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A STUDY ON THE PATHOGENESIS OF BLUETONGUE: REPLICATION OF THE VIRUS IN THE ORGANS OF INFECTED SHEEP

A. PINI, Veterinary Research Institute, Onderstepoort

ABSTRACT

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The pathogenesis of bluetongue infection was studied by the titration of the virus in tissue samples taken from sheep inoculated subcutaneously in the auricula of the ear with 76 TC ID_{50} of the plaque-purified type 10 bluetongue virus. Tissue samples were taken from individual animals killed at daily intervals over a period of 11 days. The mean incubation time was 6,9 days and the first clinical sign was pyrexia. On the 4th day, bluetongue virus was demonstrated in the lymph nodes of the cephalic area, tonsils and spleen; viraemia became demonstrable on the 6th day post-inoculation and typical macroscopic lesions due to the virus were first observed on the 8th day. It was concluded that, post-infection, the virus entered the regional lymph nodes. From there it was disseminated via the lymph and/or the blood stream to the lymphoid tissues in other parts of the body where further replication occurred. From these primary sites the virus was carried via the blood stream and infected the majority of tissues. Humoral antibody, as detected by immunofluorescence, did not appear to have a direct influence on the concentration of virus in solid tissues. Persistence of the virus in infected sheep was not demonstrated when tissues were taken 6, 8 and 16 weeks after infection.

Resumé

UNE ÉTUDE DE LA PATHOGÉNIE DE LA FIÉVRE CATARRHALE DU MOUTON: PROLIFÉRATION DU VIRUS DANS LES ORGANES DE MOUTONS INFECTÉS

L'auteur a étudié la pathogénie de la fièvre catarrhale du mouton par titrage du virus dans échantillons de tissus prélevés de moutons infectés par voie sous-cutannée dans le pavillon de l'oreille avec 76 DI_{50} CT du virus de la fièvre catarrhale du type 10 purifié sur plaques. Les échantillons de tissu ont étés prélevés d'animaux individuels sacrifiés par intervalles quotidiens au cours de 11 jours. Une période moyenne d'incubation de 6,9 jours a été observé avec la fièvre comme premier symptôme. Le 4em jour aprés l'inoculation le virus a été detecté dans les ganglions lymphatiques de la region cephalique, dans les amygdales et dans la rate; on a pu démontrer une virémie au 6em jour et des lesions macroscopiques ont apparu au 8em jour. On a tiré la conclusion que le virus de la fièvre catarrhale du mouton parvient, après l'inoculation, aux ganglions lymphatiques régionals. A partir de ces organes le virus est disséminé par voie sanguine et/ou lymphatique aux autres organes lymphatiques de l'organisme où a lieu une multiplication additionelle. Ensuite le virus est transporté de ces localités primaires par la circulation sanguine à la plupart des tissus. La presence d'anticorps humorals, révélée par l'immunofluorescence, ne semble pas avoir une influence directe sur la concentration du virus dans les tissus solides. La persistance du virus n'a pas pu être démontrée dans les tissus préleves 6, 8 et 16 semaines apres l'inoculation des moutons.

INTRODUCTION

In his review on bluetongue, Bowne (1971) stated that "little is known about the pathogenesis and nothing is known about the route the virus takes from the site of the insect bite". On the other hand, the pathological changes taking place after the virus has reached the target organs of a susceptible animal have been extensively investigated and were described by Spreul (1905), Theiler (1906), Bekker, De Kock & Quinlan (1934), Thomas & Neitz (1947), Moulton (1961) and Karstad & Trainer (1967). From the observations made by these authors it appears that the pathological lesions due to the virus are characteristic of an inflammatory process with increased vascular permeability of the mucous membranes of the digestive tract, the epithelium of the sensitive laminae of the hooves and the skeletal musculature. The sites of the lesions indicate widespread distribution of the virus. In a study on the pathogenesis of bluetongue in sheep in which immunofluorescence and histopathological techniques were employed, Stair (1968) established that the virus had an affinity for the reticuloendothelial cells, the pericytes, the endothelial and the periendothelial cells of the precapillary arterioles, venules and capillaries.

In the present investigation it was decided to study the pathogenesis of the disease on the basis of the titration of the virus in tissue samples taken from sheep killed at intervals post-infection. The aim was to obtain information concerning the virological events that occur both during the incubation period prior to the appearance of the first overt symptoms and the early stages of the disease. The persistence of the virus in the organs of recovered sheep was also investigated.

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MATERIALS AND METHODS

Virus

Type 10 bluetongue virus was used. The plaque-purified strain of virus (Howell, 1969) was passaged twice in BHK21 C13 cell cultures (Macpherson & Stoker, 1962) and a stock was prepared. The titre of the virus suspension after freeze-drying was $10^{6,0}$ 50% tissue culture infective doses ($TCID_{50}$) per ml when assayed in roller tube cultures of BHK21 C13 cells.

Cell cultures

BHK21 C13 cells which had undergone approximately 70 generations were obtained from the Experimental Virus Research Unit, Institute of Virology, Glasgow, Scotland. On arrival at the laboratory, the cells were subcultured according to the standard procedures, using Eagle's minimum essential medium (MEM) supplemented with 10% Bacto tryptose phosphate broth and 10% bovine serum. A frozen stock was prepared at the 4th passage level and, after 10 consecutive subcultures, the cells were discarded and a new batch initiated. Maintenance medium for the cell monolayers was identical to the growth medium except that the bovine serum was replaced by 2% foetal bovine serum.

Infectivity titrations in cell cultures

Serial tenfold dilutions of virus suspensions were prepared in Eagle's MEM. Confluent monolayers of BHK cells were rinsed in 3 changes of phosphate buffer saline and infected with the appropriate dilution. After incubation at 37 °C for 60 min, the inoculum was discarded and 1,5 ml of the maintenance medium added. Unless otherwise stated, 4 tubes were used for each dilution. Monolayers were observed for 10 days to detect the appearance of the cytopathic effects. The infectivity end-points per ml of fluid or g of tissue were calculated according to the method of Reed & Muench (1938).

Indirect immunofluorescence

The preparation of the conjugates and the staining procedures were carried out according to the methods described by Pini, Ohder, Whiteland & Lund (1968), using BHK cells cultivated on coverslips.

Sheep

Yearling Merino sheep were used. They were selected from a farm on which bluetongue had not been recorded and where vaccination was not practised. At the laboratory the animals were housed in an insect-proof stable. Their susceptibility to bluetongue virus was confirmed by indirect immunofluorescent tests conducted on sera collected 2 weeks after their arrival and again on the day of inoculation.

Infection of sheep

For quantitative assays, 2 sheep were inoculated subcutaneously in the auricula of the left ear with the appropriate virus dilution. Successful infection was assessed by the development of viraemia, pyrexia and specific antibody response in sera collected 30 days post-inoculation.

For the main experiment, the freeze-dried content of 5 vials of the virus stock was reconstituted in distilled water and pooled. Twenty susceptible sheep were each inoculated subcutaneously in the auricula of the left ear with 76 TC ID_{50} as estimated by titration in BHK cells. One or 2 animals were subsequently killed at 24-h intervals.

Clinical examination

Rectal temperatures were taken daily in the early morning; only those above 40,0 °C were considered febrile and recorded in the results. At the same time every animal was examined for clinical lesions of bluetongue.

Assessment of viraemia and antibody response

Following infection, 20 ml of blood was collected daily from the jugular vein of each sheep. Heparin, at a concentration of 10 units per ml of blood, was used as anticoagulant. The blood was centrifuged, the plasma separated and the blood cells resuspended in Eagle's MEM. After a 2nd centrifugation the supernate was decanted and the cells resuspended in a volume of Eagle's MEM equal to that of the plasma removed. When required, monolayers of BHK tissue culture cells were infected with serial decimal dilutions of the above blood cell suspension stored in the interim at 4 °C. Five tubes were inoculated with undiluted blood and 4 tubes with the successive dilutions. A negative result implies that no virus was isolated from a total volume of 1,0 ml of blood.

The antibody response was assessed from plasma by means of the immunofluorescent technique. The samples, stored at —20 °C, were diluted 1:10 in phosphate-buffered saline before use.

Collections of specimens

The experimental sheep used in the main experiment were killed by exsanguination. To facilitate this, each animal was inoculated intravenously with 10 000 units of sodium heparin 1 h before slaughter. During autopsy, carried out immediately after death, the following specimens were collected: lymph-nodes, epithelium of the tongue opposite the incisor teeth, portions of lung from the apical lobes, myocardium from the apical region and intestinal mucosa at the level of the ileo-caecal valve. Samples were obtained from the central portion of the trapezius muscle. Nasal swabs were taken from both nares of the sheep just before slaughtering and immersed in 2,0 ml of Eagle's MEM. The contents of the rectum as well as small volumes of urine from the bladder were collected.

Treatment of the specimens

After collection, solid tissues were individually rinsed in phosphate-buffered saline, pH 7,4 and stored at 4 °C in a 25 ml McCartney bottle filled with a 50% glycerol-buffered saline, pH 7,4. When required, enough of each specimen to make a 10% (m/v) suspension was washed in 3 changes of buffered saline, cut into small pieces and macerated in a Griffiths's tissue grinder. The intestinal content was immediately macerated and stored as a 10% (m/v) suspension. All suspensions were prepared in Eagle's MEM containing 500 i.u. of penicillin, 500 μg of streptomycin and 500 μg of Colistin (Banyu) per ml. The suspensions were centrifuged for 5 min to remove large cell debris and the supernate used as the $10^{-1,0}$ dilution. Confluent monolayers of BHK tissue culture cells were infected with serial decimal dilutions of each suspension, 5 tubes were inoculated with the $10^{-1,0}$ dilution and 4 tubes with each of the successive dilutions. The tubes were examined daily for 10 days for cytopathic effects. A negative result implied that no virus was isolated from 0,1 g of tissue.

Persistence of virus in infected sheep

Three susceptible sheep were infected with 10^6 TC ID_{50} of the virus stock used in the previous experiment. The animals were killed 6, 8 and 16 weeks post-inoculation and the tissues were collected and treated as described above.

Identification of the isolates

The identity of the virus isolates from each individual sheep was confirmed by a serum-virus neutralization test carried out according to the standard techniques used for bluetongue virus (Howell, 1969).

RESULTS

Assessment of the susceptibility of sheep to type 10 plaque-purified bluetongue virus

In order to assess the response and to determine the minimal infective dose for sheep, serial decimal dilutions of the virus stock varying from $10^{-3,0}$–$10^{-7,0}$ were inoculated into monolayers of BHK cell cultures and susceptible animals. The virus titres were $10^{6,0}$ and $10^{6,5}$ ID_{50}, respectively.

TABLE 1 Pyrexia and viraemia in sheep inoculated with decreasing concentrations of type 10 plaque-purified bluetongue stock virus

Sheep No.	Virus dilution inoculated		Days post-inoculation									
			1 to 5	6	7	8	9	10	11	12	13	14
1	10^{-3}	P	.	.	40,2	40,1	40,3	.	.	.	.	.
		V	.	+	+	+	+	+	+	.	.	.
2		P	.	.	.	40,3	.	.	.	.	.	.
		V	.	+	+	+	+	+	+	+	.	.
3	10^{-4}	P	.	.	40,3	41,1	40,1	.	.	.	.	.
		V	.	+	+	+	+	+	+	.	.	.
4		P	.	.	40,3	40,1	40,1	.	.	.	.	.
		V	.	+	+	+	+	+	+	.	.	.
5	10^{-5}	P	.	.	.	40,1	40,3	40,1	.	.	.	.
		V	.	.	+	+	+	+	+	+	.	.
6		P	.	.	.	.	.	.	.	.	.	.
		V	.	.	.	.	.	.	.	.	.	.
7	10^{-6}	P	.	.	.	.	40,3	40,3	.	.	.	.
		V	.	.	.	.	+	+	+	.	.	.
8		P	.	.	.	.	.	.	.	.	.	.
		V	.	.	.	.	.	.	.	.	.	.
9	10^{-7}	P	.	.	.	.	.	.	.	.	.	.
		V	.	.	.	.	.	.	.	.	.	.
10		P	.	.	.	.	.	.	.	.	.	.
		V	.	.	.	.	.	.	.	.	.	.

P=Pyrexia; V=Viraemia; . =Pyrexia or viraemia not demonstrated; + =Traces

The response of the animals inoculated with varying concentrations of virus is shown in Table 1. All the sheep inoculated with the $10^{-3,0}$ and $10^{-4,0}$ virus dilutions, but only 1 in each group of those inoculated with the $10^{-5,0}$ and the $10^{-6,0}$ dilutions, showed evidence of infection. The clinical signs in the animals inoculated with the $10^{-3,0}$ and $10^{-4,0}$ virus dilutions were observed on the 8th day post-inoculation and these persisted for 4–5 days. They consisted of hyperaemia of the oral cavity, slight oedema of the lips and mucous nasal discharge. Coronitis was observed in Sheep 1 and Sheep 4. In the reacting animals inoculated with the $10^{-5,0}$ and $10^{-6,0}$ virus dilutions, clinical symptoms were very mild. An antibody response 30 days post-inoculation was detected by immunofluorescence only in the animals with viraemia.

Assessment of the sensitivity of BHK cell cultures to type 10 *plaque-purified bluetongue virus following replication in sheep*

In this experiment the sensitivity of BHK cell cultures in detecting minimal concentrations of the virus present in the tissues of the infected animals was compared with that of the natural host.

Heparinized viraemic blood collected from an animal in the previous experiment and stored at 4 °C for 2 weeks was used. Decimal dilutions from $10^{-3,0}$ –$10^{-7,0}$ were inoculated into BHK cell monolayers and susceptible sheep. The virus titres estimated were $10^{5,5}$ ID_{50} and $10^{6,5}$ ID_{50}, respectively. The end point of the titration in cell cultures was obtained on the 6th day post-infection. In sheep, viraemia became demonstrable from the 6th day post-inoculation and was present in all animals receiving dilutions of virus from $10^{-3,0}$ –$10^{-5,0}$. An antibody response was detected only in animals in which viraemia was demonstrated.

Replication of bluetongue virus in sheep

For the main experiment, each of 20 sheep infected with 76 TC ID_{50} of the stock virus was clinically examined and bled daily. One or 2 animals were killed at 24-h intervals for the collection of specimens. Virus titres were determined as described under "Materials and Methods" and compared with the results of the clinical examination.

Incubation period

The first clinical sign observed was pyrexia. Only temperatures above 40 °C were considered febrile and are shown in Table 2. In 2 sheep the first temperature rise was recorded on the 6th day post-inoculation, in 7, on the 7th day, and in 1, on the 8th day. The mean incubation time was 6,9 days. The results of the infectivity titrations of blood and solid tissues taken at daily intervals are given in Tables 3 and 4. During the first stage of incubation, from Day 1 to Day 3, no virus was isolated from any of the tissues tested.

TABLE 2 Pyrexia in sheep inoculated with 76 TC ID_{50} of type 10 plaque-purified bluetongue stock virus

Sheep Number	Days post-inoculation						
	1 to 5	6	7	8	9	10	11
1 to 9	.						
10	.	41,1					
11	.	.					
12	.	.	40,6				
13	.	.	41,1	.			
14	.	.	40,6	40,1			
15	.	.	40,5	.			
16	.	.	40,2	40,2	40,3		
17	.	.	41,1	41,0	41,3	40,7	
18	.	40,4	40,5	.	.	.	
19	.	.	.	40,1	40,2	40,3	40,7
20	.	.	40,4	40,5	40,2	.	.

. =Pyrexia not demonstrated

TABLE 3 Viraemic titres of sheep inoculated with 76 TC ID_{50} of type 10 plaque-purified bluetongue stock virus

Sheep Number	Days post-inoculation						
	1 to 5	6	7	8	9	10	11
1 to 9.....	.						
10.......	.	1,6*					
11.......	.	2,6					
12.......	.	3,6	3,5				
13.......	.	4,0	4,3	4,5			
14.......	.	2,6	3,5	3,0			
15.......	.	2,6	3,5	3,3	3,0		
16.......	.	.	3,5	3,5	2,5		
17.......	.	3,6	3,5	3,6	3,0	.	
18.......	.	2,6	3,5	4,0	3,0	1,8	
19.......	.	.	3,6	4,6	4,0	3,3	1,6
20.......	.	2,6	3,6	4,0	3,0	2,0	2,0
Mean..	.	2,34	3,61	3,81	3,08	1,77	1,80

. = Viraemia not demonstrated; * = Log 10 TC ID_{50}/ml

In 1 of the 2 animals examined on Day 4, virus was isolated from the parotid, lateral retropharyngeal, mandibular, dorsal ruminal and renal lymph nodes, tonsils and spleen. In the lymphoid tissue associated with the site of inoculation and in the spleen, the virus was present in higher concentration than in the ruminal and renal lymph nodes where only trace amounts were detected. No virus was isolated from the blood.

On Day 5, virus was isolated from both sheep. The pattern of virus isolation and the virus concentrations were similar to those obtained from Sheep 7 on the previous day. On Day 4, virus was isolated from 7 different tissues, whereas on Day 5 virus was isolated from 10 tissues.

On the 6th day, viraemia became demonstrable in 9 of the 11 remaining sheep. In Sheep 16 and Sheep 19, viraemia was delayed to the 7th day. Viraemic titres varied from $10^{1,6}$–$10^{4,0}$ TC ID_{50} with a mean titre of $10^{2,3}$ TC ID_{50}. In Sheep 11 virus generalization appeared to be more advanced than in Sheep 10. Virus was not only demonstrated in the lymphoid organs but also in the lung, kidney, trapezius muscle, tongue and intestinal mucosa.

The clinical period

In the majority of the sheep, clinical signs commenced on the 7th day post-inoculation when 7 out out of the 9 animals became febrile. The duration and peaks of the febrile response were variable, the temperatures ranging between 40,1 °C and 41,3 °C. In some animals it lasted only 24 h, whereas in others it persisted for at least 4 days.

The mean viraemic titre was $10^{3,6}$ TC ID_{50} on the 7th day, reached a peak of $10^{3,8}$ on the 8th day and then gradually decreased to $10^{1,8}$ by the end of the observation period on the 11th day post-inoculation.

On Day 8, hyperaemia of the oral cavity and lips and a catarrhal nasal discharge became noticeable in all the animals. During the autopsy on Sheep 13 and Sheep 14 macroscopic pathological changes of the lymph nodes were observed. They were swollen, oedematous and congested. Complete virus generalization was attained, with a tendency for the virus concentrations to be higher than those encountered on the previous day.

Sheep 17 and Sheep 18 showed congestion of the coronets on the 9th day following inoculation. The mean viraemic titre was at a lower level and coincided with the detection of fluorescent antibodies to bluetongue virus in the plasma of the 6 animals. The correlation between viraemia and antibody response is illustrated in Fig. 1. The pathological lesions in the 2 sheep sacrificed at that time were again confined to the lymph nodes and were similar to those observed on the 8th day. Virus concentrations in most of the tissues remained constant.

TABLE 4 Titrations of the tissues of sheep killed at 24-hour intervals post-inoculation

Days post-inoculation	1 to 3	4		5		6		7	8		9		10		11	
Sheep Number	1 to 5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Lymph nodes:																
parotid.....................	.	.	3,0*	3,5	+	4,0	3,5	3,5	4,5	4,0	4,5	4,0	4,0	4,0	5,0	5,0
lat. retropharyngeal..........	.		3,5	3,0	3,5	3,5	3,5	4,5	3,5	4,5	4,0	3,5	4,0	4,0	4,0	5,0
mandibular.................	.	.	3,0	3,0	3,0	3,5	3,5	4,0	3,5	4,5	4,0	4,0	5,0	5,0	5,5	5,5
post-cervical...............	.	.	.	+	.	4,0	3,5	3,5	5,0	5,5	4,0	4,5	4,0	5,0	5,0	6,0
prescapular.................	.	.	.	.	.	.	4,0	4,0	3,5	5,0	3,5	4,0	4,0	4,5	4,0	4,0
left bronchial...............	.	.	.	.	.	.	3,5	4,5	5,0	4,0	4,5	4,0	3,5	4,5	5,0	4,0
hepatic.....................	.	.	.	.	.	3,5	.	3,0	5,0	3,5	5,0	4,0	4,0	4,0	4,0	4,0
splenic.....................	.	.	.	.	.	.	4,0	3,5	4,0	4,0	ND	4,0	4,0	4,0	3,5	4,0
dorsal ruminal..............	.	.	+	3,5	.	.	3,5	4,0	3,0	ND	4,0	3,5	4,0	4,0	5,0	4,0
caecal......................	.	.	.	+	.	.	3,5	4,0	3,0	4,5	3,0	4,5	4,0	4,0	4,0	4,0
renal.......................	.	.	+	.	3,0	3,5	3,5	3,5	4,0	4,5	4,0	4,5	4,5	5,0	5,0	6,0
deep hypogastric............	.	.	.	.	3,5	.	3,5	4,5	4,0	4,5	3,5	4,0	4,0	4,5	5,5	5,5
Tonsils......................	.	.	3,0	+	3,0	3,5	3,5	4,0	4,5	5,0	4,0	4,0	4,0	5,5	5,0	6,0
Lung.........................	.	.	.	.	.	4,5	4,0	4,0	4,5	5,5	4,0	5,0	3,5	5,0	4,5	5,5
Liver........................	.	.	.	ND	.	+	.	.	.	3,5	3,5	3,5	3,0	3,5	.	+
Spleen.......................	.	.	3,0	.	3,0	4,0	4,0	4,5	5,5	5,5	5,0	5,0	4,0	5,0	4,5	5,5
Kidney.......................	.	.	.	.	.	4,5	.	.	5,0	4,0	4,5	.	4,5	.	.	.
Trapezius muscle.............	.	.	.	.	.	.	3,0	.	3,5	3,5	3,5	3,5	3,5	4,0	4,0	4,0
Myocardium...................	.	.	.	.	.	.	.	.	4,0	.	.	.	3,0	3,5	3,5	3,5
Tongue epithelium............	.	.	.	.	.	.	3,5	3,5	4,5	.	3,5	3,5	4,0	4,0	3,5	4,5
Intestinal mucosa............	.	.	.	.	.	.	3,5	4,0	.	4,0	3,5	4,0	3,5	4,0	5,0	5,0
Thyroid......................	.	.	.	.	.	.	.	4,0	5,0	4,0	4,5	.	4,0	4,0	4,0	4,0

. = Virus not isolated; * = Log 10 TC ID_{50}/g; ND = Not done; + = Traces

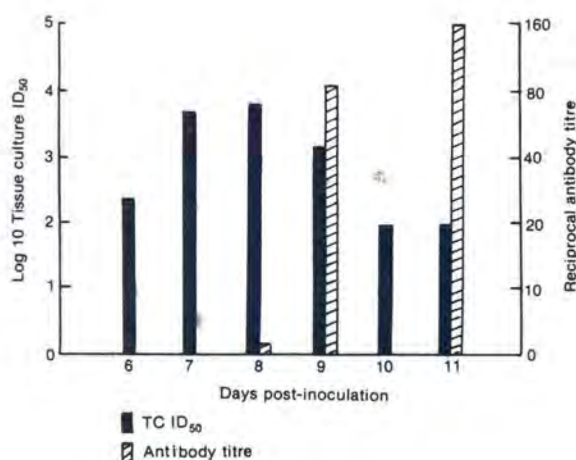

FIG. 1 Correlation between concentration of virus in blood and antibody titre

On the 10th and 11th days post-inoculation, the clinical signs were characterized by a more pronounced congestion of the oral cavity and, in Sheep 20, the coronets were inflamed. At a post mortem examination, no macroscopic lesions were observed. Virus titres in the blood decreased to low levels while higher antibody titres were recorded. The immune response did not affect the virus concentration in the organs and tissues.

No virus was isolated from the nasal discharge, urine or faeces of any of the animals examined.

Survival of the virus was not demonstrated in any of the tissues obtained from the sheep killed 6, 8 and 16 weeks post-inoculation.

Discussion

In studies on the pathogenesis of virus diseases, the method whereby the concentrations of virus in infected tissues taken from animals at fixed intervals post-inoculation are assayed, is a relatively crude technique since it does not provide information about the type of cell infected. It has nevertheless been extensively used and the work of Fenner (1948) on ectromelia is a classical example of this type of investigation. The quantitative approach makes it possible to follow events from the moment of entry of the virus into the host to the final stage of the infective process. However, because the quantitative evaluations are carried out on different animals, variation in individual susceptibility is an important factor influencing the interpretation of the results.

In this study on the pathogenesis of bluetongue, BHK cell cultures were used to assay the replication of the virus in the tissues of the infected sheep. It was shown by Pini, Coackley & Ohder (1966) that this system is suitable for the isolation of unmodified bluetongue virus and, in this investigation where a plaque-purified strain was used, the use of BHK cells appeared to be both practical and sensitive enough. This was confirmed from the results of the preliminary experiments. A comparison of the susceptibility of these cells with those of sheep showed that the ratio of TC ID_{50} to sheep ID_{50} did not exceed 1:10. The type 10 plaque-purified strain of virus maintained its pathogenicity for the animals, and lesions of bluetongue were observed when 10 or more TC ID_{50} of virus were inoculated subcutaneously. On the basis of these results, a dose of no more than 100 TC ID_{50} of the virus stock was considered to be adequate to infect consistently all the sheep to be used in the main experiment. The delay until the 6th day post-inoculation in the appearance of viraemia obtained with the virus preparation selected, together with the minimal infecting dose administered, provided favourable conditions for determining the primary sites of viral replication in the various tissues of the sheep. It was assumed that, if viraemia was not demonstrated within the first 6 days after infection, any virus detected in the tissues could be regarded as a genuine primary replication of the infective agent. The removal of plasma from the blood taken for the detection of viraemia and the washing of the blood cells were carried out in an attempt to remove antibody to bluetongue virus which, according to previous investigations by Pini *et al.* (1968) and Stair (1968), appeared in the circulation between the 6th and 9th days post-inoculation. Because bluetongue virus is cell-associated (Pini *et al.*, 1966; Luedke, 1970), the removal of plasma should not be expected to decrease the chances of successful virus isolation.

The response to the inoculation of bluetongue virus obtained in the experiments described above was comparable with that reported by Stair (1968) who used immunofluorescence. This author detected primary signs of disease, including hyperaemia of the oral cavity, as early as the 3rd day post-inoculation in the Rambouillet sheep used in his experiment. Secondary lesions became pronounced from the 7th day post-infection. Primary signs of disease were not encountered in the experiments described above nor have they been seen in analogous investigations with other strains of bluetongue virus. Their appearance could be due to various factors, since it is known that the interrelationship between bluetongue virus and the host can be influenced by the breed, individual susceptibility, environmental conditions and the stress and strain of the virus (Thomas & Neitz, 1947).

In this investigation, bluetongue virus was first demonstrated in the tissues of the infected sheep on the 4th day post-inoculation. Virus was detected in the tonsils, spleen and the lymph nodes of the cephalic area, indicating an affinity of the virus for the lymphoid tissue. On the 5th day post-inoculation, the results were similar to those obtained on the previous day. It can be assumed that the virus, after penetration into the host, associated itself with the macrophages or lymphoid cells, and thus gained access to the regional lymph nodes. From this point the virus was disseminated via the lymph and/or blood stream to the lymphoid tissue in other parts of the body where further replication took place. The gradient between virus concentrations in the lymph nodes of the cephalic area and those of other parts of the body suggests this as the mode by which the virus is disseminated. The simultaneous demonstration of antigen in the spleen suggests a low level of primary viraemia which could not be demonstrated by the techniques used.

The generalization of the virus throughout the tissues of the body was observed at the end of the incubation period, approximately 24 h before the occurrence of the febrile reaction and 48 h before the onset of overt signs of disease. Viraemia was demonstrable on the 6th day post-inoculation, approximately 48 h after virus was first detected in the lymphoid tissue.

After inoculating infective blood into sheep intravenously, Stair (1968) was able to demonstrate by immunofluorescence the affinity of the virus for the pericytes, endothelial, peri-endothelial and reticulo-endothelial cells. The virus was detectable from the 3rd day post-inoculation, reached maximum concentration between the 6th and 9th days and then showed a steady decline. Concentrations appeared to be greater in the oral mucosa than in any other tissue examined. Among the lymph nodes, those receiving afferent vessels from the tissues of the oral cavity showed the greatest intensity of fluorescence. The early localization of bluetongue virus in the lymphoid tissue of the cephalic area and in the spleen reported by Stair (1968) appears to be in agreement with the results of the present series of experiments. On the other hand, Stair (1968) detected virus simultaneously in the lymphoid tissue, oral mucosa, upper digestive and respiratory tracts. This appears to be the result of a more rapid spread of the virus throughout the host and it was probably due to the experimental procedures used by the author. From the results of the experiments reported here, bluetongue virus was detected outside the lymphoid tissue only on the 6th day post-inoculation and this coincided with the demonstration of viraemia. The demonstration of the antigen by immunofluorescence in the cells of the capillary and precapillary systems (Stair, 1968) explains the widespread secondary localization of the virus in most of the organs examined in these experiments.

Kidney and liver were the 2 organs from which virus isolation was not consistent and from which virus tended to disappear at an early stage. These results are also in agreement with those of Stair (1968), who did not detect specific fluorescence in either organ at any stage of the infective process. The isolation of the virus from renal and hepatic lymph nodes at a time when the respective organs did not produce virus indicates that the lymphoid tissue is a primary site of localization and the presence of the virus cannot be explained by the fact that the lymph node acts as a draining mechanism.

The antibody response was first detected on the 9th day post-inoculation and it coincided with a decrease in the viraemic titres from the highest level on the 8th day to the lowest on the 11th day post-inoculation. The antibody response detected by immunofluorescence did not appear to have any direct influence on the concentration of virus in the various tissues. A steady increase was observed in the virus titres in the various tissues examined from the 4th-11th days post-inoculation.

When the persistence of the bluetongue antigen in infected sheep was investigated, the fact that the virus was not isolated from any of the tissues of sheep sacrificed at the intervals of 6, 8 and 16 weeks after infection, appears to suggest that sheep do not play a role as virus carriers in the epizootiology of the disease.

References

BEKKER, J. G., DE KOCK, G. & QUINLAN, J. G., 1934. The occurrence and identification of bluetongue in cattle—the so-called pseudo-foot-and-mouth disease in South Africa. *Onderstepoort Journal of Veterinary Research & Animal Industry*, 2, 393–407.

BOWNE, J. G., 1971. Bluetongue disease. *Advances in Veterinary Science & Comparative Medicine*, 15, 1–46.

FENNER, F., 1948. The pathogenesis of acute exanthems. *Lancet*, 2, 915–930.

HOWELL, P. G., 1969. The antigen classification of strains of bluetongue virus, their significance and use in prophylatic immunization. D.V.Sc. Thesis, University of Pretoria.

KARSTAD, L. & TRAINER, D. O., 1967. Histopathology of experimental bluetongue disease in white-tailed deer. *Canadian Veterinary Journal*, 8, 247–254.

LUEDKE, A. J., 1970. Distribution of virus in blood components during viraemia of bluetongue. Proceedings of the 74th Annual Meeting, United States Animal Health Association, 9–21.

MACPHERSON, I. A. & STOKER, M. G. P., 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology*, 16, 147–151.

MOULTON, J. E., 1961. Pathology of bluetongue of sheep. *Journal of the American Veterinary Medical Association*, 138, 493–498.

PINI, A., COACKLEY, W. & OHDER, H., 1966. Concentration of bluetongue virus in experimentally infected sheep and virus identification by immunofluorescent technique. *Archiv für die gesamte Virusforschung*, 18, 385–390.

PINI, A., OHDER, H., WHITELAND, A. P. & LUND, L. J., 1968. Studies on the fluorescent and neutralizing antibodies to bluetongue virus in sheep. *Archiv für die gesamte Virusforschung*, 25, 129–136.

REED, L. J. & MUENCH, H., 1938. A simple method of estimating fifty per cent end points. *American Journal oj Hygiene*, 27, 493–497.

SPREUL, J., 1905. Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics*, 18, 321–337.

STAIR, E. L., 1968. The pathogenesis of bluetongue in sheep: a study by immunofluorescence and histopathology. Dissertation, Texas A & M University.

THEILER, A., 1906. Bluetongue in sheep. *Annual Report of the Director of Agriculture, Transvaal, for* 1904–1906, 110–121.

THOMAS, A. D. & NEITZ, W. O., 1947. Further observations on the pathology of bluetongue in sheep. *Onderstepoort Journal of Veterinary Science & Animal Industry*, 22, 27–40.

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