Bluetongue Virus: Some Relationships Among North American Isolates and Further Comparisons with EHD Virus

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SUMMARY

Seven isolates of bluetongue virus with different isolation histories and one isolate of the virus of epizootic hemorrhagic disease of deer were cloned by three consecutive plaquings in L-929 cells. The isolates were categorized on plaque size and margin. Antisera to the bluetongue virus isolates were produced in calves and antiserum to epizootic hemorrhagic disease virus in deer. Plaque reduction neutralization tests were done using the eight isolates and antisera to six of these.

The isolates could be partially categorized on plaque type. In the plaque reduction neutralization test, all of the bluetongue viruses cross reacted and although differences were frequently observed, no obvious antigenic classification was possible. Reactions between the bluetongue viruses and epizootic hemorrhagic disease virus were all within the limits of what is presently considered to be non-specific inhibition.

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RÉSUMÉ

Les auteurs ont purifié sept souches du virus de la fièvre catarrhale du mouton, obtenues de cas cliniques différents, et une souche du virus de la maladie hémorragique épizootique du chevreuil, au moyen de trois passages consécutifs dans les cellules L-929. Ils cataloguèrent ensuite ces souches d'après les dimensions et le contour de leurs plages. Ils utilisèrent des veaux pour produire des antisérums correspondant aux souches du virus de la fièvre catarrhale du mouton, et des chevreuils pour produire l'antisérum correspondant à la souche du virus de la maladie hémorragique épizootique du chevreuil. Ils employèrent aussi les huit souches de virus et les antisérums correspondant à six d'entre elles, pour effectuer les épreuves de neutralisation destinées à réduire le nombre de plages.

Ils réussirent à cataloguer partiellement les souches de virus, en se basant sur l'aspect des plages. Dans l'épreuve de neutralisation, destinée à réduire le nombre de ces plages, toutes les souches du virus de la fièvre catarrhale du mouton donnèrent une réaction croisée et, bien qu'on ait observer fréquemment des différences, il fut impossible d'en arriver à une classification antigénique définie. Les réactions entre les souches du virus de la fièvre catarrhale du mouton et celle de la maladie hémorragique épizootique du chevreuil, se situaient toutes dans les limites de ce que l'on considère présentement comme une inhibition non-spécifique.

INTRODUCTION

Several South African "strains" of bluetongue virus (BTV) have been categorized on the basis of plaque type (2) and cross neutralization (3). The former report did not indicate to what extent, if any, plaques produced by one isolate were neutralized by antisera against others. Comparison of different North American isolates (using the alpha system of neutralization) was reported prior to the availability of an acceptable cell culture system, plaquing technique, or plaque reduction neutralization test (4). The degree of resolution afforded by the latter system was necessary to work efficiently on the question of antigenic relationships.

A recent comparison of epizootic hemorrhagic disease virus (EHDV) and the BT_8 "strain" of BTV revealed them to be morphologically indistinguishable but antigenically different viruses (5).

The purpose of the present study was to compare all available North American BTV isolates and the original isolate of EHDV with each other on the basis of plaque type and antigenic make-up.

MATERIALS AND METHODS

Virus Isolates

Four BTV isolates were obtained from the USDA-ARS Animal Disease Research Laboratory in Denver, Colorado. These were the "standard" North American isolate, BT_8, and BT_{262}, BT_{318}, and BT-OX_{193} (4). The isolate designated BT_{Texas} was obtained from Dr. D. G. McKercher, School of Veterinary Medicine, Davis, California via Dr. B. C. Easterday, Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin. Two recent isolates, DBT and BHBT came from naturally occurring cases of bluetongue in a white-tailed deer and bighorn sheep respectively. The EHDV isolate was derived from the original Shope isolate (New Jersey, 1955) and has been further described (5). All viruses were cloned by three consecutive plaquings in L-929 cells. These viruses and their isolation histories are listed in Table I.

TABLE I. Bluetongue and Epizootic Hemorrhagic Disease Virus Isolates, Place and Year of Isolation, and Plaque Type in L-929 Cells

Isolate Designation	Source	Plaque Type	
		Size[a]	Margin[b]
1 BT_8	California (1953)	Large	Even
2 BT_{262}	Wyoming (1962)	Medium	Irregular
3 BT_{318}	Texas (1962)	Large	Even
4 BT-OX_{193}	Colorado (1963)	Medium	Irregular
5 BT_{Texas}	Texas (1953)	Medium	Irregular
6 EHD	New Jersey (1955)	Small	Irregular
7 BHBT	Texas (1969)	Medium	Irregular
8 DBT	Texas (1969)	Medium	Irregular

[a]Small < 1 mm
Medium 1-2 mm
Large >2 mm
[b]Plaque border (even or irregular)

Antisera

Antisera to BTV isolates 1 through 5 (Table I) were produced as follows: Each of three calves received approximately 10^2 plaque forming units (p.f.u.) of one isolate subcutaneously. This dose produced a viremia. Three weeks later the calves were bled and a serum pool prepared (equal volumes from each of the three calves, heat inactivated 30 minutes at 56°C). This is referred to as the "late infection" pool and is the one on which most of the work is based. On the day of bleeding, each calf received 10^6 p.f.u. of the same virus clone used initially and was bled again three weeks later. This pool is called the "hyperimmune" pool.

The EHDV antiserum was a pool from two young white-tailed deer inoculated with two doses of live EHDV and prepared from a bleeding approximately five weeks after the first inoculation.

Plaque Technique and Classification

The plaquing technique utilized L-929 cells and a DEAE-dextran-containing agar overlay (2,6). BTV plaques were stained and counted at four days and categorized on (i) size and (ii) margin. The size ranges used were arbitrary and overlapping (small < 1 mm; medium = 1—2 mm; large = > 2 mm). "Margin" refers to the plaque border and was classified as "even" or "irregular". Examples are shown in Fig. 1-A, B, C.

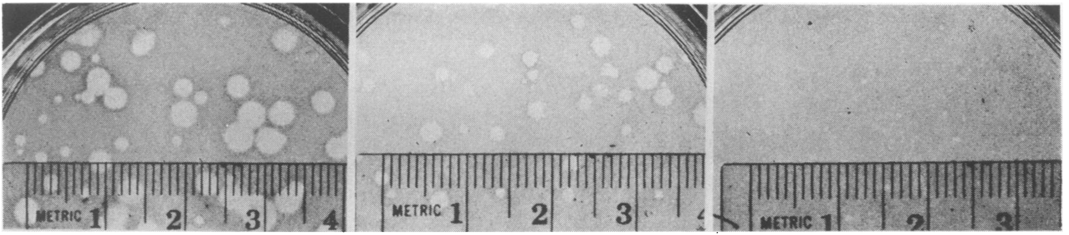

Fig. 1. Plaque Types: A. BT_8 — large, even; B. RT-OX_{193} — medium, irregular; C. EHDV — small, irregular.

Plaque Reduction Neutralization Test

One change was made from the technique previously described (6). In the present work, each plate inoculated with serum and virus (serum plate) was paired with one control plate which was inoculated with diluent and virus. Thus, each serum plate had its own control. After staining and counting, the number of plaques for each serum plate was compared with its control to estimate percent reduction (or survival) in the presence of each serum dilution. These percentages were then plotted on log probit paper and the 50% effective dose (ED_{50}) graphically estimated (6).

The five late infection BTV antisera and hyper-immune BTV sera were incubated with all eight virus isolates, as was the EHDV antiserum. Each serum-virus reaction was done twice (on different days) and the ED_{50}'s averaged.

Since low titer non-specific inhibitors of BTV and EHDV are frequently present in sera, an arbitrary cut-off point of 1:50 was chosen (i.e. all titers less than 1:50 were considered negative).

RESULTS

Plaques

All of the isolates caused plaque formation in L-929 cells and all were cytolytic (clear plaques). BTV plaques appeared in 48 hours and the numbers had stabilized by four days post inoculation, but the size continued to increase. The EHDV plaques were seen only after five days incubation, and in our system reliable results were obtained at seven days. In all cases, plaque size was quite variable and several-fold variation in diameter was often seen on one plate (Fig. 1).

On the basis of plaque type the viruses could be divided into three groups (Table I). BT_8 and BT_{318} formed large plaques with even borders (Fig. 1-A); BT_{262}, BT-OX_{193}, BT_{Texas}, DBT, and DHBT, all formed medium-sized plaques with irregular borders (Fig. 1-B); EHDV plaques were small with irregular borders (Fig. 1-C).

Antigenic Relatedness

Using the late infection sera, all seven BTV isolates cross reacted extensively (one or two way crosses) in the plaque reduction neutralization test (Table II). BT_{Texas} was not involved in a two-way cross, the virus not being neutralized by any of the antisera including its own. Antiserum prepared againt BT_{Texas} did, however, react with all other BTV isolates.

EHDV appears to be antigenically distinct from all the BTV isolates (Table II). In no case was a titer of greater than 1:50 observed for any BTV antiserum against EHDV; nor was any BTV neutralized by the EHDV antiserum. The EHDV homologous reaction was notably high, 1:3000.

When these reactions were repeated using the "hyperimmune" sera, the same general pattern appeared. However, all BTV sera then neutralized BT_{Texas} virus, all BTV reactions resulting in two-way crosses. The situation involving EHDV remained the same; no hyperimmune BTV antiserum neutralized EHDV.

Any one serum formed a spectrum of quantitatively different titers with the virus isolates. The reaction of the serum-virus mixtures was not an all-or-none phenomenon.

In some cases, the homologous reaction was less than a heterologous reaction for the same serum (some sera neutralized the virus to which they were prepared to a lesser extent than a heterologous virus) (Table II).

DISCUSSION

The virus isolates examined can be grouped according to plaque type. Among the BTV isolates, these groups do not coincide with obvious antigenic categories. It has been noted that plaque size is quite variable within a clone and extensive overlapping occurs among isolates. This heterogeneity of size was obvious at any time during plaque development. Similar results were reported by Howell using South African isolates (2). The usefulness of such a method of BTV classification remains to be demonstrated. For both plaque classification and antigenic comparisons, only one clone of each isolate was used. It might be useful to compare clones within an isolate to determine within-isolate variation.

All of the BTV isolates examined cross reacted extensively in the plaque reduction neutralization test (Table II). The quantitative differences observed fail to separate the isolates into any obvious antigenic groups. When examining the spectrum of results of any one serum against the different isolates, it seems certain that the extremes of these spectra represent real differences; some of the smaller differences among titers are undoubtedly due to chance and do not reflect any real antigenic differences.

A statistical tool is necessary to help judge which differences are real and which are not. Having been unsuccessful in finding an adequate method available, we are in the process of developing one.

No cross reactions (i.e. $> 1{:}50$) occurred between EHDV and the BTV isolates, even when the "hyper-immune" sera to the latter were used. Since EHDV apparently does not cross react with BTV antiserum in the agar gel precipitin test or the complement fixation test (5), we must conclude that the EHDV isolate is antigenically unrelated or only slightly related to the BTV isolates. Certainly the close relationships seen among the BTV isolates does not exist between EHDV and BTV.

The BTV antisera were produced in calves while the EHDV antiserum was produced in deer, a condition allowing an explanation based on species differences. Previous results have shown, however, that deer can produce neutralizing antibody against BTV as well as EHDV (5). The ability of calves to produce EHDV antibodies has not been demonstrated to our knowledge.

There is no obvious explanation for the lack of reaction of BT_{Texas} virus with any of the late infection sera including the homol-

TABLE II. Mean ED_{50}'s of Homologous and Heterologous Plaque Reduction Neutralization Tests, Using "Late Infection Serum" with Several Bluetongue Virus Isolates and the Virus of Epizootic Hemorrhagic Disease of Deer

Sera	Viruses							
	1 BT_8	2 BT_{262}	3 BT_{318}	4 $BT\text{-}OX_{193}$	5 BT_{Texas}	6 EHD	7 BHBT	8 DBT
1 BT_8	**1850**[a]	50	1200	350	Neg.[b]	Neg.	140	100
2 BT_{262}	650	**300**	1300	500	Neg.	Neg.	450	400
3 BT_{318}	110	75	**300**	70	Neg.	Neg.	100	60
4 $BT\text{-}OX_{193}$	3000	450	20,000	**4000**	Neg.	Neg.	800	400
5 BT_{Texas}	1650	180	1400	250	**Neg.**	Neg.	70	350
6 EHDV	Neg.	Neg.	Neg.	Neg.	Neg.	**3000**	Neg.	Neg.

[a]Numbers represent the mean ED_{50}'s (two tests) of the sera expressed as a reciprocal of the dilution. Homologous reactions are underlined
[b]All reactions > 50 = negative

ogous one. That the latter contained antibodies is shown by its extensive reactions with the other viruses. Also, since all hyper-immune sera neutralized BT_{Texas} the virus suspension used was quite neutralizable. It appears that the antigens to which most of the late infection sera response was directed were not antigens involved in the neutralization reaction for this virus, although they were of obvious importance for the other BTV isolates. Alternatively, this virus may be neutralizable only by immunoglobulins produced later in the antibody response (our "hyper-immune" sera).

Some of the viruses, such as BT_{262}, seem to be less neutralizable than others, such as BT_{318}. This may reflect some inherent differences in antigen distribution in the capsid. This phenomenon could also be explained as a "preparation" difference, even though care was taken to prepare all virus stocks in an identical manner. Different preparations should be used to test this possibility. To what extent the apparent covering of BT virions with host material as previously described (5) may interfere with their neutralization is unknown.

Throughout this paper, all reactions less than 1:50 were considered negative. This has been done to avoid mistaking non-specific inhibition from specific neutralization. When dealing with cross reactions in particular, some titers due to antibody are probably included in this group. Hence some of the "negative" may well be relatively low positive reactions.

The plaque reduction neutralization test, as used with American isolates of BTV, appears to be a useful procedure for the diagnostician and the epizootiologist. Contrary to implications in the literature (1, 4), it is not necessary to use the isolate to which the animal was exposed. Indeed, in some cases it would be desirable to use a different isolate (Table II). Our results suggest the use of an isolate (clone? preparation?) which is neutralized to high titer by all sera, such as BT_{318} in Table II. The advantages of such a sensitive quantitative technique need no elaboration.

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