Bluetongue Virus Genome Remains Stable throughout Prolonged Infection of Cattle

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SUMMARY

Infection of three calves with a highly plaque-purified strain of bluetongue virus (BTV) resulted in prolonged infections, during which virus and neutralizing antibodies co-circulated in peripheral blood. Oligonucleotide fingerprint analyses of the original challenge virus and of the final virus isolate obtained from each calf demonstrated the BTV genome to remain stable throughout prolonged infection as no differences in fingerprint patterns were detected. Six neutralizing monoclonal antibodies (MAbs), and a polyclonal rabbit antiserum, were produced against the challenge virus. This panel of MAbs recognized at least two distinct neutralizing epitopes as demonstrated by immune precipitation. Neutralizing epitopes remained stable through the prolonged infections, as all MAbs and the polyclonal rabbit antiserum neutralized the challenge virus and the final calf isolates to equivalent titres. These results suggest that antigenic drift is not the mechanism by which BTV is able to persist in cattle in spite of a strong humoral immune response.

Bluetongue virus (BTV) is a member of the virus family Reoviridae and the prototype virus of the genus Orbivirus (Murphy et al., 1971). The BTV genome consists of 10 individual dsRNA segments (Verwoerd et al., 1970), with segment 2 encoding virus protein 2 (VP2), a major protein of the outer capsid (Kahlon et al., 1983; Mertens et al., 1984), and the polypeptide primarily responsible for serotype specificity (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983). Outer capsid protein VP5 may also play a minor role in virus neutralization (Mertens et al., 1987). Presently 24 serotypes of BTV are recognized world-wide, as defined by neutralization with type-specific antisera (Huismans & Cloete, 1987).

The genetic and antigenic heterogeneity within the BTV serogroup has resulted from genetic drift (Sugiyama et al., 1982), as well as reassortment of genome segments during mixed infections in both the arthropod vector and the ruminant host (Samal et al., 1987a, b; Stott et al., 1987). Genome segments encoding the outer capsid proteins VP2 and VP5 are the most variable among BTV isolates, presumably due to selection of mutations as a result of immunological pressure from the infected host (Huismans et al., 1987; Mertens et al., 1987). Antigenic drift has also been described for rotavirus (Goto et al., 1986; Murakami et al., 1986), another member of the Reoviridae, as well as many other viruses including foot-and-mouth disease virus (Hyslop & Fagg, 1965) and influenza virus (Laver & Webster, 1979).

Cattle have been proposed to be a reservoir host of BTV because viraemia is usually prolonged, and the vast majority of infections are asymptomatic (DuToit, 1962; Bowne et al., 1968; Nevill, 1971). In BTV-infected cattle the virus can co-exist with neutralizing antibody in the peripheral blood for prolonged periods. This apparent ability of BTV to evade the host's immune response might be the result of antigenic variation of the virus under the immunological pressure of neutralizing antibody (MacLachlan & Fuller, 1986; MacLachlan et al., 1987).
Table 1. Viraemia and antibody response in calves experimentally infected with BTV

<table>
<thead>
<tr>
<th>Day p.i.*</th>
<th>Calf 1</th>
<th>Calf 2</th>
<th>Calf 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus titre</td>
<td>Antibody titre</td>
<td>Virus titre</td>
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<tr>
<td>0</td>
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<td>10</td>
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<td>7</td>
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<td>-</td>
</tr>
<tr>
<td>70</td>
<td>-</td>
<td>160</td>
<td>-</td>
</tr>
</tbody>
</table>

* p.i., Post-inoculation.
† Virus titres expressed as log_{10} TCID_{50}/0.1 ml washed and lysed blood cells.
‡ Neutralizing antibody titre expressed as inverse of the serum dilution providing 80% plaque reduction.
§ - , Virus not isolated from 0.1 ml of washed and lysed whole blood cells.

The objective of the present study was to investigate the genetic and antigenic stability of a highly plaque-purified strain of BTV in infected calves. Specifically, we aimed to determine whether genetic drift is responsible for prolonged viraemia in BTV-infected cattle, by comparing the cloned virus used for inoculation with virus isolates obtained late in infection using oligonucleotide fingerprint analysis and reactivity with neutralizing monoclonal antibodies (MAbs).

Three calves were obtained locally and housed in isolation facilities within 24 h of birth. Analysis of blood samples demonstrated all calves to be free of both BTV and BTV-specific antibodies prior to inoculation. At 1 week of age each calf was intravenously inoculated with 1.5 x 10^6 p.f.u. of plaque-purified BTV-10 having a passage history as previously described (MacLachlan & Thompson, 1985). Before use for inoculation, this virus was plaque-picked three more times from agar-overlaid African green monkey kidney (Vero) cells and partially purified by ether and fluorocarbon extraction as described (MacLachlan et al., 1984).

Samples of whole blood (heparin-treated) and sera were collected at weekly intervals for 10 weeks and used for virus isolation on baby hamster kidney (BHK-21) cells and for detection and quantification of virus-specific neutralizing antibodies as described (MacLachlan & Thompson, 1983). Virus was isolated from all three calves on day 7 post-inoculation, and prolonged viraemias (35 to 56 days) occurred in each (Table 1). Calves did not exhibit obvious signs of disease at any time during infection. Virus-specific neutralizing antibodies were first detected in the sera of calves 1 and 3 at day 21 post-inoculation and at day 28 post-inoculation in calf 2. Neutralizing antibodies co-circulated in blood with infectious BTV for 21, 35 and 28 days, respectively, in the calves. The final virus isolate obtained from each calf was passaged once in BHK-21 cells, and this stock was used in the oligonucleotide fingerprint analyses and neutralization assays.

Viral dsRNAs of the original challenge virus and of the final calf isolates were labelled with [32P]orthophosphate and extracted and purified from infected monolayers of BHK-21 cells essentially as described (Sugiyama et al., 1981). The individual dsRNA segments were resolved on 6% polyacrylamide gels overlaid with a 4.5% stacking gel following methods described previously (Laemmli, 1970; MacLachlan & Fuller, 1986). With the exception of genome segments 5 and 6 all segments were readily separated by this method. Segments 5 and 6 displayed similar mobilities in this system, and required excision of both segments from the gel followed by a second gel electrophoresis for adequate separation. Following electrophoresis at 72 V for 22 h the gels were exposed to film (Kodak XAR-5) for 5 min to locate separated segments. Individual segments were then excised from the gel and electro-eluted using a sample
concentrator (Isco, model 1750). Concentrated material was collected and supplemented with 100 μg tRNA (yeast) and ethanol-precipitated overnight at −20 °C. Oligonucleotide fingerprints were obtained for the 10 genome segments from each of the four viruses using the technique of two-dimensional PAGE previously described (Clewley et al., 1977; Sugiyama et al., 1981). Briefly, separated single strands of viral RNA were specifically cleaved by T1 ribonuclease and electrophoresed in the first dimension through a urea-containing 10% polyacrylamide gel at pH 3.5. The gel strip containing the separated oligonucleotides was then electrophoresed in the second dimension through a 20% polyacrylamide gel at pH 8.3. Following electrophoresis, the gel was wrapped in a thin sheet of plastic and exposed to film (Kodak XAR-5) for 48 h at −70 °C.

Several segments, particularly segments 7, 8 and 9, consistently produced patterns with faint background contamination. Background patterns were consistent between fingerprints and, therefore, did not interfere with comparisons. Analysis of fingerprints obtained for genome segments 5 and 6, one of which encodes VP5 (Mertens et al., 1987), demonstrated both to remain stable throughout the prolonged infection in each of the three calves. Fingerprints of genome segment 2, the segment encoding the outer capsid protein primarily responsible for serotype specificity, are presented in Fig. 1. Significant differences were not detected in these fingerprints nor in those of any of the other seven segments of the four viruses (data not shown).

Although oligonucleotide migration profiles varied somewhat between fingerprints of comparable segments, in no instance did sufficient genetic variation occur as to result in the addition or deletion of detected oligonucleotides. Genome analysis by this technique, therefore, demonstrated BTV to remain genetically stable throughout prolonged infection. The technique of oligonucleotide fingerprinting has been shown to be capable of detecting both gross and subtle variation in base sequences in different strains of BTV (Sugiyama et al., 1981, 1982; Collisson & Roy, 1983; Rao & Roy, 1983). Since oligonucleotide fingerprinting allows direct examination of only a minor portion of the viral genetic information, it is possible that point mutations could have gone undetected.

In addition to genomic analysis of the four viruses, the stability of the neutralizing epitopes on the challenge virus throughout prolonged infection was also studied. MAbs to the challenge virus were produced as previously described (Carter et al., 1986). Briefly, BALB/c mice were inoculated intravenously with the BTV-10 stock used for the inoculation of calves. Mice were resensitized 1 month later with 1.0 ml of a 50% (v/v) suspension of infected mouse brains (prepared from the brains of suckling mice inoculated intracranially with stock challenge BTV-10) in Freund’s complete adjuvant, administered subcutaneously at four separate sites. Subcutaneous injections at two sites were repeated with a 0.5 ml 50% (v/v) suckling mouse brain suspension in Freund’s incomplete adjuvant 2 weeks later. Mice were intravenously inoculated with stock challenge virus 3 days before fusion. In addition, polyclonal rabbit antiserum to the challenge virus was obtained by sensitizing a rabbit in a manner similar to that just described.

Fusion of splenic lymphocytes with cells from the P3-X63-Ag8.653 myeloma cell line was carried out in the presence of polyethylene glycol (M₄, 1450) in RPMI medium. Hybridomas were selected by growth on 96-well culture plates in hypoxanthine, aminopterin and thymidine medium containing 20% foetal bovine serum. Hybridomas producing neutralizing antibodies were selected with a microneutralization test performed essentially as described (Appleton & Letchworth, 1983), except that the assay was conducted with BHK-21 cells. Appropriate hybridomas were cloned at least three times by limiting dilution.

Six neutralizing MAbs, each specific for the challenge virus, were cloned. High titre ascitic fluid from each hybridoma was produced in BALB/c mice. The virus protein specificity of each MAb was determined by immunoprecipitation with [35S]methionine-labelled lysates of both infected and mock-infected BHK-21 cells, from which intact virions were first removed by ultracentrifugation, essentially as described (Mitchell et al., 1980; Appleton & Letchworth, 1983). Proteins precipitated by individual MAbs were resolved on a 10% polyacrylamide gel and identified by comparison to the proteins precipitated from BTV-infected BHK-21 cell lysates by the polyclonal rabbit antiserum. The electrophoretic mobility of the immunoprecipitated proteins was compared to M₄, markers to determine their size and identity (Fig. 2).
Fig. 1. Oligonucleotide fingerprint analyses of BTV genome segment 2 obtained from the final isolates of (a) calf 1, (b) calf 2, and (c) calf 3 and (d) the original challenge virus.
The virus protein specificity of five MAbs was shown to reside on VP2. Immunoprecipitation with MAb 041, however, was unsuccessful when the ultracentrifuged antigen source, which contained predominantly individual soluble virus proteins, was used. Binding of virus by MAb 041 was demonstrated by using labelled lysates that had not been ultracentrifuged and thus contained intact virions (Fig. 2). The epitope bound by MAb 041, therefore, requires virus proteins in their virion-associated conformation. Though the virus protein specificity of MAb 041 has not been determined, it clearly recognizes an epitope that is distinct from that bound by any of the other five MAbs.

Reactivity of the MAbs and of the polyclonal rabbit antiserum with the final calf isolates was assessed to determine whether neutralization sites were altered during prolonged infection in the calves. Neutralization titres were determined using the microneutralization assay described earlier. All MAbs neutralized the original challenge virus and the final calf isolates to similar titres, as did the polyclonal rabbit antiserum (Table 2), indicating that the epitopes recognized by these antibodies remained stable throughout the prolonged infection of the calves. At least
Table 2. Neutralization titres of MAbs against challenge virus and three final calf isolates

<table>
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<tr>
<th>Antibody</th>
<th>Challenge virus</th>
<th>Final isolates</th>
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<tbody>
<tr>
<td></td>
<td>Calf 1</td>
<td>Calf 2</td>
</tr>
<tr>
<td>Monoclonal</td>
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</tr>
<tr>
<td>016</td>
<td>800*</td>
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<td>290†</td>
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<td>–</td>
</tr>
<tr>
<td>044‡</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Polyclonal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

* Neutralization titres are expressed as the inverse of the final dilution of ascitic fluid or rabbit antiserum that provided at least 50% protection of BHK-21 cell monolayers from 250 TCID₅₀ of virus.
† MAbs specific for BTV protein VP7.
‡ MAbs specific for BTV protein NS2.
§- Failure to neutralize virus.

Results from these experiments suggest, therefore, that in spite of the considerable genetic diversity of BTV in nature, the virus remained genetically stable throughout the prolonged viraemia that occurred in three experimentally infected calves. Oligonucleotide fingerprint analysis demonstrated that the predominant population of circulating virus detected just before clearance is indistinguishable from the challenge virus administered up to 56 days previously. In addition, virus that is antigenically equivalent to the challenge virus, as determined by reactivity with neutralizing MAbs, was demonstrated to co-circulate with virus-specific neutralizing antibodies in the peripheral blood for up to 35 days. Experimental findings presented here do not discount BTV-infected cattle as a source of genetic variation of BTV, as genetic mutations must certainly be expected to occur. They do suggest, however, that genetic drift does not appear to occur either at a sufficient rate or at the sites necessary to allow the virus consistently to evade the host’s immune response during infection.

Thus, antigenic drift of BTV does not appear to be the mechanism responsible for the prolonged viraemia that occurs in BTV-infected cattle, and the role of infected cattle in the process of genetic drift of BTV remains unclear. As antigenic drift has not been demonstrated to occur, an alternative mechanism for the prolonged viraemia must be implicated. It has been suggested that BTV may reside within a circulating cell type and remain protected from the effects of neutralizing antibody (Luedke, 1970; MacLachlan et al., 1987). A similar mechanism has been identified for Colorado tick fever virus, also a member of the Orbivirus genus (Emmons et al., 1972). This virus resides within erythrocytes, presumably as a result of infecting erythocyte precursors in the bone marrow, and produces a prolonged infection despite the presence of neutralizing antibody in serum. The possibility that prolonged BTV infections in cattle are produced by a similar mechanism is presently being investigated in our laboratory.

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Short communication


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