Comparison of Bluetongue Type 20 with Certain Viruses of the Bluetongue and Eubenangee Serological Groups of Orbiviruses

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SUMMARY

The genome of bluetongue virus type 20 consists of 10 segments of double-stranded RNA each of which contains unique sequences as determined by oligonucleotide mapping. The 10 polypeptide products of the virus genome were detected in virus-infected cells, and in pulse–chase experiments there was no secondary cleavage of the primary gene products. Using stringent conditions for RNA–RNA reassociation, no significant homology could be detected between the genomes of bluetongue type 20 isolated in Australia and representative serotypes isolated in other geographic regions. The results suggest sequence divergence between geographically isolated viruses and not the recent introduction of a bluetongue virus into Australia.

INTRODUCTION

Viruses of the bluetongue serological group of orbiviruses have been isolated in Africa, the Middle East, the Mediterranean region, North America and Australia. The multiplicity of bluetongue virus serotypes was first recognized by Neitz (1948) and it was shown subsequently that common virus antigens were detectable in complement-fixation tests (Kipps, 1956), in agar gel precipitin tests (Klontz et al., 1962) and in fluorescent antibody tests (Ruckerbauer et al., 1967). The specificity of the serum neutralization test in defining bluetongue virus serotypes was demonstrated by Howell (1970) who found no cross-reactions between serotypes. However, Thomas et al. (1979) reported a spectrum of cross-reactivity between bluetongue virus isolates from virtual identity to clear antigenic differences and they suggested that bluetongue virus isolates might form an antigenic continuum.

Certain viruses of the Eubenangee and epizootic haemorrhagic disease of deer (EHD) serological groups of orbiviruses have been shown to cross-react with bluetongue viruses in complement-fixation tests (Borden et al., 1971; Moore & Lee, 1972; Gorman & Taylor, 1978) and in agar gel precipitin tests (Moore, 1974). The certification of animals as free of bluetongue virus is based on negative reactions in group-reactive serological tests so that the relationships between these viruses have economic as well as taxonomic significance.

Bluetongue virus type 20 was isolated from a mixed pool of species of Culicoides at Beatrice Hill in the Northern Territory of Australia in 1976 (St George et al., 1976). Verwoerd et al. (1979) reported some cross-reaction in serum neutralization tests between type 20 and types 4 and 17 while Della-Porta et al. (1981) using similar tests suggested that type 20 is closely related to type 4 and could be regarded as a subtype of type 4.

Serological tests alone are inadequate in assessing the relationships between orbiviruses. Orbiviruses contain 10 segments of double-stranded RNA (dsRNA) each of which is the gene
for a single protein (for recent reviews, see Verwoerd et al., 1979; Gorman, 1979). The group-reactive antigens in bluetongue viruses are found mainly on the core protein of purified virions and the type specificity is determined mainly by antigens located on one of the surface proteins (Verwoerd et al., 1979). Thus, the conventional serological tests do not allow a detailed comparison of each of the 10 proteins of bluetongue and related viruses.

We have studied the relationships between structural components of bluetongue virus type 20 and selected viruses of the bluetongue and Eubenangee serogroups of the orbiviruses in an attempt to define the molecular basis for the serological reactions between them and also to detect possible relationships between the viruses which may not be detected in conventional serological tests.

**METHODS**

**Cell cultures.** Viruses were grown in baby hamster kidney cells (BHK21) or in a line of pig kidney cells (PS-EK) and assayed for plaque-forming units in pig kidney cells by methods described previously (Gorman et al., 1975).

**Viruses.** Bluetongue type 20 (CSIRO 19) was obtained from CSIRO Division of Animal Health Laboratories, Brisbane as the 5th passage of the pool of insects in BHK cells, was passaged in BHK cells and is referred to as uncloned. A further pool was obtained as the 6th passage in suckling mouse brain and was assayed for plaques in pig kidney cells. Small and large plaques were selected and cloned separately at least three times. Strains of bluetongue virus serotypes exotic to Australia were used only at the Animal Virus Research Institute, Pirbright. Bluetongue virus types 1 (isolate 494, South Africa), 4 (isolate 922, South Africa), 10 (isolate 1926, South Africa), 15 (isolate 133/60, South Africa) and 17 (Wyoming isolate 2790 U.S.A.) were obtained from the reference collection held there and passaged without further cloning in BHK cells.

Eubenangee (In 1074) and Tilligerry (NB 7080) viruses were plaque-cloned three times in pig kidney cells (Gorman & Taylor, 1978). Isolates CSIRO 20, 23 and 36 of the Eubenangee serological group were obtained from CSIRO Division of Animal Health, Brisbane as pools in suckling mouse brain and were plaque-cloned three times in pig kidney cells.

**Extraction and purification of double-stranded (ds)RNA.** Virus-infected cultures were extracted with 3% diethyl pyrocarbonate and 1% SDS in 0.05 M-HEPES pH 7.55 (Summers, 1970; Gorman et al., 1978). The extract was adjusted to 2 M in lithium chloride and allowed to stand at 4 °C for 18 h to precipitate single-stranded (ss)RNA (Avital & Elson, 1969). The supernate was applied to a cellulose (Whatman CF11) chromatography column in 0.1 M-NaCl, 0.001 M-EDTA, 0.01 M-tris-HCl pH 6.9 (TSE) + 15% ethanol. RNA which eluted in TSE + 15% ethanol was discarded and dsRNA then eluted with TSE (Franklin, 1966).

**Extraction and purification of single-stranded RNA.** Cell cultures were infected with between 1 and 10 p.f.u./cell and from 16 h after infection the medium was replaced with Eagle's minimum essential medium (MEM) containing 50 μg actinomycin D per 10⁸ cells. After 2 h the medium was changed to Eagle's MEM containing 500 μCi [³H]uridine and 50 μg actinomycin D per 10⁸ cells. After a further 2 h the medium was removed, the cells lysed in 20 ml 0-05 M-sodium acetate buffer pH 5 containing 1% SDS and 20 mg heparin and extracted with an equal volume of water-saturated phenol at 60 °C for 5 min. The mixture was chilled in an ice bath, 0-5 vol. chloroform added and the phases separated by centrifugation at 2000 g for 15 min. The aqueous phase was extracted once more with phenol and chloroform, twice with ether and the residual ether removed by bubbling the aqueous phase with nitrogen. The aqueous phase was adjusted to 2 M in lithium chloride and after 18 h at 4 °C the ssRNA collected by centrifugation at 10000 g for 30 min at -10 °C. The pellet was dissolved in distilled water and the ssRNA again precipitated with 2 M-lithium chloride.
The second precipitate was dissolved in TSE and applied to a cellulose column (Whatman CF11) in TSE + 35% ethanol. The fraction eluting in TSE + 35% ethanol was discarded and the ssRNA was eluted with TSE + 15% ethanol (Franklin, 1966).

Electrophoresis of RNA. Double-stranded RNA was electrophoresed in slab gels of 7.5% polyacrylamide (Gorman et al., 1977). The gels were immersed in 7.5% acetic acid for 15 min, the RNA stained for 1 h in 0.4% methylene blue in 0.2 M-sodium acetate pH 4.7 and destained in distilled water. The location of 3H in the gels was determined by exposure of pre-flashed Fuji RX X-ray film to gels after impregnation with the scintillator PPO (Laskey & Mills, 1975) or with sodium salicylate (Chamberlain, 1979).

Oligonucleotide fingerprinting of dsRNA. Methods for the preparation of individual genome segments of bluetongue virus type 20, digestion with RNase T1, and two-dimensional electrophoretic analysis of the oligonucleotides produced have been described by Walker et al. (1980).

RNA–RNA reassocation assay. Single-stranded RNA radiolabelled with [3H]uridine was mixed with increasing amounts of unlabelled dsRNA in a total volume of 40 μl 0.01 M-tris–HCl pH 7.8, 0.001 M-EDTA, 0.01 M-NaCl and heated in a boiling water bath for 3 min. After addition of 10 μl 1.5 M-NaCl the mixture was incubated at 72 °C for 30 min. To 25 μl were added 1% SDS and 5% sucrose and the solution applied to a slab gel of 7.5% polyacrylamide for electrophoresis. The remaining 25 μl was diluted to 1 ml in ice-cold 0.01 M-tris–HCl pH 7.8, 0.001 M-EDTA, 0.3 M-NaCl and divided into four equal amounts. Two samples were each treated with 2 μg ribonuclease A at 37 °C for 15 min (Worthington Biochemical Corp.) and two remained untreated. Yeast RNA previously treated with diethyl pyrocarbonate was added to 100 μg as carrier and the acridine-precipitable material determined after addition of trichloroacetic acid to 10%. For treatment with nuclease S1 (Aspergillus oryzae; P.-L. Biochemicals) the samples were diluted in 1 ml ice-cold 0.02 M-sodium acetate pH 4.5, 0.001 M-ZnCl2, 0.3 M-NaCl and 5% glycerol (Dodgson & Wells, 1977) and incubated at 37 °C for 2 h with 1 unit of enzyme per ml mixture. The reaction was stopped by addition of EDTA to 0.01 M, 100 μg yeast RNA and trichloroacetic acid to 10% for determination of precipitable material.

Virus-induced protein synthesis in infected cells. Cultures of 5 × 10⁶ cells were infected with from 1 to 10 p.f.u./cell. After 12 h the medium was replaced with Eagle's MEM without methionine. After a further 30 min the medium was changed to Eagle's MEM without normal amounts of methionine but containing 100 μCi [35S]methionine for 10 min, the medium then removed and the cells lysed by addition of 1 ml 0.0625 M-tris–HCl pH 6–8 containing 2% SDS, 2% β-mercaptoethanol, 0.5 M-urea and 10% glycerol (Laemmli, 1970). For pulse-chase experiments, after removal of the medium containing [35S]methionine the cultures were incubated for further periods in Eagle's MEM with added unlabelled methionine, before lysis of cells.

Electrophoresis of protein. Virus-induced proteins were analysed by electrophoresis in polyacrylamide gels in the discontinuous system described by Laemmli (1970).

RESULTS

Analysis of the dsRNA and polypeptides induced in cells infected with bluetongue virus type 20

Double-stranded RNA isolated from cells (BHK21 or PS-EK) infected with bluetongue virus type 20 was separated into 10 segments by electrophoresis in 7.5% polyacrylamide gels (Fig. 4). The pattern of separation into four molecular size classes is characteristic of bluetongue virus RNA (Verwoerd et al., 1970) and of the RNA of most orbiviruses (Gorman, 1979). Individual genome segments were eluted from preparative polyacrylamide
Fig. 1. Ribonuclease T₁ fingerprints of individual double-stranded RNA genome segments of bluetongue type 20. Electrophoresis in the first dimension was from right to left and in the second dimension from bottom to top. (a) Segment 1; (b) segment 4; (c) segment 7; (d) segment 8; (e) segment 9; (f) segment 10.

gels denatured to ssRNA and treated with RNase T₁. The cleavage products were separated in two dimensions by polyacrylamide gel electrophoresis to generate oligonucleotide fingerprints. Fingerprints of segments 1, 4, 7, 8, 9 and 10 are shown in Fig. 1. Segments 2 and 3 were difficult to separate (Fig. 4). The fingerprint for segment 3 is shown in Fig. 2 (a) and a composite fingerprint of segments 2 and 3 in Fig. 2 (b). Similarly, segments 5 and 6 were difficult to resolve and the fingerprint for segment 5 is shown in Fig. 2 (c) and a composite in Fig. 2 (d). The pattern of large oligonucleotides in the fingerprint of each genome segment was unique, indicating that each segment contained different sequence information. No high mol. wt. polyadenylic acid or polypyrimidine tracts were detected.

Bluetongue virus type 20 depressed host cell protein synthesis so that from 12 h after infection [35S]methionine was incorporated mainly into 10 polypeptides which were separated by electrophoresis in slab gels of 8% polyacrylamide (Fig. 3). Little radioisotope was incorporated into cellular protein and the virus-induced polypeptides are labelled in order of decreasing mol. wt. but each is not necessarily the gene product of the correspondingly numbered RNA segment. Pulse–chase experiments showed that no secondary proteolytic cleavage of the virus-induced polypeptides occurred (Fig. 3).

Comparison of electrophoretic migration patterns of the RNA of bluetongue type 20 and other viruses of the bluetongue and Eubenangee serogroups

RNA extracted from cells infected with bluetongue serotypes 1, 4, 10, 15, 17 and 20 were electrophoresed in 7.5% polyacrylamide gels (Fig. 4). The electrophoretic separation of the
Comparison of BTV 20 and other orbiviruses

Fig. 2. Ribonuclease T1 fingerprints of segments of bluetongue type 20. (a) Segment 3; (b) mixture of segment 2 and segment 3; (c) segment 5; (d) mixture of segment 5 and segment 6. Arrows on (b) and (d) indicate those oligonucleotides derived from segment 2 and from segment 6 respectively.

10 genome segments was distinct for each bluetongue virus serotype. The electrophoretic patterns of separation could not be used to infer relationships between viruses. Bluetongue virus type 20 is more closely related by serum neutralization tests to types 4 and 17 than to any other serotype (Verwoerd et al., 1979; Della-Porta et al., 1981). By co-electrophoresis of RNA of bluetongue type 20 with each of the other serotypes (Fig. 5) only 3 and 4 segments of type 20 have the same electrophoretic mobilities as segments of types 4 and 17 respectively while 6 segments of types 10 and 20 have the same electrophoretic mobilities.

The electrophoretic separation of RNA genome segments of bluetongue virus type 20 and five viruses of the Eubenangee serological group are shown in Fig. 6. Each virus produced a distinct pattern of separation and despite the distant serological relationship between the bluetongue and Eubenangee group viruses it was not possible to identify an Eubenangee group RNA pattern distinct from a bluetongue virus RNA pattern. The heterogeneity in genome segment sizes of Eubenangee and Tilligerry viruses, which are recognized as
Fig. 3. PAGE of proteins induced by bluetongue virus type 20 BHK cells. (a) Virus-infected cells treated with methionine-free Eagle's MEM + [35S]methionine for 10 min at 12 h post-infection. (b to d) As for (a) with removal of [35S]methionine and incubation in Eagle's MEM + excess unlabelled methionine for (b) 15 min, (c) 40 min and (d) 75 min. (e) Uninfected cells labelled for 10 min with [35S]methionine. Electrophoresis in an 8% polyacrylamide slab gel was by the method of Laemmli (1970).

Fig. 4. Separation of dsRNA genome segments of serotypes 10, 15, 4, 17, 1, and 20 of bluetongue virus. Electrophoresis was performed in a downward direction in a slab gel of 7.5% polyacrylamide and the RNA stained with methylene blue (Gorman et al., 1977).

Fig. 5. Co-electrophoresis of dsRNA of bluetongue type 20 with dsRNA of types 10, 15, 4, 17 and 1. Electrophoresis was performed in a slab gel of 7.5% polyacrylamide and the RNA stained with methylene blue.
Comparison of BTV 20 and other orbiviruses

Comparison of BTV 20 and other orbiviruses

Comparison of the genome segments of bluetongue virus type 20 and viruses of the bluetongue and Eubenangee serogroups by RNA–RNA reassociation assay

Radiolabelled ssRNA isolated from cultures infected with bluetongue virus type 20 was mixed with increasing amounts of unlabelled homologous dsRNA, heated in a boiling water bath and allowed to reanneal at 72 °C in the presence of 0.3 M-sodium chloride. The reassociation of radiolabelled single strands with complementary strands of the dsRNA genome was detected by electrophoresis of the mixtures in 7.5% slab gels. Single-stranded RNA which did not reassociate with a complementary strand of dsRNA was excluded from the gel (Fig. 7). Ten double-stranded radiolabelled molecules were observed in each of the mixtures of ssRNA with from 1 to 12 µg of unlabelled dsRNA (Fig. 7). The pattern of separation coincided with that of the dsRNA segments detected by staining the gel with methylene blue (not shown), indicating that the electrophoretic mobility of each reassociated double strand was equivalent to each of the corresponding original double strands.

Radiolabelled ssRNA isolated from cultures infected with bluetongue virus type 20 was tested for reassociation with 5 µg unlabelled dsRNA isolated from certain viruses of the Eubenangee serological group. Reassociation of ssRNA with a complementary strand of the virus RNAs of the Eubenangee serogroup was not detected by electrophoresis of the mixtures...
B. M. GORMAN AND OTHERS

Fig. 8. Electrophoresis in a slab gel of 7.5% polyacrylamide of reassociation products after melting 5 μg dsRNA of bluetongue virus serotypes 1, 4, 10, 17, 20L (large plaque clone), 20S (small plaque clone), 20 (uncloned) and CS 23 (Eubenangee serogroup) and reannealing in the presence of radiolabelled ssRNA isolated from cells infected with bluetongue virus type 20. The mixtures were treated with ribonuclease A immediately before electrophoresis. Reassociated strands were detected by fluorography (Laskey & Mills, 1975).

Table 1. Reassociation of ssRNA labelled with [3H]uridine of bluetongue virus type 20 with certain other bluetongue virus serotypes and a virus of the Eubenangee serological group

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>Ct/min reassociated</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bluetongue type 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>2432*</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>180</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>3</td>
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<td>1</td>
</tr>
<tr>
<td>1</td>
<td>352</td>
<td>14</td>
</tr>
<tr>
<td>Eubenangee (CS 23)</td>
<td>192</td>
<td>8</td>
</tr>
</tbody>
</table>

*ssRNA isolated from cells infected with bluetongue type 20 labelled with [3H]uridine (2934 ct/min) heated with 5 μg unlabelled dsRNA and, after reannealing, treated with pancreatic ribonuclease. The values of the ribonuclease-resistant material in three samples were averaged, giving a value of 83% for homologous annealing. The reactions for heterologous dsRNA were then expressed as a percentage of the homologous value taken as 100%.

in 7.5% polyacrylamide gel slabs. To detect reassociation within genome segments resulting in partial hybrids which might be excluded from 7.5% gels, the mixtures were treated with nuclease S1 and nuclease-resistant materials precipitated with trichloroacetic acid. No significant reassociation of radiolabelled ssRNA from bluetongue type 20 with complementary sequences in the genomes of the Eubenangee viruses was detected. In similar experiments using dsRNA from bluetongue serotypes 1, 4, 10, 17 and 20 reassociation occurred between radiolabelled ssRNA from bluetongue type 20 and unlabelled dsRNA isolated from plaque-cloned and uncloned stocks of bluetongue type 20 but not with dsRNA from the other bluetongue virus serotypes (Fig. 8). A small fraction of radioactivity in the heterologous mixtures was ribonuclease-resistant (Table 1) but the significance of this fraction was not determined.
DISCUSSION

The genome of bluetongue virus type 20 consists of 10 segments of dsRNA each of which by oligonucleotide mapping contains unique sequences. Ten polypeptides have been detected in virus-infected cells and in contrast to reoviruses (Joklik, 1974) there appears to be no secondary cleavage of the primary gene products (Fig. 3).

The isolation of the virus from biting midges, in the Northern Territory of Australia (St George et al., 1976), led to considerable disruption in animal movement and financial loss due to trade embargoes despite lack of evidence of bluetongue disease in Australia. Several orbiviruses had been isolated previously in Australia (for review, see Gorman, 1979) and although none was directly related to bluetongue virus, there were inconclusive data suggesting a distant relationship between viruses of the Eubenangee and bluetongue serogroups (Borden et al., 1971; Gorman & Taylor, 1978). Furthermore, Della-Porta et al. (1979) reported that antiserum prepared against the isolate CSIRO 23 of the Eubenangee serogroup neutralized bluetongue type 1 in plaque-inhibition tests although CSIRO 23 was not neutralized by antiserum prepared against bluetongue type 1.

Clearly, serological methods alone are inadequate to characterize these viruses and we have attempted to establish the relationships between them by comparing their RNA genomes. It has been suggested by others (Payne et al., 1977; Rodger & Holmes, 1979) that cytoplasmic polyhedrosis viruses and rotaviruses could be classified by the electrophoretic separation patterns of their dsRNA genomes. Our previous work had shown extensive heterogeneity in electrophoretic patterns of RNA of serotypes of other orbivirus groups (Gorman et al., 1977, 1978; Gorman & Taylor, 1978) and recently we have shown by analysis of oligonucleotide fingerprints of each of the RNA genome segments of serotypes of the Wallal group of orbiviruses, that substantial sequence heterology is not restricted to segments with different electrophoretic mobilities (Walker et al., 1980). Segments with identical mobility produced dissimilar fingerprint patterns and classification according to electrophoretic analysis of whole genome segments can be misleading. The heterogeneity in the patterns of separation of RNA of bluetongue viruses allows no conclusion to be made on possible relationships between the viruses. Indeed, it was not possible to distinguish viruses of the Eubenangee serogroup from a bluetongue virus by electrophoresis of genome RNA (Fig. 4 and 6).

We have not compared oligonucleotide maps from equivalent segments of bluetongue type 20 and the other viruses. We have previously shown (Walker et al., 1980) that equivalent segments of serotypes of the Wallal group of orbiviruses produce distinct oligonucleotide maps and in general it has been observed that viruses which can be distinguished by serological techniques share few if any large ribonuclease T1-derived oligonucleotides (El Said et al., 1979; Vezza et al., 1980). Furthermore, the lack of sequence homology detected by the RNA–RNA reassociation technique between bluetongue type 20 and the other viruses suggested that no useful comparisons could be made by oligonucleotide mapping techniques.

Despite the extensive sequence homology between bluetongue viruses isolated in South Africa reported by Huismans & Howell (1973) we have failed to show significant reassociation of mRNA(+) derived from bluetongue virus type 20 with strands of opposite (−) polarity of bluetongue virus types 1, 4 and 10 isolated in South Africa and type 17 from the U.S.A. (Table 1) but since the smallest genome segments each represent less than 5% of the total nucleic acid, we analysed the mixtures by electrophoresis in 7.5% polyacrylamide gels. Single-stranded RNA does not enter these gels and no double-stranded duplexes could be detected in the mixtures. It is possible that short regions of homology could have escaped detection by the methods used here but the results indicate a considerable sequence divergence between the virus isolated in Australia and bluetongue virus serotypes isolated in other
geographic regions. Our experiments also showed no significant homology between viruses of the Eubenangee serogroup isolated in Australia and bluetongue type 20.

Failure to detect the common nucleotide sequences which are the basis of the group-specific serological test between the bluetongue viruses raises the possibility that the conditions used in the reassociation assay were inadequate to detect homologous regions. In a recent study, Gaillard & Joklik (1980) found that the antigenic determinants on most proteins encoded by three reovirus serotypes were similar as judged by precipitation of proteins from virus-infected cell extracts with antisera from each serotype. This was true even for the proteins of serotype 2 the genome of which is related to those of serotypes 1 and 3 by no more than 10% as judged by RNA–RNA reassociation. According to Howley et al. (1979) the evaluation of DNA homology under stringent hybridization conditions (Tm – 25 °C) can be misleading and fail to detect clearly related regions of DNA. They found that only DNA molecules with 85% base matching were detected using standard conditions. Rentier-Delrue & Young (1980) found that genome homologies of Sindbis virus isolates were maximized by the use of non-stringent reassociation conditions. We are currently investigating the use of non-stringent reassociation conditions to detect homologous sequences in related orbiviruses.

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Comparison of BTV 20 and other orbiviruses


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