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THE CYTOPATHOGENIC ACTION OF BLUETONGUE VIRUS ON TISSUE CULTURES AND ITS APPLICATION TO THE DETECTION OF ANTIBODIES IN THE SERUM OF SHEEP.

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The need for an *in vitro* test to demonstrate the presence or absence of immune bodies to bluetongue virus will be appreciated by all who have studied the disease. Therefore, attempts were made to propagate bluetongue virus in tissue culture with the object of developing a serum-virus neutralization test in which convalescent sheep serum could be used. In this report an account is given of the cytopathogenic activity of egg-propagated bluetongue virus on cultures of sheep kidney cells, and the inhibition of this activity by serum from bluetongue-immune sheep.

Materials and Methods.

Virus Strains.—Five strains of bluetongue virus were employed in the study. All had been given one or more passages in sheep before they were adapted to propagation in embryonated eggs.

Cyprus.—The Cyprus strain of bluetongue virus was originally obtained from a natural case of bluetongue in a sheep on the island of Cyprus. Virus at the first, second, third, fourth, and one hundred and fourth egg passage levels was used.

Estantia.—The Estantia strain of bluetongue virus was obtained from a natural case of bluetongue in a sheep in the Eastern Transvaal. This virus was used at the 72nd egg passage level.

Ermelo.—The Ermelo strain was obtained from a sheep in the Eastern Transvaal in 1955. The strain was used at the 72nd serial egg passage level.

Vlak.—This strain was recovered in 1953 from a natural case of bluetongue in immunized sheep in the Eastern Transvaal. It was used at the 72nd serial egg passage level for the studies reported herein.

California II.—The California II strain was originally obtained from an outbreak of bluetongue among sheep in California (McKercher 1954). When used in this study it was at the 56th serial egg passage level.

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It is of interest to note that the Estantia, Ermelo and Vlak strains of virus were isolated from sheep, previously immunized with routine egg adapted virus-vaccine, that were reacting to natural infection.

Sera.—Convalescent sera were obtained from sheep that had recovered approximately two months previously from experimental infection with unmodified strains of Cyprus and Estantia bluetongue virus. Control serum was taken from a sheep in a group subsequently proven to be susceptible to bluetongue. The sera were stored at -15° C. They were inactivated at 56° C. for 25 minutes immediately before use and were then diluted 1 in 5 for use in the *in vitro* neutralization tests.

Diluent.—All dilutions of virus and serum were made in buffered balanced saline prepared according to the method of Dulbecco (1954).

Cells.—Kidneys were obtained from freshly slaughtered lambs three to five months of age and the roller tube cultures of trypsin-dispersed kidney cells were set up according to the method of Younger (1954). During the period of active cell growth the cells were bathed in a medium consisting of Hank's solution containing 0·5 per cent lactalbumin hydrolysate (Melnick, 1955), and 5 per cent sheep serum. During this phase of cell propagation the tubes were not rolled. At the time of inoculation of the cultures with virus, the medium was replaced with fresh medium in which the 5 per cent sheep serum was replaced by 2 per cent cattle serum. The cultures at that stage had been preincubated for 8 to 16 days and had not been rolled. After infection some tubes were rolled others not. The temperature of incubation throughout was ± 36° C.

Experimental.

The virus strains used in this study were stored under dry ice refrigeration in the form of infected chicken embryo emulsions. A 10^{-2} suspension of each strain was prepared and 0·2 ml. amounts were added to sheep kidney cell cultures, using four tubes for each strain.

Table 1.

Cytopathogenic Effect of Different Strains of Bluetongue Virus on Sheep Kidney Cells.

Strain.	Egg Passage Level.	Day of Incubation.					
		1.	2.	3.	4.	5.	6.
Cyprus	104	—	—	····			
Estantia	72	—	—	···	····		
Ermelo	72	—	—	···	····		
Vlak	72	—	—	··	····		
California II	56	—	—	··	···	····	

Legend: — No apparent change.
· to ···· Approximately 25, 50, 75 and 100 per cent of cells affected.

Result.

From Table 1 it is seen that of the strains studied, all produced a cytopathogenic effect in sheep kidney cell cultures. This change was produced most rapidly by the Cyprus strain and was somewhat delayed in the case of the California II strain of virus. Unfortunately, the virus titre of the inocula was not determined in hen's eggs. It is therefore possible that the difference in rate of cytopathogenesis was due to difference in titre of the seed material.

Since the Cyprus strain appeared to produce the strongest cytopathogenic effect, the tissue culture-propagated virus was titrated in sheep kidney cells. Its titre was found to be 10^{-5}.

Nature of the Cell Changes.

In the early stages of the infection it appeared that cells or groups of cells in areas scattered throughout the sheet became swollen and ill-defined. Later these changes spread to adjacent areas until the entire sheet, with the exception of small islands of epithelial-like cells, which retained their normal appearance, exhibited this effect. The affected cells became granular and underwent variable degrees of shrinkage. Later most became detached from the glass. Eventually the islands of normal appearing cells underwent the same changes. It appeared that fibroblast-like cells were rather more resistant and survived longer than the epithelial-like cells.

The Induction of Cytopathogenic Activity in Bluetongue Virus by Egg Passage.

On two separate occasions attempts were made to propagate unmodified bluetongue virus in sheep kidney cells, using serum from sheep infected with either the Cyprus or Estantia strains of virus as inoculum. No cellular changes were seen over the ten day period during which the cultures were kept under observation. A single sub-passage in tissue culture was made in each case but in neither instance were cytopathogenic effects observed. At this stage it was decided to investigate this point as a separate study; subinoculations of the tissue culture material into sheep to determine whether virus was present or had multiplied were not made, and further serial sub-passage in tissue culture was discontinued.

Since it had been shown that the egg adapted and propagated bluetongue virus is pathogenic for sheep kidney cells in culture, an experiment was undertaken to determine at which passage level this property is acquired by the virus.

Virulent Cyprus virus obtained from a sheep was adapted to propagation in eggs after the method of Alexander (1947). Embryos that died during each serial passage were harvested and, after maceration, were stored in the refrigerator. When this strain had been passaged four times, serial tenfold dilutions were made from material from each passage level. Two tubes of sheep kidney cell culture were seeded with each dilution of virus. The results of this experiment are summarized in Table 2.

TABLE 2.

Development of Cytopathogenic Activity in Cyprus Strain of Bluetongue Virus by Serial Egg Passage.

Egg Passage Level.	Dilution.	Day of Incubation.						
		1.	2.	3.	4.	5.	6.	7.
1	10^{-1}	—	—	—	·	··	···	
	10^{-2}	—	—	—	—	—	—	—
2	10^{-1}	—	—	—	—	··	···	
	10^{-2}	—	—	—	—	—	—	··
3	10^{-1}	—	—	··	···	····		
	10^{-2}	—	—	—	··	···	····	
4	10^{-1}	—	—	··	····			
	10^{-2}	—	—	··	····			

Result.

From the above table it is seen that virus at the first egg passage level was capable of producing cytopathogenic effects in sheep kidney cells, but this effect was detectable not before the fourth day of incubation and was well defined only on the fifth day; in addition it was produced only by the higher of the two concentrations of virus used. At the second egg passage level cytopathogenesis was equally delayed, but with the 10^{-2} dilution of seed virus appeared on the seventh day. By the fourth serial passage cytopathogenesis was well marked on the third day and complete by the fourth day, even with the hundred-fold dilution of seed virus.

Serum Neutralization.

A series of tests was carried out to determine whether the cytopathogenic effect produced by the bluetongue virus could be neutralized by serum from sheep that had recovered from the disease.

In a preliminary trial, infected chick embryos in which two different strains of virus had been propagated separately, were used. This material had been stored under dry ice refrigeration. Serial ten-fold dilutions were made and mixed with equal amounts of serum that had been inactivated at 56° C. for 25 minutes. Sheep cell cultures were then inoculated. Two tubes were used for each dilution of serum and virus.

In view of the fact that the titre of both antigens used did not exceed 10^{-3} in the presence of negative serum, the results of this test were regarded as unsatisfactory. Nevertheless, it was apparent that there was no cross-neutralization between the two strains of virus although in each case a high degree of homologous neutralization occurred.

A second experiment was then made using as the cytopathogenic agent for the tissue cultures freshly prepared emulsions of culture propagated virus.

The results are shown in Table 3.

TABLE 3.

In vitro *Neutralization of Tissue Culture Passaged Virus.*

Strain and Passage Level.	Dilution.	Serum.	Day of Incubation.						
			1.	2.	3.	4.	5.	6.	7.
Cyprus 104 egg, 2 T.C.	10^{-1}	Cyprus.	—	—	—	—	—	—	—
			—	—	—	—	—	—	—
	10^{-2}		—	—	—	—	—	—	—
			—	—	—	—	—	—	—
	10^{-3}		—	—	—	—	—	—	—
			—	—	—	—	—	—	—
	10^{-3}	Estantia.	—	—	....				
			—	—	....				
	10^{-4}		—	—	....	..	...		
			—	—	....	...	....		
	10^{-5}		—	—	—	—	—	—	—
			—	—	—	—	—	—	—
	10^{-3}	Normal Control.	—	—	....				
			—	—	....				
	10^{-4}		—	—	..	....			
			—	—	...	....			
	10^{-5}		—	—	—	—	—	—	—
			—	—	—	—	.	....	
Estantia 72 egg, 2 T.C.	10^{-4}	Cyprus.			—	..	...	....	
					—	..	...	....	
	10^{-5}				—	—	..	....	
					—	—	..	....	
	10^{-6}				—	—	.	....	
					—	—	—	—	—
	10^{-1}	Estantia.			—	·?	·?	·?	·?
					—	·?	·?	·?	·?
	10^{-2}				—	—	—	—	—
					—	—	—	—	—
	10^{-3}				—	—	—	—	—
					—	—	—	—	—
	10^{-4}	Normal Control.			..	....			
					..	...			
	10^{-5}				—	.	....		
					—	.	....		
	10^{-6}				—	—	—	....	
					—	—	—	....	

Results.

From Table 3 it is seen that the Cyprus virus was cytopathogenic in the presence of non-immune serum to a titre of 10^{-5}. This effect was inhibited completely by the homologous convalescent serum, but less than one log of virus was neutralized by the heterologous serum.

In the case of the Estantia virus a clear-cut end-point to the virus titration in the presence of normal serum was not obtained. From the delay in the production of cytopathogenesis an estimate of the end point as being a dilution slightly higher than 10^{-6} is permissible. If this is conceded the homologous serum neutralized approximately 6 logs of virus whereas the heterologous serum neutralized somewhat less than 1 log, accompanied by a slight delay in the appearance of cytopathogenic effect.

Conclusion.

Bluetongue immune sheep serum is capable of inhibiting the cytopathogenic effect of the homologous tissue culture adapted virus with slight but possibly insignificant effect upon the heterologous virus in the case of the two strains investigated.

In a subsequent experiment using Cyprus virus at the 104 egg, 4 tissue culture passage level, Estantia virus at the 72 egg, 4 tissue culture passage level and the same sera the above result was confirmed within the limits of experimental error.

Discussion.

Each of five egg-adapted strains of bluetongue virus that were tested was found to possess cytopathogenic activity for sheep kidney cells in culture. Reciprocal cross neutralization tests between two of the strains indicate that this effect is specific. The fact that the five strains of virus originated from widely separated areas (South Africa, Cyprus and the U.S.A.) strongly suggests that cytopathogenic activity is a property of all strains of bluetongue virus.

On the basis of observations made on one strain, Cyprus, it would appear that this activity is not a property of the unmodified virulent sheep virus but is associated with adaptation to propagation in eggs. However, the fact that one egg passage is adequate to induce the demonstrability of this activity, indicates that it is not correlated with the phenomenon of attenuation. No data are available on the effect of adaptation to multiplication in tissue culture without intervening egg adaptation.

Demonstration of cytopathogenesis by bluetongue virus and the inhibition or neutralization of this activity by the homologous antibody in sheep serum will prove an excellent *in vitro* test for studying various aspects of immunity to the disease, particularly the plurality of virus strains and the identification of strains causing natural infection in the field. As a means to determine the extent of infection in nature it has its limitations. In conjunction with a complement fixation test for bluetongue which is currently being investigated at Onderstepoort, a test which has a wide specificity, the serum-virus neutralization test described above should provide a useful technique for studies on bluetongue.

Summary.

Cytopathogenic activity by egg adapted strains of bluetongue virus for sheep kidney cells in culture, and the neutralization of this activity by homologous antiserum are described. The possible applications of this phenomenon to studies on various phases of bluetongue is discussed briefly. Reference is made to preliminary studies which indicate that the property of cytopathogenesis is not associated with virulent, unmodified, bluetongue virus.

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