

VARIATION IN COMPLEMENT-FIXING ACTIVITY OF *RICKETTSIA BURNETI* DURING EGG ADAPTATION

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INTRODUCTION

Evidence that strains of *Rickettsia burneti* differ in complement-fixing activity was put forward by Robbins, Rustigian, Snyder & Smadel (1946) and Topping, Shepard & Huebner (1946). These workers compared strains isolated in Europe, America and Australia, and found that they could be divided into two groups: (a) strains such as the Italian 'Henzerling' strain which reacted in high dilution with both homologous and heterologous antisera; and (b) those such as the American 'Dyer' and the Panama strains which showed little complement-fixing activity, except at low dilution, with any antisera. Topping and his colleagues found similar differences between the antigens in their sensitivity to agglutination by antisera, but agglutination cross-absorption tests failed to give clear evidence of more than one antigenic fraction.

These studies demonstrated the superiority of the Henzerling strain for detection of complement-fixing antibodies, and this is now widely used for the preparation of antigen for routine diagnosis. Subsequently, however, Wolfe & Kornfeld (1949) found no antigenic difference between the Henzerling strain and the American 'Nine Mile' strain (in contrast to the American Dyer strain) so that Nine Mile strain antigen is also used for routine diagnosis.

Two strains of *R. burneti* isolated in Britain appeared at first to resemble the Dyer strain because antigens prepared from infected yolk sacs failed to fix complement with homologous antisera. After several egg passages, however, the strains were found to react almost as well as the Henzerling strain with these antisera (Stoker, 1950). It seemed unlikely that this was due to accidental contamination with the Henzerling strain because the freshly adapted strain still differed from the Henzerling strain in pathogenicity for chick embryos. The increase in complement-fixing activity of the strains after egg adaptation, was independent of numbers of rickettsiae in the antigens and raised the possibility of a change in antigenic structure. Herzberg & Urbach (1951) subsequently reported similar findings with German strains which increased in complement-fixing activity after fourteen or fifteen passages in eggs.

This paper describes further studies on the change in behaviour of a single strain of *R. burneti* during egg adaptation. First, it seemed desirable to test antigens from each successive passage to find out when the alteration in complement-fixing activity occurred and whether it was sudden or gradual. Antigens of each type, from an early and a late passage, were then compared in more detail with each other and with Henzerling strain antigen. In these comparisons the conglutinating complement absorption test was used to find out if failure of

fixation by early passage antigen extended to other types of complement besides haemolytic complement.

The reaction was also investigated by the agglutination and the antiglobulin sensitization tests. The latter detects the presence of adsorbed antibody globulin on an antigen by addition of an antiglobulin serum, and it does not depend on any observable reaction in the primary antibody antigen union (Coombs & Stoker, 1951). This test was used to find out if the inability of the early passage antigen to fix complement was in fact due to failure to unite with antibody.

METHODS

(1) *Strains*. The Christie strain, which was the main subject of this investigation, was isolated in London by MacCallum, Marmion & Stoker (1949) and its characteristics were reported by Stoker (1950). Except for the behaviour as a complement-fixing antigen, this strain of *R. burneti* closely resembled the classical Henzerling strain and there was reciprocal cross-immunity in guinea-pigs between the two strains.

After the original isolation in guinea-pigs, the Christie strain was passaged once to further guinea-pigs and a suspension of spleen and lung from one of the latter was used as starting material for adaptation of the strain to eggs for the experiments described below. The classical Italian Henzerling strain has already been adapted to eggs by an unknown number of passages since the first isolation in 1945.

(2) *Egg inoculation*. Fertile White Leghorn hen's eggs were incubated for 6 or 7 days at 37° C. before inoculation into their yolk sacs of 0.25 ml. quantities of infected material. Subsequent incubation was at 35–36° C. with candling twice daily. After 3 days yolk sacs were harvested from the eggs as they died and from survivors 14 days after inoculation. Impression smears were stained by Giemsa stain, and tests for bacterial sterility made by aerobic culture on blood agar slopes.

Most of the infected yolk sacs from each passage were stored in glass bottles at –22° C. until they were used for antigen preparation, but two or three were selected for preparation of seed suspension for inoculation of the next passage. These yolk sacs were also stored at –22° C. until the day of inoculation when seed suspension was prepared by shaking them with an equal volume of 10 % horse serum broth and glass beads. After light centrifugation, the supernatant suspension (diluted 5–20 %) was used for inoculation of the next batch of eggs.

(3) *Antigen preparation*. After nine passages, antigens were prepared from the stored yolk sacs of each passage of the Christie strain excluding the first two passages (see below), and also from yolk sacs infected with the Henzerling strain. After weighing, the pooled yolk sacs of each batch were emulsified, to give 20 % (w/v) suspensions, in 0.5 % formol saline buffered at pH 7.0. These suspensions were stored for 48 hr. at +4° C. and then centrifuged for 30 min. at 10,000 r.p.m. at room temperature. The deposits were resuspended, shaken with an equal volume of ethyl ether and allowed to separate at +2° C. overnight. The aqueous phases were removed, centrifuged, and overnight ether extraction of the resuspended deposits repeated. The aqueous phases were now subjected to repeated extractions with ether for 1 hr. at room temperature until a clear interface was

seen between ether and aqueous phases. After removal of ether under low pressure, the rickettsiae in the aqueous phase were washed three times by centrifugation and resuspension and finally resuspended in one-fifteenth the original volume in 0.25 % formol saline buffered at pH 7.0 and stored at +2° C.

Smears from each antigen were stained by Giemsa and Macchiavello's stains and some of the antigens were also examined by electron microscopy. The antigens were standardized by dilution to equal opacity as judged visually in strong indirect light against a dark background.

(4) *Antisera*. Homologous human antiserum was used for most of this study of the Christie strain. This was obtained, 4 months after the onset of his illness, from the patient who had been the original source of the Christie strain. This serum (and in our hands, most other human Q fever antisera) showed poor ability to agglutinate Q fever antigens despite a high titre of complement-fixing antibody. Agglutination and agglutinin-absorption tests were, therefore, carried out with a specimen of whey from a naturally infected cow, which was known to agglutinate Q fever antigens strongly. The whey was prepared from milk by the technique described by Stoker & Marmion (1952). A few tests were also done with convalescent sera from guinea-pigs infected with Henzerling, Christie and naturally occurring bovine strains of *R. burneti*, which were bled 28–35 days after inoculation.

All sera and whey were inactivated at 56° C. for 30 min. before testing.

(5) *Serological techniques*. The haemolytic complement-fixation test, the conglutinating complement-absorption test, the direct agglutination test and the anti-globulin sensitization test were all performed using techniques described elsewhere (Stoker & Marmion, 1952). It must be emphasized, however, that in the haemolytic complement-fixation test, two exact units of complement are used, and these are determined by previous overnight titration of complement in the presence of antigen in the dilution or dilutions to be used in the final test. One unit of complement is taken as the quantity in the last tube showing about 75 % haemolysis (read after centrifugation). This is only a little less than the quantity of complement required to produce complete haemolysis, because with 1% sheep cells and five minimal haemolytic doses of haemolysin, the end-point of the complement titration is sharp.

RESULTS

Adaptation of Christie strain to yolk sacs

The Christie strain was passaged nine times successively in yolk sacs, and large numbers of rickettsiae were seen in smears from each passage. The eggs of the first passage inoculated with the original guinea-pig suspension, although they were heavily infected, were alive when harvested 14 days after inoculation. Most of the second-passage eggs died between the 11th and 14th day, but from the third to the ninth passage deaths occurred regularly around the end of the first week with a mean for successive passages between 6.3 and 8.6 days after inoculation.

In order to avoid the possible influence of the age of the embryo upon the antigenicity of the rickettsiae, antigens were only prepared from yolk sacs of each of passages 3 to 9 (inclusive) in which deaths had all occurred at approximately

the same age of embryo. These antigens were called C1, C2, C3, etc., according to the passage number. An antigen was also prepared from yolk sacs infected with the Henzerling strain of *R. burneti*. The technique was the same except that yolk sacs from living as well as dead embryos were used.

Smears from all these antigens showed highly purified suspensions of rickettsiae except for passage 4 antigen, in which there was also an occasional bacterial contaminant. The Christie antigens were all approximately equal in opacity, but the Henzerling antigen was less dense, and presumably contained fewer rickettsiae per g. of original yolk sac. The antigens were diluted to a standard equal opacity as follows:

Christie passage 3 antigen (C3)	1:10
" " 4 " (C4)	1:10
" " 5 " (C5)	1:10
" " 6 " (C6)	1:10
" " 7 " (C7)	1:10
" " 8 " (C8)	1:8.4
" " 9 " (C9)	1:12.4
Henzerling antigen	1:4

The matched antigens were then reckoned as 1:10 dilutions of a standardized suspension and dilutions were made up and expressed in terms of this standard.

Preliminary overnight titrations of guinea-pig complement in the presence of these antigens revealed no anticomplementary action at 1:10. Ascending dilutions of each antigen were then titrated in chequerboard fashion with ascending dilutions of Christie antiserum, and the results are shown in Table 1.

The Henzerling antigen gave good fixation with high serum and antigen titres. The antigens prepared from the Christie strain at the third and fourth egg passages, however, failed to fix complement at all, except in the presence of high concentrations of the homologous antiserum. There was a sudden increase in complement-fixing ability of the antigen at the fifth passage and little further change in behaviour of antigens of later passages which all resembled the Henzerling strain antigen.

Comparison of C3, C9 and Henzerling antigens

After this preliminary experiment more detailed comparisons were limited to Henzerling strain antigen and Christie strain antigens of egg passages 3 and 9. Fresh batches of these three antigens were prepared in larger quantities and standardized by opacity as before. This time the C3 antigen was twice as dense as the C9 and the Henzerling antigens. The C3 antigen was, therefore, diluted with an equal volume of saline to match the other two antigens, and all dilutions have been expressed in terms of these standardized suspensions. Electron microscopy of these antigens showed very little extraneous material, and there was no obvious morphological difference between the rickettsiae.

It should be noted that these variations in rickettsial concentration for different batches of antigen do not affect the results. If dilutions of antigen are expressed in terms of the original unmatched suspensions, the differences between the antigens are not substantially altered.

Conglutinating complement-absorption test

C3, C9 and Henzerling antigens were now compared by the haemolytic complement-fixation test and the more sensitive conglutinating complement-absorp-

Table 1. *Haemolytic complement-fixation tests with Christie strain human antiserum and Christie strain antigens from successive yolk-sac passages, as well as Henzerling strain antigen.*

Yolk-sac passage no. of Christie antigen	Standardized antigen dilution (reciprocals)	Dilution of Christie serum (reciprocals)							Antigen control (units of complement)		
		10	20	40	80	160	320	640	2	1	0.5
3	5	4	2	0	0	0	0	0	0	2	4
	10	2	0	0	0	0	0	0	0	2	4
	20	0	0	0	0	0	0	0	0	2	4
	40	0	0	0	0	0	0	0	0	2	4
4	10	2	0	0	0	0	0	0	0	1	4
	20	2	0	0	0	0	0	0	0	1	4
	40	1	0	0	0	0	0	0	0	1	4
	80	0	0	0	0	0	0	0	0	1	4
5	10	4	4	4	4	4	1	0	0	1	4
	20	4	4	4	4	4	1	0	0	1	4
	40	4	4	3	3	2	0	0	0	1	4
	80	4	2	1	0	0	0	0	0	1	4
6	10	4	4	4	4	4	0	0	0	2	4
	20	4	4	4	4	4	0	0	0	1	4
	40	4	4	4	4	2	0	0	0	1	4
	80	4	3	1	2	0	0	0	0	1	4
7	10	4	4	4	4	4	0	0	0	1	4
	20	4	4	4	4	3	0	0	0	1	4
	40	4	4	4	4	2	0	0	0	2	4
	80	3	1	1	1	0	0	0	0	2	4
8	10	4	4	4	4	4	1	0	0	3	4
	20	4	4	4	4	4	0	0	0	1	4
	40	4	4	4	4	3	1	0	0	1	4
	80	4	3	1	0	0	0	0	0	2	4
9	10	4	4	4	4	3	0	0	0	1	4
	20	4	4	4	4	4	1	0	0	1	4
	40	4	4	4	4	4	0	0	0	1	4
	80	4	4	4	2	2	0	0	0	1	4
Henzerling antigen	10	4	4	4	4	4	2	0	0	2	4
	20	4	4	4	4	4	3	0	0	2	4
	40	4	4	4	4	4	1	0	0	1	4
	80	4	4	4	4	3	1	0	0	1	4
Serum control	.	0	0	0	0	0	0	0	.	.	.

(All antigens were of approximately equal rickettsial concentration before dilution. 4 denotes complete fixation of complement, 3, 2, 1 denote degrees of partial fixation of complement, 0 denotes no fixation of complement. The same designation applies to succeeding tables.)

tion test, to find out whether the C3 antigen fixed conglutinating complement despite its failure to fix haemolytic complement with the homologous antiserum. The results appear in Table 2.

In the haemolytic test, as with previous antigens, C9 closely resembled Henzerling antigen, and differed markedly from C3. There was some reaction between C3 antigen and Christie serum at 1:10 only but this was present even when the antigen was diluted to 1:80.

The conglutinating complement-absorption test was carried out with horse complement and the intrinsic sensitivity of the test was further enhanced by the relatively small amount of free complement as compared to that in the haemolytic test. As seen in Table 2, serum titres were higher with all three antigens but there was still a marked difference between that observed with Henzerling and C9 on the one hand, and C3 on the other. Fixation between C3 antigen and Christie anti-serum, in fact, showed the same pattern of high antigen and relatively low serum titre as was demonstrated in the haemolytic complement-fixation test.

It thus appeared that rickettsial suspensions prepared during the early stages of egg adaptation of the Christie strain failed to fix either complement with high dilutions of homologous antiserum. The next step was to determine if this was due to a failure of the antigen to unite with antibody at all under these conditions or whether union took place but failed to fix complement.

Antiglobulin sensitization test and agglutination reaction

The antiglobulin sensitization test was used to find out if antibody globulin was adsorbed on to the surface of the rickettsiae, and for this test the titre of the Christie serum was expressed as the highest dilution which so sensitized the rickettsiae that they were strongly agglutinated by antiglobulin serum. It will be seen from the results in Table 3 that all three antigens adsorbed globulin from Christie serum at high dilution, despite the difference in complement-fixing ability. From this it would appear that the early passage antigen reacts well enough with antibody in the higher dilutions of serum but fails to fix complement.

As already mentioned, Christie antiserum gives poor direct agglutination of any Q fever antigens tested. When the serum was fresh an agglutination titre of 1:20 was observed with Henzerling antigen, but after 2 years storage at -20° C. agglutinins were no longer detectable with Henzerling or Christie antigens. Q fever antibodies in whey from the milk of infected cows, however, agglutinate rickettsial suspensions strongly, and for this reason a selected specimen of whey was used to compare C3, C9 and Henzerling antigens for susceptibility to agglutination and ability to fix complement. The results (Table 4) show that there was agglutination and complement fixation up to a whey dilution of 1/160 with C9 and Henzerling antigens, but although C3 antigen was strongly agglutinated by whey diluted to 1:80, there was only partial fixation of complement at low dilutions of whey.

Other sera tested

Haemolytic complement-fixation tests were carried out with another human convalescent serum and with pools of sera from guinea-pigs inoculated with naturally infected milk, with Henzerling strain yolk sac suspension and with Christie strain yolk sac suspension. The latter suspension was from fourth passage eggs in a series different from that described above and in which the change in

Table 3. *Antiglobulin sensitization tests using Christie strain human antiserum with Christie strain antigens from passages 3 and 9, and Henzerling strain antigen*

Antigen	Treated antigen resuspended in	Dilution of Christie human serum used for treating antigen (reciprocals)								Un-treated antigen controls
		5	10	20	40	80	160	320	640	
C3, 1:5	Normal rabbit serum, 1:20	++	++	+	-	-	-	-	-	-
	Rabbit antiglobulin serum, 1:20	++	++	++	++	++	++	-	-	-
C9, 1:5	Normal rabbit serum, 1:20	++	++	+	-	-	-	-	-	-
	Rabbit antiglobulin serum, 1:20	++	++	++	++	++	++	+	-	-
Henzerling, 1:5	Normal rabbit serum, 1:20	+	+	-	-	-	-	-	-	-
	Rabbit antiglobulin serum, 1:20	++	++	++	++	++	++	+	-	-

(++ denotes complete or almost complete agglutination of rickettsiae by antiglobulin serum; + denotes partial agglutination; - denotes no agglutination.)

Table 4. *Reaction between Q fever antibodies in whey and Christie strain antigens from passages 3 and 9 and Henzerling strain antigen. Comparison of haemolytic complement-fixation test and agglutination reaction*

Haemolytic complement-fixation test											
Antigen	Standardized antigen dilution (reciprocals)	Dilutions of whey (reciprocals)						Antigen control (units of complement)			
		5	10	20	40	80	160	320	2	1	0.5
C3	5	3	3	2	1	0	0	0	0	1	4
	10	1	2	2	0	0	0	0	0	1	4
	20	0	1	1	0	0	0	0	0	1	4
	40	0	0	0	0	0	0	0	0	1	4
C9	5	4	4	4	4	4	4	0	0	1	4
	10	4	4	4	4	4	1	0	0	1	4
	20	0	0	1	2	1	0	0	0	1	4
	40	0	0	0	0	0	0	0	0	1	4
Henzerling	5	4	4	4	4	4	4	0	0	1	4
	10	4	4	4	4	4	4	1	0	1	4
	20	0	0	3	3	3	3	1	0	1	4
	40	0	0	0	0	0	0	0	0	1	4
		0	0	0	0	0	0				

Agglutination reaction									
Antigen	Standardized antigen dilution (reciprocals)	Dilution of whey (reciprocals)							Antigen control
		5	10	20	40	80	160	320	
C3	10	++	++	++	++	++	-	-	-
C9	10	++	++	++	++	++	+	-	-
Henzerling	10	++	++	++	++	++	++	-	-

(++ denotes complete or almost complete agglutination; + denotes partial agglutination; - denotes no agglutination.)

behaviour of complement-fixing antigen occurred between the sixth and eleventh passages. These sera resembled the Christie serum and the whey in that much higher titres of complement-fixing antibody were observed with Henzerling and C9 antigens than with C3 antigen.

Attempts to differentiate antigenic components

The experiments suggested that the antigens from early egg passages of Christie strain combined with Q fever antibody, but in so doing were unable to fix complement, at least in low antibody concentration. After egg adaptation antigens combined with antibody and fixed complement as well, and resembled Henzerling strain antigen in this respect. One possible explanation was that the antigen was partially masked in some way in the C3 suspension, allowing absorption of antibody but not of complement, and that this masking effect was lost in further passage. Alternatively, an entirely different (non-complement-fixing) antigen might occupy the surface of the C3 rickettsiae and be replaced by the usual complement-fixing antigen on passage.

Attempts to alter the antigen or to differentiate two antigens if present, were made by treatment with heat. C3 and Henzerling antigens were heated for 30 min. at 56, 70 and 100° C. When these treated antigens were tested by the antiglobulin test and haemolytic complement-fixation test with Christie antiserum, however, there was no alteration of the ability of either C3 and Henzerling antigens to absorb antibody globulin, or of Henzerling antigen alone to fix complement with antiserum. Heated C3 antigen, however, still failed to fix complement except with low dilutions of serum.

C3, C9 and Henzerling antigens were also made from infected yolk sacs by centrifugation and adsorption by Celite without the use of ether, to find out if the latter had any effect on the action of the antigens. The Celite-treated antigens contained more extraneous material than the other ether-treated antigens and were unsuitable for antiglobulin tests, but in haemolytic complement-fixation tests with Christie antiserum they behaved like ether-treated antigens, and C9 and Henzerling, but not C3 antigen-fixed complement with Christie antiserum.

Further attempts to differentiate antigenic components were made by absorbing Christie serum and whey by C3, and C9 and Henzerling antigens, but there was no clear evidence of more than one antigenic component. When absorbed Christie serum was tested by the complement-fixation test, C9 antigen behaved like Henzerling strain antigen. Antiglobulin tests on the absorbed serum, however, showed an important distinction between the Christie antigens on the one hand and the Henzerling antigen on the other. Both C3 and C9 suspensions absorbed antibody fractions reacting with C3 and C9, but there was little effect on the antibody fraction reacting with Henzerling antigen.

This suggests that even after egg adaptation the Christie strain remains antigenically distinct from the Henzerling strain despite its resemblance to the latter as a complement-fixing antigen. It is, therefore, unlikely that the change in serological behaviour of the Christie strain is due to contamination by the Henzerling strain.

DISCUSSION

These experiments show that, during the early stages of egg adaptation, the Christie strain of *R. burneti* can combine readily with Q fever antibody but cannot fix complement unless the antibody is in high concentration. The ability to fix complement appeared suddenly after four passages of this series and thereafter the strain resembled the classical Henzerling strain. In preliminary experiments (Stoker, 1950), the change took place between the sixth and eleventh passages but in that series there had been some difficulty with the first few egg passages. Another English strain (Stoker, 1950), and two German strains (Herzberg & Urbach, 1951), have also been shown to undergo the change in complement-fixing activity. The repeated observation of the phenomenon, the abruptness of the change, and the difference in antigenicity between C9 and Henzerling strains demonstrated in the cross-absorption tests, make it extremely unlikely that the phenomenon is due to accidental contamination by the Henzerling strain.

Such a change might be due to loss of a non-specific component which hinders absorption of complement but not antibody, or it might possibly be due to a true antigenic variation with successive dominance of two antigens, the earlier of which fixes complement poorly despite union with antibody. Attempts to differentiate antigenic fractions by heating and by antibody absorption produced no definite evidence one way or the other. Most of these tests were performed on Christie strain human serum and it is possible that more conclusive results would be obtained by using guinea-pig sera prepared against the Henzerling strain and the Christie strain at successive stages of egg adaptation.

Observations by various workers have suggested that the serological behaviour of *R. burneti* is not altogether simple. Differences between strains of the Henzerling type and those of the Dyer type reported by Topping *et al.* (1946) and Robbins *et al.* (1946) have already been mentioned. The latter also observed the development of two types of complement-fixing antibody in guinea-pigs inoculated with the Henzerling strain. Before the 40th day after inoculation antibodies fixed complement with the homologous Henzerling antigen alone but between the 40th and 70th day the guinea-pig developed the ability to fix complement with Dyer antigen as well. Berge (personal communication, 1951) has made detailed studies of ten strains of *R. burneti* from various sources and he finds variation in sensitivity of these strains as complement-fixing antigens and in their ability to absorb complement-fixing antibody, as well as alteration in the specificity of antibodies produced at various stages after infection.

It is possible that some of the antigenic differences reported may be due to variations in the stage of egg adaptation undergone by the different strains, or alternatively, that strains vary in the number of egg passages required to bring about the change in behaviour as complement-fixing antigens.

Whether or not the variation in complement-fixing activity is associated with an alteration in virulence or any other property of the strain remains to be seen. At present the main interest lies in the ability of the rickettsial antigen to combine with antibody without fixing complement, and this is of practical importance for

preparation of diagnostic complement-fixing antigen from some freshly isolated strains.

SUMMARY

1. The haemolytic complement-fixation test and the conglutinating complement-absorption test, as well as the agglutination reaction and the antiglobulin sensitization test, have been used to study a change which occurs during egg adaptation of the Christie strain of *R. burneti*.

2. Antigens were prepared from yolk sacs of the third to the ninth passages inclusive. Irrespective of numbers of rickettsiae, antigens from the third and fourth passage failed to fix complement with homologous (human) antiserum, unless the latter was in very low dilution. From the fifth passage, however, antigens fixed complement with high dilutions of the same antiserum and thus resembled the classical Henzerling strain antigen.

3. Third-passage antigen failed to fix conglutinating as well as haemolytic complement with high serum dilutions. The agglutination reaction and antiglobulin test, however, showed that third-passage antigen absorbed antibody almost as well as ninth-passage and Henzerling strain antigens.

4. It was not possible to find out if the change in behaviour was due to a true antigenic variation or to non-specific hindrance of complement absorption. Heating failed to alter the behaviour of the antigens and the results of absorption tests were inconclusive.

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