Neutralizing epitopes of the serotypes of bluetongue virus present in the United States

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Neutralizing epitopes present on the five serotypes of bluetongue virus (BTV) which have been isolated in the United States were investigated with a panel of monoclonal antibodies (MAbs). Neutralizing MAbs were raised against the U.S. prototype viruses of BTV serotypes 2, 10, 11, 13 and 17, and were reacted with each virus in both neutralization and immune precipitation assays. All MAbs neutralized and precipitated VP2 of the virus against which they were raised. Five MAbs raised against BTV-10 also precipitated VP2 of the prototype strain of BTV-17, and four of these MAbs neutralized BTV-17. To characterize further the neutralizing epitopes of BTV, the MAbs raised against BTV-10 and BTV-17 were reacted by immune precipitation and neutralization assays with four field strains each of BTV-17 and BTV-10 isolated from ruminants in the U.S. All MAbs raised against BTV-10 both precipitated VP2 and neutralized the four field isolates of BTV-10, whereas none of the MAbs raised against BTV-17 reacted with these viruses. By contrast, all seven MAbs raised against BTV-17 and four of the seven MAbs raised against BTV-10 precipitated VP2 of the four BTV-17 field isolates. Another MAb raised against BTV-10 precipitated VP2 of three of the four field isolates of BTV-17. Whereas neutralization of the BTV-17 field isolates by several MAbs was inconsistent, all 10 isolates of BTV-10 and BTV-17 were neutralized by three MAbs raised against BTV-10. Results of this and other studies indicate that multiple neutralizing epitopes exist on each serotype of BTV. Some of these epitopes are conserved whereas others apparently vary in their significance to the neutralization of individual field isolates of BTV-17 and perhaps other BTV serotypes. These findings have implications for the future development of efficacious subunit vaccines to prevent BTV infection of ruminants.

Introduction

Bluetongue virus (BTV) is a member of the family Reoviridae and prototype virus of the genus Orbivirus (Murphy et al., 1971). The BTV particle consists of an icosahedral core composed of viral proteins VP1, VP3, VP4, VP6 and VP7, a diffuse outer protein capsid of VP2 and VP5, and a genome of 10 segments of dsRNA (Huismans & Van Dijk, 1990). Some 24 distinct serotypes of BTV have been identified (Huismans & Cloete, 1987), four of which (serotypes BTV-10, BTV-11, BTV-13 and BTV-17) are endemic in the United States (Barber, 1979). An additional serotype, BTV-2, was recently isolated in Florida (Gibbs et al., 1983). Epitopes responsible for the neutralization of BTV have been identified on only the major outer capsid protein VP2 (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983; Letchworth & Appleton, 1983a; Mecham et al., 1986; Huismans et al., 1987; Roy et al., 1990) although the other outer capsid protein, VP5, might be indirectly involved in virus neutralization by its conformational influence on VP2 (Cowley & Gorman, 1989; Mertens et al., 1989; Roy et al., 1990). Multiple neutralizing epitopes exist on BTV (Letchworth & Appleton, 1983b; Gould & Eaton, 1990; Greider & Schultz, 1990; White & Eaton, 1990), and at least some of these epitopes are intimately associated on VP2 and contribute to a single, larger immunogenic domain (Heidner et al., 1990).

Epitopes responsible for virus neutralization are apparently not unique to each serotype as neutralization tests with polyclonal antisera frequently do not clearly distinguish all BTV serotypes (Della-Porta et al., 1981; Huismans & Bremmer, 1981, Huismans & Erasmus, 1981; Campbell, 1985; Thomas, 1985). Furthermore, recent studies with neutralizing monoclonal antibodies (MAbs) raised in mice against BTV-13 identified a neutralizing epitope also common to BTV-2 (Ristow et al., 1988). Neutralizing MAbs produced against Australian serotype BTV-1 bound but did not neutralize a variety of heterologous serotypes, suggesting that epitopes responsible for the definition of one BTV serotype may be preserved on other serotypes but not be involved in their neutralization (White & Eaton, 1990).
The goal of this study was to characterize further the neutralizing epitopes present on the five serotypes of BTV present in the U.S. Acquisition of such information is a prerequisite to the development of efficacious vaccines which might be used to prevent BTV infection of ruminants. A panel of neutralizing MAbs was generated to each of the prototype strains of the U.S. serotypes of BTV, and the MAbs were then characterized by neutralization and immune precipitation assays using the five U.S. prototype strains of BTV. Selected MAbs were further characterized with four field isolates each of BTV-17 and BTV-10.

Methods

Viruses. Prototype U.S. strains of BTV-2, -11, -13 and -17 were obtained from Dr J. L. Stott (University of California, Davis, Ca., U.S.A.) and twice plaque-picked from agar-overlaid Vero cells. The passage history of the prototype strain of BTV-10 used has been previously described (MacLachlan et al., 1987). Four field isolates of BTV-17, designated 17-B80Z, 17-B81U, 17-C81X and 17-O79Y, and four field isolates of BTV-10, designated 10-B81U, 10-B81I, 10-O80Z and 10-O80R, were twice plaque-picked from Vero cells prior to use in this study. The field isolates of BTV-17 were isolated from cattle (17-B80Z, 17-B81U), sheep (17-O79Y) and a goat (17-C81X) in California, whereas the isolates of BTV-10 were isolated from cattle in California (10-B80Z) and Louisiana (10-B81U), and sheep in California (10-O80Z) and Idaho (10-O80Z) in the years 1979 to 1981 (Osburn et al., 1981; Heidner et al., 1991). Virus stocks were prepared by passage in baby hamster kidney (BHK-21) cells and stored at -70°C. The serotype of each prototype virus was confirmed at the National Animal Disease Center, Ames, Iowa, U.S.A.

Neutralizing MAbs. Neutralizing MAbs were produced to BTV-2, -10, -11, -13 and -17. The production and characterization of the MAbs to BTV-10 have been described previously (Heidner et al., 1988). The MAbs to BTV-2, -11, -13 and -17 were generated using the mouse myeloma P3X Ag8.653 cell line, essentially as described previously (Heidner et al., 1988). All MAbs included in the panel were cloned at least twice prior to characterization. Purified IgG stocks of each hybridoma were prepared from exhaustion supernatants by precipitation with 50% saturated ammonium sulphate and purification on either Protein A-agarose for IgG2 isotypes or bulk ion-exchange chromatography on ABX 40 μm ion-exchange resin (J. T. Baker) for IgG1 isotype MAbs. All MAbs were characterized by both immune precipitation and virus neutralization assays.

Neutralization assays. The neutralizing activity of MAbs against each BTV serotype was determined by a microneutralization assay using BHK-21 cells (Heidner et al., 1988), and the MAbs raised against BTV-10 and BTV-17 were also evaluated by plaque-reduction assay using Vero cells and a methyl cellulose overlay (MacLachlan & Thompson, 1985). Titres in the plaque-reduction assay are reported as the reciprocal of the final twofold dilution of MAb, from an initial concentration of 0.1 mg/ml, that caused a greater than 75% reduction in the number of plaques compared to the number in control wells. Rabbit antisera to each of the five BTV serotypes were prepared essentially as described previously (Heidner et al., 1988), and their neutralizing titres were determined by plaque-reduction assay. Titres of each antiserum are reported as the reciprocal of the final serum dilution that produced a greater than 75% reduction in plaque numbers.

Results

All rabbit antisera neutralized the virus against which they were raised. The antisera to BTV-11 also weakly neutralized BTV-10, BTV-13 and BTV-17, and the antisera to BTV-13 weakly neutralized BTV-2 and BTV-11. The other rabbit antisera exhibited no cross-reactivity. The weak cross-neutralization of heterologous serotypes by the polyclonal antisera to BTV-11 and BTV-13 is consistent with previous reports that polyclonal antisera often do not clearly distinguish all BTV serotypes (Della-Porta et al., 1981; Campbell, 1985; Thomas, 1985). Although polyclonal antisera exhibited no cross-reactivity between BTV-10 and BTV-17, several MAbs raised against BTV-10 strongly neutralized both viruses. Similarly, BTV-2 and BTV-13 share a common neutralizing epitope that was not detected previously by neutralizing polyclonal antisera to these serotypes (Ristow et al., 1988), whereas we demonstrated a weak cross-neutralization of BTV-2 by rabbit antiserum to BTV-13. The source and titre of antisera raised against individual BTV serotypes are possibly important in determining whether or not cross-neutralization occurs.

A panel of neutralizing MAbs was raised against each of the five U.S. serotypes of BTV, and included six MAbs to BTV-2, seven to BTV-10, five to BTV-11, two to BTV-13 and seven to BTV-17. All MAbs neutralized the virus against which they were raised and immune-precipitated soluble VP2 of that virus. The MAbs to BTV-2, -11, -13 and -17 did not cross-react with other serotypes in either the microneutralization or immune precipitation assays. In contrast, five of seven MAbs raised against BTV-10 cross-reacted with BTV-17 by either neutralization or immune precipitation assay (Table 1). Three MAbs raised against BTV-10 (MAbs 020, 024 and 034) strongly precipitated VP2 and neutralized BTV-17. By contrast, MAbs 016 and 039 precipitated VP2 of BTV-17 only weakly. MAb 016 also weakly neutralized BTV-17, whereas MAb 039 did not neutralize BTV-17. All four MAbs that neutralized both BTV-10 and BTV-17 neutralized BTV-10 to higher titre (titres 128 to > 512) than they did BTV-17 (titres 2 to 64). These data clearly demonstrate that neutralizing epitopes common to BTV-10 and BTV-17 exist, but that these epitopes vary in their significance to the neutralization of U.S. prototype strains of BTV-10 and BTV-17.
To evaluate further the neutralizing epitopes of BTV-17, four field isolates each of BTV-17 and of BTV-10 were compared to the prototype strains of BTV-10 and BTV-17. Viruses were compared by plaque-reduction and immune precipitation assays, using each of the MAbs raised against BTV-10 and BTV-17 (Table 1; Fig. 1). The field isolates of BTV-10 were neutralized by all MAbs raised to BTV-10, with neutralizing titres comparable to those of the prototype (titres ranged from 64 to >512), whereas these viruses were not neutralized by any MAb raised against BTV-17 (data not shown). Similarly, all seven MAbs raised against BTV-17 precipitated VP2 of each of the four field isolates of BTV-17, whereas none of the MAbs raised against BTV-17 precipitated VP2 of these viruses (data not shown). In contrast, the seven MAbs raised against BTV-17 and five MAbs (016, 020, 024, 034 and 039) raised against BTV-10 precipitated VP2 of at least three of the four field isolates of BTV-17 (Table 1). The MAbs 17.82, 17.83, 17.85, 17.87, 17.813, 17.815, 17.816, 17.818, 17.819, 020, 024 and 034 strongly precipitated VP2 of all field strains of BTV-17, whereas MAbs 17.87, 016 and 039 only weakly precipitated VP2 of one or more of the field strains. MAb 039 failed to precipitate VP2 of virus 17-C81X. The MAbs that only weakly precipitated VP2 of individual viruses invariably either failed to neutralize those viruses or only weakly neutralized them. MAbs that strongly precipitated VP2 from individual viruses, however, also sometimes failed to neutralize those viruses. MAb 17.816, for instance, precipitated VP2 from all four field isolates of BTV-17, yet it failed to neutralize three of them (illustrated with field isolate 17-B80Z in Fig. 1). Similarly, MAb 17.813 precipitated VP2 from all four field isolates of BTV-17 but neutralized only two of them. Neutralization titres obtained with different field isolates and individual MAbs varied considerably, although the MAbs raised against BTV-17 rarely neutralized the field isolates as efficiently as they did the parental strain of BTV-17 (Table 1). These data indicate that epitopes responsible for the neutralization of one field isolate of BTV-17 may be preserved on VP2 of another isolate but not be responsible for neutralization of the latter virus.

The MAbs raised against BTV-17 recognize at least four distinct neutralizing epitopes, as determined by their patterns of neutralization of a large number of field isolates of BTV-17 (MacLachlan et al., 1992). The seven BTV-10-specific MAbs also recognize at least four distinct epitopes on the prototype strain of BTV-10, as determined by competitive immune precipitation assay. MAbs 016, 020, 024 and 034 were not distinguished by competitive binding assay, whereas MAbs 039, 041 and 045 define another three distinct epitopes (Heidner et al., 1990). Three of these MAbs to BTV-10 (020, 024 and 034) consistently neutralized and precipitated VP2 of the five BTV-17 viruses evaluated in this study, whereas MAb 016 weakly precipitated and either failed to neutralize

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### Table 1. Neutralization and immune precipitation of U.S. prototype BTV-10 and BTV-17 and field isolates of BTV-17

<table>
<thead>
<tr>
<th>Virus</th>
<th>BTV-10*</th>
<th>BTV-17*</th>
<th>17-B80Z†</th>
<th>17-B81U†</th>
<th>17-C81X†</th>
<th>17-O79Y†</th>
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<td>MAb</td>
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<td>+</td>
<td>16</td>
<td>+</td>
</tr>
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<td>17.83</td>
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<td>-</td>
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<td>8</td>
<td>+</td>
</tr>
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<td>-</td>
<td>&gt;512</td>
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<td>128</td>
<td>+</td>
<td>16</td>
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<td>17.815</td>
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<td>-</td>
<td>64</td>
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<td>4</td>
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<td>-</td>
<td>64</td>
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* Indicates U.S. prototype strain.
† Indicates field isolate of BTV serotype 17.
‡ PN indicates antibody titre as determined by plaque reduction assay.
§ PPT indicates immune precipitation of VP2 by each MAb where + is precipitation, +w is very weak precipitation and - is no precipitation of VP2.

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or only weakly neutralized all BTV-17 viruses; only 17-O79Y virus is both neutralized and strongly precipitated by MAb 016 (Table 1). These data indicate that the epitope on BTV-17 recognized by MAb 016 either is distinct from that recognized by MAbs 020, 024 and 034, or the binding affinity of MAb 016 to BTV-17 is usually less than that of the other three MAbs.

**Discussion**

Despite numerous studies which demonstrate the VP2 gene of BTV to be serotype-specific and the most variable gene between serotypes (Fukusho *et al.*, 1987; Huismans & Cloete, 1987; Mertens *et al.*, 1987; Gould, 1988; Ritter & Roy, 1988; Yamaguchi *et al.*, 1988; Heidner *et al.*, 1991), this and earlier studies confirm that epitopes may be conserved between serotypes in a neutralizing or non-neutralizing conformation (Ristow *et al.*, 1988; White & Eaton, 1990). The results also indicate that field isolates of BTV-17 vary considerably in their patterns of neutralization by individual neutralizing MAbs (Letchworth & Appleton, 1983b; Greider & Schultz, 1990; MacLachlan *et al.*, 1992). Expression of epitopes in a conformation that results in virus neutralization could be influenced by a variety of complex interactions between individual BTV proteins, including that of VP2 with itself, as well as the interaction of VP2 with outer capsid protein VP5 and core proteins such as VP7. VP5 has been proposed previously to affect neutralization by its conformational influence on VP2 (Cowley & Gorman, 1989; Mertens *et al.*, 1989), and it has been shown recently that VP7 is associated with the outer capsid of BTV and may be expressed in small amounts on the surface of the virus (Hyatt & Eaton, 1988; Lewis & Grubman, 1990). Sequencing of the VP2 gene, and perhaps other genes, from viruses used in this study will be necessary to characterize fully the obvious differences amongst the neutralizing epitopes of field isolates of BTV-17.

The presence of neutralizing epitopes common to BTV-10 and BTV-17 is consistent with the considerable nucleic acid homology of the gene encoding VP2 of these viruses (Yamaguchi *et al.*, 1988). MAbs 020, 024 and 034 recognize a single epitope which apparently is conserved in a neutralizing conformation on BTV-10 and BTV-17. The highly conserved nature of the neutralizing epitope recognized by MAbs 020, 024 and 034 on BTV-10 and
BTV-17 has been confirmed recently in an extensive study of the neutralizing epitopes of field isolates of U.S. serotypes of BTV (MacLachlan et al., 1992).

The data indicate that most neutralizing epitopes recognized by the MAb panel on the five U.S. serotypes of BTV are serotype-restricted, but that one-way cross-neutralizations occur with BTV-17 and some MAbs raised to BTV-10. Furthermore, it was clearly demonstrated that MAbs may precipitate VP2 of a given field isolate of BTV-17 but not neutralize that virus, which is consistent with the hypothesis that the neutralizing domain of each BTV serotype contains many distinct epitopes which vary in their significance to the neutralization of individual viruses. This considerable plasticity of neutralizing epitopes amongst field isolates of BTV-17 must be adequately characterized and considered in the context of production of efficacious subunit vaccines.

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