then analysed by SDS–PAGE and autoradiography. Extracts from uninfected BHK-21 cells were immunoprecipitated with the antisera to distinguish cellular proteins from viral proteins.

To clearly isolate VP2 and VP3 which often migrate very closely together in PAGE, infected cellular extracts were first immunoprecipitated with heterologous antiserum to precipitate all viral proteins except VP2. The cellular extract was reacted with homologous antiserum to precipitate VP2. The precipitates were analysed by PAGE and autoradiography to locate the proteins of interest. In this way VP2 and VP3 were isolated without cross-contamination.

Production of serotype-specific antibody to BTV. Young adult New Zealand white rabbits weighing 2 to 3 kg were injected intramuscularly with 107 to 108 p.f.u. of partially purified BTV in complete Freund's adjuvant (1:1). BTV had been purified from BHK-21 lysates by extraction with Genosolv and Tris–HCl buffer. Rabbits were rested 5 months, injected intradermally with the same concentration of virus without adjuvant and bled for neutralizing serum antibodies 5 to 7 days later. Subsequent injections with half the initial concentration of virus in incomplete Freund's adjuvant and bleedings were at 2 month intervals.

Peptide mapping of viral proteins. Viral proteins were peptide-mapped by partial proteolysis similarly to the method of Cleveland et al. (1977). The proteins were located and cut from a preparative gel following SDS–PAGE and autoradiography. Densitometric scans of the autoradiographs were performed to standardize the amount of each protein to approximately 4000 to 6000 c.p.m. except for VP1 and VP4 which were standardized to approximately 1000 to 1500 c.p.m. The preparative gel was re-autoradiographed to establish that the correct protein had been cut from the gel. The plugs which had been cut from the gel were soaked for 30 min in 0.125 M-Tris–HCl pH 6.7 containing sucrose and were wedged into the wells of a 15% gel and overlaid with a solution of 1% agarose (Seakem) in 0.125 M-Tris–HCl pH 6.7.

Five μg of V-8 protease was added to each well and the samples were electrophoresed at 80 V until the bromophenol blue dye front had migrated to the stacking gel–resolving gel interface. At that time the power was turned off for 30 min to allow digestion to occur in the gel. Electrophoresis was then continued at 120 V to the end of the run and the peptide fragments were analysed by autoradiography.
Antigenicity and protein structure of BTV

RESULTS

Purification of all five U.S. serotypes of BTV permitted comparison of their structural proteins. All of the serotypes possessed at least seven structural proteins (Fig. 1) which are designated VP1, VP3, VP2, VP4, VP5, VP6 and VP7. The order of VP2 and VP3 has been reversed in our gel system by using N,N'-diallyltartardiamide as a cross-linker as compared to gel systems in which bisacrylamide is used as a cross-linker (Huismans, 1979; Appleton & Letchworth, 1983). The identification of VP2 and VP3 was made by immune precipitation with homologous and heterologous polyclonal antisera. VP2 was immune-precipitated best with the serotype-specific homologous antiserum, whereas VP3 was immune-precipitated by heterologous as well as homologous antiserum (see below). The apparent mol. wt. for the structural proteins ranged from 121,600 to 41,200. The migration profiles of VP1, VP3 and VP4 were similar among the five serotypes. VP6 and VP7 were similar among the serotypes except for BTV-13 which had a slightly larger VP6 and a slightly smaller VP7. VP2 and VP5 showed the greatest heterogeneity in mol. wt. among the five serotypes. Two protein bands were sometimes observed in the VP2 region of BTV-17.

RIP of infected cell extracts with monospecific polyclonal antisera revealed the presence of the structural proteins seen in the purified virions (Fig. 1). In addition, three non-structural proteins were readily observed. Based upon their relative positions, concentrations and peptide maps (see below) these proteins are designated NS1, NS1a and NS2 according to the nomenclature of Mertens et al. (1984) for similar proteins from BTV-1-infected cells. The relative amount of NS1a varied from infection to infection and it was usually absent from cells infected with BTV-2. The mol. wt. of these non-structural proteins from BTV-10, 11 and 17 were very similar, but some variation was observed for serotypes 2 and 13.

There was a small protein(s) which migrated near the dye front in some of the infected cell extracts. This protein may be analogous to the small viral proteins identified in cells infected with BTV-1 and BTV-17 and designated 8/8a or VP10 respectively (Mertens et al., 1984; Grubman et al., 1983). However, it was not always present in all of the virus-infected cell extracts and was not further characterized.
Peptide mapping by partial proteolysis of the seven structural and three major non-structural proteins of the five U.S. serotypes of BTV allowed us to define further the relationships of the viruses. These results are shown in Fig. 2, 3 and 4. The peptide maps for VP1, VP3 and VP4 were very similar among the five serotypes (Fig. 2). The largest peptide fragment from VP6 and VP7 of BTV-13 was slightly smaller than the corresponding fragment from the other four serotypes (Fig. 3). The peptide maps of VP5 allowed the U.S. serotypes to be divided into two groups: the maps of VP5 from BTV-2 and BTV-13 were similar to each other, but were different from the peptide maps of VP5 from BTV-10, BTV-11 and BTV-17 which were all similar (Fig. 3). Peptide mapping of VP2 indicated that this protein is unique for each of the five serotypes examined (Fig. 2). Peptide mapping of NS1, NS1α and NS2 (Fig. 4) indicated that these proteins are quite highly conserved among the five serotypes with some differences noted in NS2. NS1 and NS1α appeared to be related as previously described by Mertens et al. (1984). The results of peptide mapping were reproducible using various amounts of V-8 protease.
Antigenicity and protein structure of BTV

Fig. 4. Peptide mapping of (a) NS1, (b) NS1a and (c) NS2 from the five U.S. serotypes of BTV. Protease digestion and analysis of the viral proteins were carried out as described in Fig. 2.

Fig. 5. Radioimmune precipitation of BTV-infected BHK-21 cell extracts by homologous (*) and heterologous rabbit antisera: A, anti-BTV-2; B, anti-BTV-10; C, anti-BTV-11; D, anti-BTV-13; E, anti-BTV-17; F, normal rabbit serum. Arrowheads indicate the position of VP2.

Immunoprecipitation of virus-infected cell extracts (Fig. 5) showed that VP3 and VP7 reacted equally well with either homologous or heterologous antisera. NS1, NS1a and NS2 were also precipitated by both homologous and heterologous antisera. In those instances where VP1, VP4, VP5 and VP6 were detectable no differences could be seen on precipitation with either homologous or heterologous antisera. In all cases, VP2 was precipitated best by the homologous antiserum, although some precipitation by heterologous antisera was sometimes noted. A doublet in the region of VP2 of BTV-17 was sometimes noted following immunoprecipitation. These proteins appeared to be identical as determined by peptide mapping (data not shown).

DISCUSSION

The present studies have expanded our understanding of similarities and differences among the five U.S. serotypes of BTV. Peptide mapping of structural and non-structural proteins has allowed us to show relationships and differences among these viruses. As shown in Table 1, the five virus strains possess proteins that are highly conserved, moderately conserved and unique
Table 1. Summary of peptide maps of proteins of five U.S. serotypes of bluetongue virus

<table>
<thead>
<tr>
<th>Conserved proteins</th>
<th>Partially conserved proteins</th>
<th>Unique proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>VP5</td>
<td>VP2</td>
</tr>
<tr>
<td>VP3</td>
<td>VP6</td>
<td></td>
</tr>
<tr>
<td>VP4</td>
<td>VP7</td>
<td></td>
</tr>
<tr>
<td>NS1</td>
<td>NS2</td>
<td></td>
</tr>
<tr>
<td>NS1a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

among the serotypes. As determined by peptide mapping, BTV-2 and BTV-13 are more closely related to each other than to the other three serotypes (BTV-10, BTV-11 and BTV-17) which are more closely related to each other.

The data obtained from peptide mapping indicate that all the virus serotypes differ in VP2. This structural protein is located on the surface of the virion and possesses antigenic determinants responsible for serotype specificity, virus neutralization and host protection (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983; Letchworth & Appleton, 1983a, b; Huismans et al., 1983, 1985). The RIP data in which both homologous and heterologous antisera were used suggest that this protein contains dominant serotype determinants. We have produced a battery of type-specific neutralizing monoclonal antibodies to VP2 of BTV-10, which do not immunoprecipitate VP2 of the other four U.S. serotypes (unpublished results). Reassortant studies with BTV-10 and BTV-11 have also implicated this protein as being responsible for serotype specificity (Kahlon et al., 1983).

The other structural protein which is located on the surface of the virus, VP5 (Martin et al., 1973; Van Dijk & Huismans, 1980), shows less structural variation than VP2. BTV-2 and BTV-13 are related as are BTV-10, BTV-11 and BTV-17 as shown by peptide mapping of this protein. We do not have any serological evidence suggesting that this protein plays a role in determining serotype specificity. However, the presence of group-specific determinants could preclude detection of serotype-specific determinants (on the same protein) in RIP assays using polyclonal antiserum. A panel of monoclonal antibodies specific for multiple determinants on this protein should help to define its biological role.

Several BTV proteins have been shown to contain group-specific antigenic determinants. VP7 possesses strong group-specific determinants as determined by immune precipitation using polyclonal antisera (Huismans & Erasmus, 1981). Based upon relative size, the non-structural protein designated NS1 and the structural protein, VP7, in our system are probably analogous to P5a (Huismans & Els, 1979) and VP7 (Appleton & Letchworth, 1983) which are immunoprecipitated by using both polyclonal and monoclonal antisera. Based upon the results of experiments in which monoclonal antibodies were used, a viral protein which, according to its size, may be analogous to NS2 in our system appeared to contain group-specific determinants (Appleton & Letchworth, 1983). Structurally, these proteins are strongly conserved among the serotypes as determined by peptide mapping. Our cross-RIP results with polyclonal antisera confirm the antigenic relatedness of NS1, NS2 and VP7 among the five U.S. serotypes of BTV. Squire et al. (1985) have cloned the genome segment which codes for VP7 from BTV-17. This probe hybridized completely with BTV-10, 11 and 17, but incompletely with BTV-13. These results are consistent with our results from peptide mapping showing that VP7 of BTV-13 is slightly different in the largest peptide fragment from VP7 of the other four serotypes.

Our results indicate that VP3 is conserved structurally and contains group-specific antigenic determinants shared by the five U.S. serotypes. In addition, monoclonal antibody to VP3 of BTV-10 also precipitates VP3 of the other four serotypes (unpublished results). Roy et al. (1985) have cloned the RNA genome segment of BTV-17 which codes for VP3 and shown that it hybridizes with RNA from the other U.S. serotypes and with other BTV isolates from different parts of the world. Our peptide mapping results contrast with those of Whistler & Newman (1986) who reported differences in the peptide maps of VP3 from a number of BTV isolates. However, since VP2 and VP3 migrate closely together and may change their relative positions in
Antigenicity and protein structure of BTV

PAGE, it is possible that these authors have presented peptide map data for VP2 rather than for VP3. Peptide mapping of both these proteins from their BTV isolates would help resolve this question.

The other structural proteins (VP1, VP4 and VP6) which appear by peptide mapping to be highly conserved among the U.S. serotypes may also contain group-specific antigenic determinants. However, we have failed to show good immunoprecipitation of these proteins. This is probably a reflection of their relative amounts in infected cell extracts when compared to the other viral proteins.

In conclusion, our results have shown a correlation between antigenic reactivity and protein structure among the five U.S. serotypes of BTV. The most structurally variable of the viral proteins, VP2, appears to contain dominant serotype-specific determinants. Other BTV proteins are more highly conserved structurally and contain group-reactive antigenic determinants. This information should be of value for both diagnostic and epidemiological application.

REFERENCES


\textbf{(Received 18 March 1986)}