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Analysis of Mixed Infection of Sheep with Bluetongue Virus Serotypes 10 and 17: Evidence for Genetic Reassortment in the Vertebrate Host

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Two seronegative sheep were infected intravenously with 10^9 PFU each of bluetongue virus (BTV) serotype 10 and BTV serotype 17. One animal experienced a mild bluetongue-like disease, and both experienced a short-duration viremia and developed neutralizing immune responses to both virus serotypes. Progeny virus was isolated from venous blood from each animal by using conditions in which reassortment could not have occurred during isolation. Electropherotypes were determined for the progeny viruses from the infected sheep, yielding strikingly similar results for the two animals. In both sheep, serotype 10 dominated among the progeny, accounting for 92% of the progeny. Serotype 17 was rarely isolated and accounted for 3% of the progeny analyzed. The remaining 5% of the progeny clones were reassortant and derived genome segments from both serotypes 10 and 17. Analysis of the parental origin of genome segments in the small number of reassortant progeny analyzed suggested that selection of specific genome segments may have occurred in the infected sheep. These data indicate that reassortment of genome segments occurs, at low frequency, in sheep mixedly infected with BTV.

Bluetongue virus (BTV) is an orbivirus, a member of the family *Reoviridae*, with 23 serotypes recognized worldwide (17, 20). Serotypes 2, 10, 11, 13, and 17 have been recovered from animals in the United States (1, 7). BTV possesses a genome that is composed of 10 segments of double-stranded (ds) RNA (32). The dsRNA segments can be separated by polyacrylamide gel electrophoresis into distinct electrophoretic patterns or electropherotypes. Electrophoretic polymorphisms have been reported for different serotypes (10, 13, 16) and for different strains within a single serotype (16, 28). These polymorphisms have provided useful biochemical markers for the analysis of genetic reassortment in in vitro crosses of BTV (11, 15; S. K. Samal, Ph.D. thesis, Texas A&M University, College Station, 1985).

BTV is an arthropod-borne virus, with biting midges of the genus *Culicoides* serving as the primary vector in the United States (5). The vertebrate hosts include sheep, goats, cattle, and wild ruminants. In sheep and wild ruminants the disease is acute and morbidity is often high, whereas in cattle and goats the disease is usually a clinically inapparent infection. Cattle experience a prolonged viremia which is thought to provide a reservoir for the dissemination of BTV. In contrast, the viremia in sheep is of short duration (4).

Most BTV epizootics are associated with a single serotype. Epizootiological studies in the western United States, however, indicate that up to one-third of all BTV-infected herds had more than one serotype on the premises (22). Moreover, multiple serotypes have been recovered from individual infected sheep and cattle (21, 29). The potential for intertypic genetic interactions in mixedly infected hosts is unknown, but results from in vitro mixed infections suggest that reassortment of genome segments should be expected to occur at high frequency (15; Samal, Ph.D. thesis). The notion that reassortment among BTV serotypes occurs in nature is supported by evidence for naturally occurring reassortment between BTV serotypes 10 and 11 (30) and within serotype 11 (31). The host for these naturally occurring reassortment events remains undefined. The potential for reassortment among BTV serotypes has prevented the use of polyvalent vaccines in the United States, although such vaccines have been used in South Africa and Israel (2, 12).

High-frequency in vivo reassortment has recently been documented in rotavirus (9) and reovirus (33). In this report we describe the simultaneous infection of sheep with two serotypes of BTV and provide unequivocal evidence for the reassortment of BTV genome segments in the vertebrate host.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Vero; ATCC CCL-81) were used throughout this study. Cells were maintained as monolayers in medium 199 (1× 199), which was prepared as described previously (24), containing 5% fetal bovine serum. After infection cells were maintained in 1× 199 containing 2% fetal bovine serum.

Wild-type BTV serotype 10 (strain BT-8) and serotype 17 (strain 262) were obtained from the Arthropod-Borne Animal Disease Research Laboratory, Denver, Colo., as infected sheep blood. Each strain was adapted to growth in Vero cells and plaque purified three times. After two passages in Vero cells, the viruses were again plaque purified three times and passaged three times in Vero cells to produce the high-titer stocks that were used to inoculate the animals (26). Serotypes 10 (strain BT-8) and 17 (strain 262) were chosen for this study because they allowed electropherotype determination by electrophoresis for all genome segments except 1 and 3, which comigrated.

Virus plaque assays. Infectious BTV was quantitated by plaque assay. Serial 10-fold dilutions of virus were made in

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serum-free 1× 199. Confluent monolayers of Vero cells in six-well dishes (Costar, Cambridge, Mass.) were inoculated with 0.1 ml of the appropriate virus dilution and allowed to adsorb for 1 h. After adsorption, 3 ml of overlay medium was added to each well. Overlay medium consisted of equal parts of (i) 2× 199, which was supplemented with the following, in grams per liter: glutamine, 0.6; $NaHCO_3$, 4.125; penicillin, 0.16; streptomycin, 0.25; gentamicin, 0.025 and with 25,000 U of mycostatin and 4% (vol/vol) FBS; and of (ii) 2% (wt/vol) agar. The plates were incubated at 34°C in a 5% CO_2 atmosphere. On day 4 postinfection 1 ml of overlay medium containing 0.01% neutral red was added, and the plaques were counted on days 5 and 6 postinfection.

Sheep. Two yearling Rambouillet mutton sheep (ear tag no. 278 and 593), which were shown to be free of serum-neutralizing antibodies to BTV serotypes 2, 10, 11, 13, and 17 by plaque-reduction neutralization test (neutralization titer, <1:5), were used for this study. Animals were housed in screened rooms at the Texas Agricultural Experiment Station, San Angelo, Tex. Each animal was inoculated intravenously with a total of 10 ml of infected cell lysate containing 10^9 PFU each of BTV serotypes 10 and 17. The high virus dose was used to promote mixed infection of cells in the infected sheep. The animals were observed daily for clinical signs and macroscopic lesions, and rectal temperatures were recorded twice daily. Samples of venous blood were collected on odd-numbered days for the first 21 days and at weekly intervals for the next month. Whole blood samples for virus isolation were collected in two tubes, one in heparin (5 U/ml) and the other in OCG (potassium oxalate, 5 g; glycerol, 500 ml; H_2O, 500 ml; 1 part OCG to 1 part blood). Blood for serological tests was collected without anticoagulant and was allowed to clot, and the serum was separated. All samples were transported on melting ice, on the day of collection, to the Baylor College of Medicine. On receipt the OCG blood and serum samples were stored at 4 and −20°C, respectively. For virus isolation the heparinized blood samples were processed immediately on receipt by preparation of the buffy coat. The buffy coat was removed and sonicated for 15 s, and dilutions were prepared and used to inoculate plaque assays. OCG blood samples were used for virus isolation subsequent to analysis of heparinized blood samples if additional isolates were required. One milliliter of OCG blood was mixed with an equal volume of serum-free 1× 199. The mixture was centrifuged for 10 min at 1,000 × *g*, and the supernatant was discarded. The sediment was suspended in 1 ml of serum-free 1× 199, sonicated for 15 s, and used to inoculate plaque assays. Parental and reassortant viruses were isolated from both heparinized and OCG-preserved blood samples.

Isolation and characterization of progeny virus clones. Heparinized and OCG blood samples were plated on Vero cell monolayers at dilutions appropriate to yield well-isolated plaques and were incubated at 34°C. At six days postinfection (dpi) individual well-isolated plaques were picked irrespective of size and morphology. Each plaque was used to inoculate a monolayer of Vero cells in a 25-cm^2 flask, and high-titer stocks were prepared by growth at 34°C. The electropherotype of each resulting progeny virus clone was determined as follows. Confluent monolayers of Vero cells in dishes (diameter, 35 mm) were infected with 1 ml of virus stock and allowed to adsorb for 1 h at 34°C. Following adsorption, the inoculum was removed and replaced with 2 ml of serum-free 1× 199 that contained 50 μCi of $^{32}P_i$ per ml and 5 μg of actinomycin D per ml, and incubation was continued at 34°C. At 48 h after infection, infected cells were scraped into the medium and pelleted for 10 min at 1,000 × *g*. The cell pellet was lysed into 1 ml of Nonidet P-40 (NP-40) buffer (3) containing 0.5% NP-40. The cell lysates were extracted once with NP-40 buffer-saturated phenol. The aqueous phase was adjusted to 0.25 M NaCl, 3 volumes of ethanol were added, and the dsRNA was precipitated by incubation for 18 h at −20°C. The precipitated dsRNA was collected by centrifugation for 1 h at 1,000 × *g*. The dsRNA pellet was dried and dissolved in 0.05 ml of 2× sample buffer (18). The resulting ^{32}P-labeled dsRNAs were subjected to electrophoresis in 6% gels (9, 18) in slabs that were 45 cm long and 0.75 mm thick. The gels were run in a Poker-Face apparatus (Hoefer) for 24 h at 350 V. After electrophoresis, gels were dried and exposed to x-ray film. The origin of each genome segment of each progeny clone was determined by comparison with parental markers that were run in the same gel. The parental origin of genome segments 1 and 3 of serotypes 10 and 17, which comigrated under these conditions of electrophoresis, could not be determined.

Plaque-reduction neutralization tests. Serum samples from infected sheep were inactivated by incubation for 30 min at 56°C. Neutralizations were performed as described previously (27), and surviving virus was quantitated by plaque assay. An 80% reduction of 100 PFU was used to determine serum neutralization endpoint titers.

RESULTS

Clinical response of sheep to BTV mixed infection. One sheep (278) that was mixedly infected with BTV serotypes 10 and 17 showed mild clinical symptoms. Sheep 278 developed pyrexia (41.8°C) that persisted for 6 h on day 1 after infection. Pyrexia developed again at 6 dpi and persisted for 3 days, reaching 41.3°C. Other signs included erythema of the skin of the ear and muzzle areas. The other sheep (593) developed a short-duration pyrexia on the day of inoculation that appeared to be unrelated to infection. Otherwise, rectal temperatures remained within normal limits and no other clinical signs were observed.

Characterization of viremia. To characterize the viremia that developed in the sheep that were mixedly infected with BTV serotypes 10 and 17, buffy coats were prepared from heparinized blood samples immediately after arrival, and titers of the sonicates of these buffy coats were determined by plaque assay. This method of direct plaque assay appeared to give an accurate quantitation of the viremia. Although a large amount of infectious virus was inoculated (2×10^9 PFU per sheep), no virus could be detected in blood samples taken 6 h postinfection (Fig. 1), suggesting that the virus was taken up by circulating cells and converted to a noninfectious form or adsorbed to noncirculating cells. At 1 dpi significant virus was detected in sheep 278, whereas sheep 593 had no detectable viremia (Fig. 1). At 3 dpi both sheep showed significant viremia, and titers of circulating virus continued to increase through 7 dpi. The viremia peaked in both sheep at 7 dpi and fell rapidly thereafter until no virus could be detected in sheep 593 at 11 and 15 dpi in sheep 278 (Fig. 1). Viremia remained undetectable for the remainder of the sampling period, except for the isolation of one plaque from sheep 278 and two plaques from sheep 593 at 28 dpi.

Subsequent to analysis of heparinized blood samples, titers of blood samples preserved in OCG were determined. The rates of virus detection in OCG-preserved samples were low, with significant titers being observed only at 7 dpi. This result suggests that preservation of blood in OCG at 4°C for

approximately 90 days was not compatible with good virus survival.

Immune response to mixed infection. Both sheep developed serum-neutralizing antibody to both inoculated virus serotypes (Fig. 1). Circulating antibody to both serotypes 10 and 17 was first detected at 5 dpi. Titers continued to increase reaching a peak at 15 to 21 dpi. Thereafter, the antibodies persisted at those high levels until at least 43 dpi. The antibody response to serotype 10 appeared to be slightly more vigorous than that to serotype 17. This may reflect the apparent better growth of serotype 10 in these mixedly infected sheep (see below).

Characterization of virus progeny from mixedly infected sheep. Individual well-isolated plaques from assays of heparinized or OCG-preserved blood samples were picked and used to prepare high-titer progeny virus clones. The dsRNAs of these clones were labeled with ^{32}P and subjected to gel analysis to determine the electropherotype of each clone (Fig. 2). This direct plaque isolation procedure made genomic reassortment during virus isolation unlikely, whereas reassortment would be likely with other standard methods of BTV isolation. Only 1 clone, of 311 clones that were analyzed, gave rise to an electropherotype with more than 10 segments. This clone was excluded from the analysis because it apparently arose from a mixed plaque.

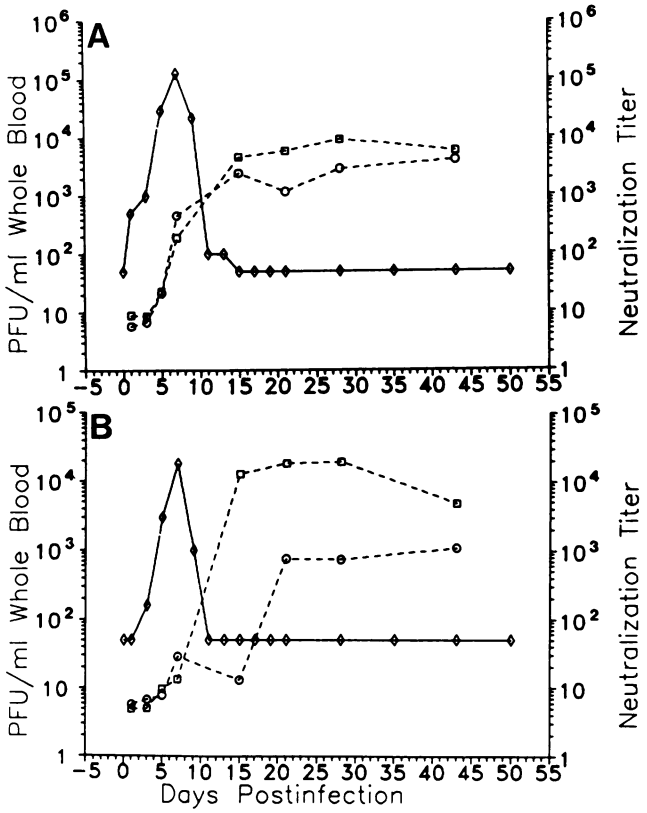

FIG. 1. Viremia and neutralizing responses in sheep mixedly infected with BTV serotypes 10 and 17. Viremia was determined by direct plaque assay of the buffy coat from venous blood. The limit of detection in these assays was 50 PFU/ml. Neutralizing responses were determined by plaque-reduction neutralization tests with the respective viruses. (A) Sheep 278; (B) sheep 593. Symbols: ◇, PFU/ml of whole blood; □, neutralizing titer to serotype 10; ○, neutralizing titer to serotype 17.

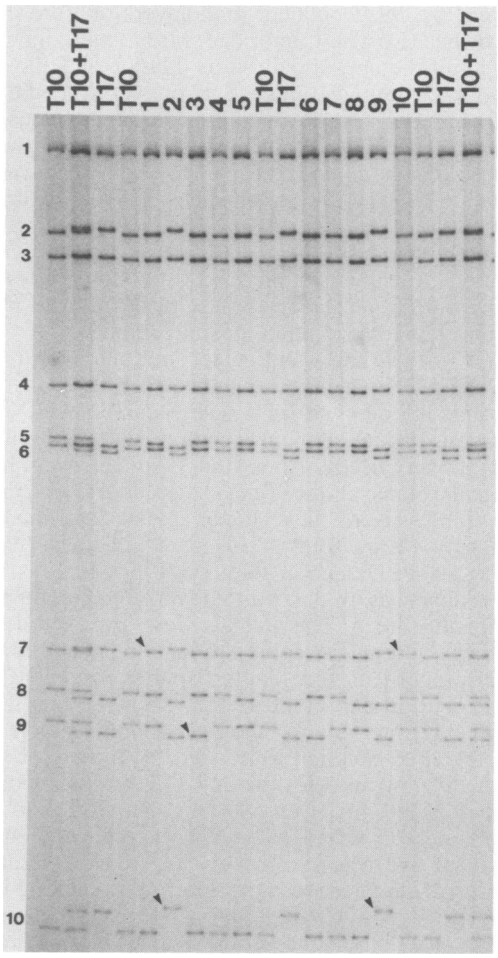

FIG. 2. Electropherotype determination of selected progeny clones isolated from sheep mixedly infected with BTV serotypes 10 and 17. The genomic dsRNAs of progeny and control viruses were labeled with ^{32}P and resolved in 6% Tris-glycine gels (thickness, 0.75 mm; length, 45 cm), as described in the text. The genome segments are numbered according to increasing electrophoretic mobility on the left. Lanes labeled T10 and T17 are BTV serotypes 10 and 17 parental controls, respectively. The lanes labeled T10+T17 are results of coelectrophoresis of T10 and T17. The numbered lanes represent parental and reassortant progeny clones isolated from mixedly infected sheep. Arrowheads indicate segments in some progeny clones that have slight mobility alterations relative to the cognate segments of the T17 parent.

A total of 310 clones were analyzed: 177 from sheep 278 and 133 from sheep 593. The numbers of parental and reassortant genotypes isolated from each animal at various times postinfection are summarized in Table 1. At all times except 28 dpi, serotype 10 predominated among the progeny. Serotype 17 was rarely isolated; at most times the presence of serotype 17 in the animals was revealed only by its contribution of genome segments to reassortants. Overall, each sheep yielded virtually identical proportions of parental and reassortant genotypes, with 92% of the progeny being serotype 10, 3% serotype 17, and 5% reassortants. Because only 8 of the 10 genome segments of the serotype 10-serotype 17 pair could be reliably scored on gels, however, the frequency of reassortant isolation must be considered to be a minimum estimate. The only striking difference between the two sheep was the isolation of the majority of reassort-

TABLE 1. Electropherotypes of virus clones isolated from sheep mixedly infected with BTV serotypes 10 and 17

Sheep no.	Time postinfection (days)	No. of clones examined	No. (%) of clones with the following electropherotype[a]:		
			Serotype 10	Serotype 17	Reassortant
278	1	21	18 (85)	2 (10)	1 (5)
	3	38	31 (82)	2 (5)	5 (13)
	5	22	22 (100)	0 (0)	0 (0)
	7	57	55 (96)	0 (0)	2 (4)
	9	23	23 (100)	0 (0)	0 (0)
	11	12	11 (92)	0 (0)	1 (8)
	13	2	2 (100)	0 (0)	0 (0)
	15	1	1 (100)	0 (0)	0 (0)
	28	1	0 (0)	1 (100)	0 (0)
593	1	4	4 (100)	0 (0)	0 (0)
	3	22	22 (100)	0 (0)	0 (0)
	5	25	24 (96)	1 (4)	0 (0)
	7	59	54 (92)	0 (0)	5 (8)
	9	21	19 (90)	0 (0)	2 (10)
	28	2	0 (0)	2 (100)	0 (0)

[a] Cumulative percents for serotypes 10 and 17 and the reassortant clone were 92, 3, and 5%, respectively.

ants from sheep 278 on or before 7 dpi and the isolation of reassortants from sheep 593 on or after 7 dpi.

During the gel analysis of progeny clones, occasional clones were observed in which one or two of the genome segments had aberrant electrophoretic mobility (Fig. 2). These slight alterations in electrophoretic mobility were generally seen in segment 9 (20 clones), but were also observed for segments 5 (3 clones), 6 (1 clone), 7 (4 clones), 8 (2 clones), and 10 (1 clone). In all cases the aberrant mobility represented a very slight mobility shift relative to the mobility of the cognate segment from serotype 17. Therefore, these aberrant genome segments were assigned a serotype 17 parent of origin. Because the aberrant migrations were reproducible from gel to gel, this result suggested that the serotype 17 virus used to infect the sheep contained a slight heterogeneity in mobility for these genome segments. Examination of the electropherotypes of 25 subclones derived from each parent did not reveal any subclones with segments with aberrant mobility (data not shown). This result suggests that if the aberrant segments are derived from heterogeneous parental viruses, the heterogeneity present is below the limit of detection of this experiment. Alternatively, the aberrant segments may have resulted from spontaneous mutations that occurred during virus growth in vivo.

Electropherotypes of reassortant clones. Among the 16 reassortant clones, 10 unique electropherotypes were identified (Table 2). Six electropherotypes were isolated twice. Seven of the electropherotypes contained only a single serotype 17 segment and three electropherotypes contained two serotype 17 segments. The majority of the genome segments of the reassortants were derived from the serotype 10 parent, a result that fits with the apparent preferential replication of serotype 10 in the mixedly infected sheep

TABLE 2. Electropherotypes of reassortants isolated from sheep mixedly infected with BTV serotypes 10 and 17

Sheep no.	Time postinfection (days)	Clone no.	Parental origin of the following genome segment[a]:									
			1	2	3	4	5	6	7	8	9	10
278	1	8b18	?	10	?	10	10	10	10	10	17	10
	3	12b10	?	10	?	10	10	10	17	10	10	10
	3	12b11	?	10	?	10	10	10	10	17	10	10
	3	12b14	?	17	?	10	10	10	10	10	10	10
	3	12b29	?	10	?	17	10	10	10	10	10	10
	3	12b30	?	10	?	17	10	10	10	17	10	10
	7	17b11	?	10	?	10	10	10	17	10	17	10
	7	17b12	?	10	?	10	10	10	10	10	17	10
	11	26b07	?	10	?	10	10	10	17	10	10	10
593	7	19b01	?	17	?	17	10	10	10	10	10	10
	7	19b03	?	17	?	17	10	10	10	10	10	10
	7	19b13	?	10	?	10	10	17	10	10	10	10
	7	19b22	?	10	?	10	10	17	10	10	10	10
	7	19b34	?	10	?	10	10	10	10	17	10	10
	9	21b06	?	10	?	10	10	10	17	10	17	10
	9	21b17	?	10	?	17	10	10	10	10	10	10
Segregation ratio (T17/T10)			?/?	3/13	?/?	5/11	0/16	2/14	4/12	3/13	4/12	0/16

[a] A question mark indicates that parental origin of segment could not be scored from gels. Numbers indicate serotype 10 or 17 as parent of origin for the segment.

(Table 1). However, serotype 17 was able to contribute all of its segments, except for segments 5 and 10, to reassortants. Although these data suggest that serotype 17 segments 5 and 10 may be preferentially selected against in mixedly infected sheep, the number of reassortants examined was too small to support any conclusions about possible selection.

DISCUSSION

The segmented nature of the BTV genome has long been recognized to provide the potential for evolution of the virus through a mechanism of reassortment. Indeed, the potential for reassortment in tissue culture cells has been demonstrated (15; Samal, Ph.D. thesis), and indirect evidence for reassortment in nature has been obtained (30, 31). Here we have demonstrated that the potential for reassortment is realized in one of the vertebrate host species, sheep. The importance of sheep as a host in which evolution can occur remains to be determined, however.

In addition to an examination of reassortment, these experiments were designed to examine several other features of the BTV system that are critical to a detailed genetic analysis of infection in a natural host. Classically, the most sensitive method for isolation of BTV from infected blood samples has been the intravenous inoculation of embryonated chicken eggs (8). Such a method would not be satisfactory for the isolation of single virus clones as required for genetic analysis. In an attempt to bypass this problem, we adapted BTV to culture in Vero cells and obtained virus stocks that were plaque purified a total of six times and passaged in culture five times. When these culture-adapted strains were inoculated into sheep, a mild bluetongue-like disease was observed and was accompanied by a significant viremia. These results suggest that the culture-adapted BTV strains remain capable of replication and pathogenesis in the natural host. By using culture-adapted virus for infection, however, it was possible to directly reisolate single clones of the virus from blood samples by plaque assay on Vero cell monolayers. The disease observed with these culture-adapted viruses was mild, as reported by others (6, 23). The viremia observed was of relatively short duration, peaking at 7 dpi and rapidly decreasing thereafter. Similar growth kinetics for BTV in sheep have been reported previously (19). Although both sheep examined showed similar kinetics, the titers attained at the peak of viremia differed significantly. Similar differences in susceptibility of individual sheep to BTV infection have been reported (19). Both of the animals inoculated with culture-adapted virus developed type-specific neutralizing responses to both of the viruses that were inoculated. The humoral immune response to both serotypes developed with similar kinetics. These results are similar to those from reports in which individual animals responded to multiple serotypes that were inoculated simultaneously (14). Thus, the use of culture-adapted virus allowed the rapid isolation of individual virus clones, as will be necessary for genetic analysis of infection and pathogenesis.

The detailed analysis of the electropherotypes of progeny clones isolated from two mixedly infected sheep was striking in the similarity of the results obtained with the two individuals. The serotype 10 parental virus apparently replicated preferentially in sheep as compared with serotype 17, which was isolated only rarely. This was reflected in the serotype 10 parental electropherotype that predominated among the progeny and that contributed the majority of the genome segments to reassortant viruses. Reassortant progeny were isolated with equal frequency (5%) from both sheep. The isolation of a few reassortant electropherotypes multiple times suggests that 5% may overestimate the frequency of reassortment because multiply isolated reassortants may have been siblings from a single reassortment event. The inability to score parental origin of two genome segments in clones analyzed, however, may have led to 5% being an underestimate of actual reassortment frequency. Thus, a 5% reassortment frequency appeared to be a good estimate, given the limitations of these experiments. The ability to isolate reassortants indicates that individual cells are mixedly infected and that genetic interactions occur in those cells. It seems likely that some of the reassortants may have a selective growth advantage, resulting in a predominance of reassortant genotypes at late times. This was not observed and may have been due to the emergence of the humoral immune response at about the same time that the viremia peaked, resulting in a clearance of infectious virus from the blood. Relatively few reassortants were isolated, and 10 different electropherotypes appeared among the 16 reassortant isolates. Examination of the segregation of genome segments among the reassortants suggests that certain genome segments of the serotype 17 parent are selected against. The small number of reassortants isolated, however, precludes a meaningful analysis of possible selection.

The isolation of a few progeny clones containing genome segments with aberrant mobility in gels was unexpected. All of these altered genome segments appeared to be derived from the serotype 17 viral parent, as they had subtle differences in migration relative to the cognate segment from serotype 17 markers in gels. Because all of the aberrant segments appeared to be derived from serotype 17, a slight migrational heterogeneity of these segments in the serotype 17 virus stock appears to be the most likely explanation. The aberrant segments were likely to be present in minor amounts in the serotype 17 stock because they were not observed in gels of the parental serotype 17, and analysis of subclones derived from the parental viruses did not reveal the presence of heterogeneity. When these aberrant segments were cloned in the progeny, however, their aberrant migration was easily detectable. An alternative explanation for the origin of aberrant segments was that they reflected the accumulation of mutations in those segments during viral growth in vivo. Point mutations have been shown to occur during natural BTV infection (25).

Finally, others have shown indirectly that reassortment between and within BTV serotypes occurs in nature (30, 31), although the host within which the reassortment occurred was not identified. In this study we identified sheep as a natural host within which reassortment of BTV genome segments can occur. At this time, however, the relative importance of sheep as a reassortment-permissive host cannot be assessed. We have evidence that reassortment is much more frequent in the vector *Culicoides variipennis* (42%) than in sheep (5%) (S. K. Samal, A. M. El-Hussein, B. J. Beaty, F. R. Holbrook, and R. F. Ramig, unpublished data). This observation suggests that the mixedly infected vector may generate and spread reassortant viruses much more efficiently than do sheep. Likewise, one can speculate that mixedly infected cattle, with prolonged viremia, may generate reassortants at frequencies that are significantly higher than those observed in sheep. Our results demonstrate, however, that reassortment of genome segments can occur in vertebrates (sheep) that are mixedly infected with BTV and emphasize that reassortment must be considered when designing vaccination strategies to be used to combat bluetongue disease.

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