OBSERVATIONS ON THE ANTIGENIC RELATIONSHIP BETWEEN THE VIRUS OF ENZOOTIC ABORTION IN EWES AND VIRUSES OF THE PSITTACOSIS-LYMPHOGRANULOMA GROUP.

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RECENTLY Stamp and his co-workers (Stamp, McEwen, Watt and Nisbet, 1950) demonstrated a virus in the foetal membranes of ewes, and showed that it was responsible for cases of abortion and premature lambing in these animals. The infection is known as enzootic abortion of ewes (E.A.E.).

In its morphology and staining reactions the virus showed a resemblance to those of the psittacosis-lymphogranuloma group and could readily be grown in embryonated eggs. Preliminary investigations in this laboratory (Barwell and Bishop, 1951), using heated yolk-sac suspensions of E.A.E. virus in complement-fixation tests, showed that it will cross-react to titre with human sera from cases of psittacosis and lymphogranuloma venereum (L.G.V.). It was, therefore, considered desirable to investigate more fully the antigenic relationship between these three viruses, and this paper records the results of complement-fixation tests using absorbed and unabsorbed sera.

MATERIALS AND METHODS.

Virus strains.

The E.A.E. strain was that isolated by Stamp *et al.* (1950). Psittacosis virus, strain MOH. 154, was isolated by Professor S. P. Bedson in 1938 from a parrot. The J.H. strain of L.G.V. virus was supplied by Dr. G. Rake.

Sera.

Antisera to E.A.E. virus came from experimentally infected ewes and were kindly sent by Mr. Stamp ; some were also prepared in this laboratory by immunizing guinea-pigs. Human sera from cases of psittacosis and L.G.V. were used.

Antigens.

Yolk-sacs of developing hens' eggs inoculated between the 4th and 6th day of incubation were harvested as soon as most of them were found to be dead. This time, in the case of psittacosis virus, was between 2 and 3 days, and for the other two about 5 days. Each yolk-sac was placed in 2 ml. of M/50 phosphate buffer at pH 7·6 and allowed to stand overnight at 4°. Much of the virus is liberated into the hypotonic solution. The suspension was clarified by light centrifugation and pooled. The virus was then deposited by centrifuging for 1½ to 2 hours in an angle centrifuge at 4500 r.p.m. and was re-suspended in saline,

usually to half the original volume in the case of L.G.V. virus and to the full volume in the case of psittacosis and E.A.E. viruses. The amount of saline used depended on the quantity of virus seen in smears stained by Castaneda's method. If time permitted, the suspension was left overnight in the refrigerator so as to allow flocculation and removal of some extraneous material. The antigen prepared by this method appeared to be at least as good as that made in the usual way after grinding up the yolk-sacs in TenBroeck tubes ; rather it appeared to be purer in that it contained much less cell débris. Antigens were prepared from infected mouse lung or mouse spleen, as described above, from suspensions made by grinding the tissue in buffer by means of a TenBroeck tube. These were used in testing guinea-pig sera which, after immunization, contained antibodies to yolk-sac. Unheated antigens were used without preservative and as fresh as possible. For use as heated antigens, suspensions were placed in a boiling water-bath for 20 minutes, sodium azide was added to 0·3 per cent as a preservative and the product was finally titrated against dilutions of a known positive serum.

Complement.

Pooled guinea-pig serum stored in a dry ice unit was used. It was titrated on the day of the test and 2 M.H.D. was used in all tests.

The test.

This was done in the manner described by Bedson, Barwell, King and Bishop (1949). Appropriate doubling dilutions of serum, 2 M.H.D. of complement and an optimal dilution of the antigen, each in 0·1 ml. volume, were mixed and, after the addition of 0·5 ml. of saline, allowed to react at room temperature for $\frac{1}{2}$ hour and at 37° for $\frac{1}{2}$ hour. Serum controls and normal tissue antigen controls were included. Sheep cells, 0·2 ml. of a 5 per cent suspension sensitized with 5 M.H.D. of amboceptor, were added to each tube, and readings were made after incubation at 37° for $\frac{1}{2}$ hour and again after standing overnight at room temperature. Complete fixation was recorded as ++++, and degrees of partial fixation by a smaller number of + signs roughly indicating the amount of cells not haemolysed.

Absorption of sera.

Serum at a concentration 8 times that at which it showed definite but partial complement-fixation with the heated antigen was absorbed with the washed deposit from an equal volume of the virus suspension. After standing overnight in the refrigerator the absorbed serum was separated by angle centrifugation for 2 hours at 7000 r.p.m.

RESULTS.

Boiled or unheated suspensions of the three viruses were used to absorb in turn the different antisera studied. When the absorbed sera were tested against various heated and unheated antigens it was found in general that absorption with heated virus gave satisfactory and consistent results while those obtained with unheated virus were irregular.

Absorption with boiled virus.

Tables I and II show examples of absorption with heated, heterologous virus suspensions. In the first of these experiments serum from E.A.E. infected sheep 776 was tested, unabsorbed and after absorption with boiled psittacosis virus, against both boiled and fresh E.A.E. and psittacosis virus preparations. Absorption removed the antibody against both heated suspensions more or less completely and against the unheated heterologous virus. The absorbed serum still contained an antibody giving complement-fixation with the unheated homologous virus. It will be seen that the unheated antigens were used at twice the concentration of the boiled preparations and even then usually failed to react as strongly. The same results as those shown in Table I were obtained when heated E.A.E.

TABLE I.—*Absorption of E.A.E. Sheep Serum with Heated Psittacosis Virus.*

Serum. Name.	Dilution.	Antigens + 2 M.H.D. complement. Heated (100°) 1 in 4. Psittacosis.	Heated (100°) 1 in 4. E.A.E.	Unheated 1 in 2. Psittacosis.	Unheated 1 in 2. E.A.E.	Normal yolk-sac 1 in 2.	Saline.
E.A.E. serum 776, unabsorbed	1/8	+ + + +	+ + + +	+ + +	+ + + +	−	−
		+ + + +	+ + + +	+ +	+ + +	−	−
	1/16	+ + + +	+ + + +	+ + +	+ + + +		
		+ + + +	+ + + +	+ +	+ + +		
	1/32	+ + + +	+ + + +	+ + +	+ + + +		
		+ + + +	+ + + +	+ +	+ + +		
	1/64	+ + +	+ + +	+ +	+ + +		
		+ + +	+ + +	+ +	+ +		
E.A.E. 776, absorbed with heated psittacosis virus	1/8	−	+	−	+ + + +	−	−
		−	±	−	+ + +	−	−
	1/16	−	±	−	+ + +		
		−	−	−	+ +		
	1/32	−	−	−	+ + +		
		−	−	−	+ +		
Pooled normal guinea-pig serum	1/4	−	−	−	−	−	−
		−	−	−	−	−	−
Saline		−	−	−	−	−	−
		−	−	−	−	−	−

Readings after 30 min. at 37° (upper row) and after standing overnight at room temperature (lower row).

virus was used for absorption. Similarly, absorption of human sera from cases of L.G.V. (Table II) or psittacosis with heated E.A.E. virus removed antibody to all heated suspensions and to unheated heterologous virus preparations, while fixation with fresh homologous virus was only slightly reduced. Except in so far as different batches of fresh partially purified suspensions of the same virus vary in their complement-fixing potency, the results obtained by absorbing a given serum with any of these heated virus preparations were consistently the

same. Whatever combination of serum and heated virus was tested for absorption, antibody only to the unheated homologous suspension was left.

Absorption with unheated virus.

Absorption with the different unheated virus suspensions has, however, been found to give somewhat irregular and unpredictable results. Examples of the findings in some of these experiments are given in the next two tables, but it

TABLE II.—*Absorption of Human L.G.V. Serum with Heated E.A.E. Virus.*

Serum.		Antigens + 2 M.H.D. complement.				
		Heated (100°) 1 in 4.	Unheated 1 in 2.		Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	L.G.V.	L.G.V.	E.A.E.		
L.G.V. serum P. unabsorbed	1/16	++++ . ++++	++++ . ++++	++++ . ++++	— . —	—
	1/32	++++ . ++++	++++ . ++++	++++ ++++		
	1/64	++++ . ++++	+++ . +++	+++ +++		
L.G.V. serum P. absorbed with heated E.A.E. virus	1/16	++ . ++	++++ . ++++	+ . ±	— . —	—
	1/32	± . —	+++ . ++	— . —		
	1/64	— . —	± . —	— . —		
Normal human serum	1/4	— . —	— . —	— . —	— . —	—
Saline		— . —	— . —	— . —	— . —	—

must be emphasized that these illustrate results which are by no means consistent. In Table III E.A.E. sheep serum 771 has been absorbed with unheated psittacosis virus, and slight but equal reduction of fixing power for the heated and for the two unheated suspensions has resulted. At other times heterologous absorption of this kind effectively removes the group antibody, and leaves the specific one as shown in Table IV in the columns referring to the behaviour of serum P. (L.G.V.) after treatment with unheated E.A.E. or psittacosis viruses. These two tables also show the results which may be obtained when a serum is absorbed with the unheated homologous virus. The usual effect is that set out in Table III, where both types of antibody have been completely or almost completely removed (Bedson, 1936). In Table IV the homologous, unheated virus, which was expected to absorb both antibodies from serum P., has only partially reduced the group antibody, while the specific antibody seems to have been more selectively absorbed.

DISCUSSION.

The results consistently obtained by absorbing sera with heated virus provide further support for the idea that these agents possess at least two antigens, one heat-

resistant and group-reactive, the other heat-labile and specific (Bedson, 1936; Barwell, 1948; Bedson *et al.*, 1949). The direct complement-fixation and cross-absorption experiments clearly show that E.A.E. virus possesses the heat-stable group antigen in common with the viruses of the psittacosis-lymphogranuloma group, and that it too has a specific component which is destroyed by heat. Absorption of sera with unheated virus gives irregular and unpredictable results.

TABLE III.—*Absorption of E.A.E. Sheep Serum with Unheated E.A.E. and Psittacosis Viruses.*

Serum.		Antigens + 2 M.H.D. complement.					
		Heated (100°) 1 in 4.		Unheated 1 in 2.		Normal yolk-sac 1 in 2.	Saline.
Name.	Dilution.	E.A.E.	Psittacosis.	E.A.E.	Psittacosis.		
E.A.E. serum 771, unabsorbed	1/8	++++ . ++++	++++ . ++++	++++ . ++++	++++ . +++	— . —	— . —
	1/16	++++ . ++++	++++ . ++++	++++ . ++++	++++ . +++		
	1/32	++++ . ++++	++++ . ++++	++++ . ++++	+++ . +++		
	1/64	++++ . +++	++++ . +++	++++ . +++	+++ . +++		
E.A.E. 771, absorbed with unheated psittacosis virus	1/8	++++ . ++++	++++ . ++++	++++ . ++++	++++ . ++++	— . —	— . —
	1/16	++++ . +++	+++ . +++	++++ . ++++	++++ . ++++		
	1/32	— . —	± . —	± . —	± . —		
E.A.E. 771, absorbed with unheated E.A.E. virus	1/8	— . —	— . —	++ . +	± . —	— . —	— . —
	1/16	— . —	— . —	± . ±	— . —		
	1/32	— . —	— . —	— . —	— . —		
Normal human serum	1/4	— . —	— . —	— . —	— . —	— . —	— . —
Saline		— . —	— . —	— . —	— . —	— . —	— . —

Using the homologous virus the usual result is the removal of both types of antibody, though occasionally this treatment may absorb most of the specific antibody whilst producing only a moderate reduction in the group antibody. Heterologous absorption with unheated virus in some experiments had the effect of reducing partially and to a comparable extent the titre for heated and unheated virus suspensions, whereas at other times there was a selective and more or less complete removal of group antibody. These irregular effects of absorbing with

fresh virus preparations are not necessarily at variance with the concept of two antigenic components in these viruses. Experience in this laboratory suggests that the serological activity of the specific antigen is much the weaker of the two ; and it is for this reason that the fresh suspensions are used as antigens at a higher concentration than the heated ones. However fresh the virus preparation, it is impossible to predict with certainty that the specific antigen will be present in a fully serologically reactive state. Furthermore it is labile, and preparations which when first prepared reacted specifically to full titre may lose this character on storage at 4° in three or four weeks' time. This deterioration of the specific antigen may or may not be accompanied by enhanced activity of the group

TABLE IV.—*Absorption of Human L.G.V. Serum with Unheated Homologous and with Two Unheated Heterologous Viruses.*

Serum.		Antigens + 2 M.H.D. complement.							
		Heated 100° 1 in 4.	Unheated 1 in 2.			Normal yolk-sac 1 in 2.	Saline.		
Name.	Dilution.	Psitta-cosis.	L.G.V.	E.A.E.	Psitta-cosis.				
L.G.V. serum P., unabsorbed	1/16	+ + + +	+ + + +	+ + + +	+ + + +	—	—		
		+ + + +	+ + + +	+ + + +	+ + + +	—	—		
	1/32	+ + + +	+ + + +	+ + + +	+ + + +				
		+ + + +	+ + + +	+ + + +	+ + + +				
	1/64	+ + + +	+ + + +	+ + + +	+ + + +				
		+ + + +	+ + +	+ + +	+ + + +				
L.G.V. serum P., absorbed with unheated L.G.V. virus	1/16	+ + + +	+ +	+ + + +	+ + + +	—	—		
		+ + + +	+ +	+ + + +	+ + + +	—	—		
	1/32	+ + + +	±	+ +	+ + +				
		+ + + +	—	+ +	+ + +				
	1/64	±	—	—	±				
		—	—	—	—				
L.G.V. serum P., absorbed with unheated E.A.E. virus	1/16	+	+ + + +	—	+	—	—		
		+	+ + + +	—	+	—	—		
	1/32	—	+ + + +	—	—				
		—	+ + +	—	—				
	1/64	—	+ +	—	—				
		—	+	—	—				
L.G.V. serum P., absorbed with unheated psittacosis virus	1/16	—	+ + + +	—	—	—	—		
		—	+ + + +	—	—	—	—		
	1/32	—	+ + +	—	—				
		—	+ + +	—	—				
	1/64	—	+ +	—	—				
		—	+	—	—				
Normal human serum	1/16	—	—	—	—	—	—		
		—	—	—	—	—	—		
Saline		—	—	—	—	—	—		
		—	—	—	—	—	—		

TABLE V.—*Human L.G.V. Serum Tested at Intervals against Unheated or Boiled Fractions of an L.G.V. Suspension.*

L.G.V. antigen J.H. 15. Prepared 23.vii.47. Diluted 1 in 4.

Serum. Name.	Dilution.	Unheated. Tested on— 1.viii.47.	15.viii.47.	19.viii.47.	Heated at 100° on— 23.vii.47. Tested on— 1.viii.47.	15.viii.47.	19.viii.47.	19.viii.47. Tested on— 19.viii.47.	Normal yolk-sac.	Saline.
L.G.V. serum GRE.	1/4	++++	+++	++	++++	++++	++++	++++	—	—
		++++	++	+	++++	++++	++++	++++	—	—
	1/8	++++	+	+	++++	++++	++++	++++		
		++++	±	±	++++	++++	++++	++++		
	1/16	++++	+	∓	++++	++++	++++	++++		
		++++	±	—	++++	++++	++++	++++		
	1/32	++++	±	—	++++	++++	++++	++++		
		++++	∓	—	++++	++++	++++	++++		
	1/64	++++	∓	—	++++	++++	++++	++++		
		+++	—	—	++++	++++	++++	++++		
Normal human serum	1/4	—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—
Saline		—	—	—	—	—	—	—	—	—
		—	—	—	—	—	—	—	—	—

antigen. In other words the labile component may have little or no serological activity, while still being capable of interfering with the group antigen. This phenomenon was observed by Bedson (1936) with psittacosis virus, and that the same thing may occur with L.G.V. virus is shown by the findings presented in Table V. The explanation of this is not known, but it could be accounted for by some qualitative change in the specific antigen itself. The possibility that absorption of tissue components present in the suspension may play a part has not been excluded.

It appears, however, that fresh virus may react with sera containing both types of antibody in one of three ways. Both specific and group antigens may be available and react with their respective antibodies, and this is what happens most commonly. Sometimes the fresh virus behaves mainly as a specific antigen, and on other occasions it may have little or no activity unless heated when, of course, it behaves as a group antigen. In view of this it is not surprising that the results of absorbing sera with unheated viruses of this group tend to be unpredictable, and further work on the serological behaviour of these agents is required to gain a further insight into their antigenic complexity. However, this in no way detracts from the use which can be made of sera absorbed with heated virus in the study of unidentified viruses belonging to this group, and this technique can enhance the value of tests on patients' sera.

SUMMARY.

The virus of enzootic abortion in ewes has been studied in complement-fixation tests using as antigens, unheated and boiled virus suspensions.

Sera against psittacosis and lymphogranuloma venereum viruses and similar preparations of these agents were used in cross-absorption experiments.

The sheep virus was found to possess the heat-stable group antigen common to the group as well as a heat-labile, specific component.

The antigenic behaviour and relationships of these viruses are discussed.

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