DIRECT CONGLUTINATION WITH *RICKETTSIA BURNETI*, THE CAUSAL AGENT OF Q FEVER

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(With 4 Figures in the Text)

INTRODUCTION

After infection or hyperimmunization of an animal with *Rickettsia burneti*, antibodies can be demonstrated by several *in vitro* tests. At present the haemolytic complement-fixation test (Bengtson, 1941) is the most satisfactory of these tests and is commonly used. In the early work on Q fever the agglutination test (Burnet & Freeman, 1939) was extensively used but, in this laboratory, this test was found to be less sensitive for the detection of antibodies to *R. burneti* than the haemolytic complement-fixation test. Thus, human sera with high titres of complement-fixing antibody to *R. burneti* but little or no reaction in the agglutination test were frequently observed. Such a difference in sensitivity between the two reactions is uncommon with other rickettsiae. For example, with washed suspensions of *Rickettsia prowazeki* and *Rickettsia mooseri* the agglutination test is of equal or even greater sensitivity than the haemolytic complement-fixation test for the detection of antibody. That the failure of sera containing complement-fixing antibody to *R. burneti* to agglutinate the organism is not a failure of antibody adsorption by the rickettsia was shown by Coombs & Stoker (1951). These workers found that suspensions of *R. burneti* which were partially or totally inagglutinable in human sera containing complement-fixing antibody were, nevertheless, agglutinated when an antiserum against human globulin was added. These results suggested that the insensitivity of the agglutination reaction was a failure of the rickettsiae to aggregate after the antibody had combined with them. In investigations into problems of antibody reaction with *R. burneti* various methods of enhancing the degree of aggregation of organisms which had combined with antibody have been tried. The direct conglutination reaction has also been studied.

Conglutination was the name used by Bordet & Streng (1909) to describe a particular reaction which resulted in the clumping of red blood cells. In their experiments erythrocytes previously sensitized by antibody were clumped by the addition of a non-haemolytic complement and by a substance present in normal bovine serum which they called 'conglutinin'. Streng (1909) also described the clumping of bacteria sensitized by antibody using both haemolytic and non-haemolytic complement and conglutinin, and Lucas, Fitzgerald & Schorer (1910) investigated conglutination of Flexner and Shiga strains of the dysentery bacillus sensitized by antibody in the presence of guinea-pig complement and bovine serum. This reaction was named the 'direct conglutination reaction' by Hole &

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https://doi.org/10.1017/S0022172400000528 Published online by Cambridge University Press

Coombs (1947*a*). The principle of the reaction is that when a particulate antigen combines with its antibody so that the antigen is sensitized the complex so formed adsorbs complement. These antigen-antibody-complement complexes react with and are bound by conglutinin, thereby forming aggregates which, as a rule, are bigger, more compact and easier to detect than those resulting from agglutination.

Unlike complement, conglutinin resists exposure to 56° C. for 30 min. It reacts with complement adsorbed on to a surface but not with complement in solution (Coombs & Coombs, 1953). Conglutinin may be likened to an 'antibody' to adsorbed complement. In the experiments now to be described, the action of complement and conglutinin on *R. burneti*, previously sensitized with antibody, were studied to see if it would enhance clumping of the organisms by converting weak agglutination into conglutination.

EXPERIMENTAL

Direct conglutination tests, haemolytic complement-fixation tests and agglutination tests (subsequently called the direct agglutination test) were done on various sera from human subjects who had recovered from Q fever, or from volunteers inoculated with Q-fever vaccine. The direct conglutination tests were done mainly with guinea-pig complement and bovine serum, but conglutination with equine complement and bovine serum was also tried.

(1) *Materials*

(*a*) *Sera.* One hundred and eighty specimens of serum came from eight human volunteers who were bled at frequent intervals after inoculation with Q-fever vaccine (Henzerling strain Q-fever vaccine, batch 7–1100–16A, Lederle Laboratories, Division of American Cyanamid Co., dose 0·5 ml. subcutaneously). Thirty-five samples were from five patients with Q fever and were taken at various times after the onset of illness. One of these patients was a research worker engaged in research with *R. burneti* who, although vaccinated, developed Q fever. The remaining nine serum specimens of the total of 224 were from healthy blood donors; these sera showed no haemolytic complement-fixing antibodies or agglutinins to *R. burneti* at dilutions of 1/2 or greater, and were included as control sera for the direct conglutination reaction.

Sera were stored without preservative at −20° C. until required. Unless otherwise stated, all sera were heated for 30 min. at 60° C. before testing to destroy complement activity.

(*b*) *Antigens.* Purified suspensions of the egg-adapted American 'Nine Mile' and of the egg-adapted Italian 'Henzerling' strains of *R. burneti* were used. These suspensions were made from infected yolk sacs by the technique described by Stoker (1953).

The optimal dilution of the Q-fever antigens for use with the haemolytic complement-fixation test and with the direct conglutination reaction were determined by titrating increasing (twofold) dilutions of antigen against doubling dilutions of a human serum known to contain antibodies to *R. burneti*. A titration of this sort,

where increasing dilutions of one reagent were tested against increasing dilutions of another, is referred to throughout this paper as a 'chessboard titration'.

The optimal dilution of Nine Mile antigen for both the haemolytic complement-fixation test and the direct conglutination reaction was found to be 1/160. A working dilution of 1/100 of this antigen was used in all subsequent haemolytic complement-fixation tests to ensure a slight excess of antigen. For the direct conglutination reaction and for the direct agglutination test Nine Mile antigen was used at a dilution of 1/50. This concentration of antigen, twice that used in the haemolytic complement-fixation test, was used because more clear-cut clumping of the rickettsiae was observed with it than with the weaker concentration of 1/100. The use of the same antigen concentration for the direct conglutination reaction and the direct agglutination test ensured that a comparable number of particles was exposed in each test.

Similar titrations were done with Henzerling antigen, and from these the working dilutions were determined.

Preliminary experiments were done with Henzerling antigen, but as Nine Mile antigen was more plentiful and gave almost identical results, it was used for all the subsequent tests. A few specimens from every member of the vaccinated group and all samples from the Q-fever patients were also examined with Henzerling antigen.

Control antigen. A purified suspension of *R. mooseri* (Lederle) was used as a control antigen. As this organism is antigenically distinct from *R. burneti* (Bengtson, 1941) it was used to detect non-specific reactions including latent anti-complementary effects. For the haemolytic complement-fixation test this antigen was used at a dilution of 1/160, a concentration of similar opacity to that of Nine Mile antigen diluted 1/100. For the direct conglutination reaction the murine typhus antigen was used diluted 1/80. The control antigen was not used with all the sera tested, the following procedure being adopted.

One or more samples of sera from each member of the vaccinated group were checked with *R. mooseri* antigen by the haemolytic complement-fixation test and the direct conglutination reaction (Fig. 3). Every serum from the convalescent group was also examined by the haemolytic complement-fixation test with *R. mooseri* antigen. As none of these sera showed any reaction with the control antigen in the haemolytic complement-fixation test, only late specimens from each of the patients were tested with *R. mooseri* by the direct conglutination reaction (Fig. 4).

(*c*) *Complement.* Unheated guinea-pig serum was used as a source of complement both for the haemolytic complement-fixation tests and for the direct conglutination reactions; unheated equine serum was also tried for the direct conglutination reaction. All the complement sera were stored at $-20°$ C. without preservative.

For the haemolytic complement-fixation test two 'full' units of complement were used. This was determined by titrating increasing dilutions of unheated guinea-pig serum mixed with the working dilution of antigen. These mixtures were kept overnight at 4° C. and the haemolytic system was added the following morning. The highest dilution of guinea-pig serum which caused more than 75 %

haemolysis of the erythrocyte suspension sensitized with haemolysin was deemed to contain one full unit of complement.

For the direct conglutination reaction the same amount of complement as for the haemolytic complement-fixation test was used. This concentration was within the optimal range when decreasing dilutions of an immune human serum were titrated against decreasing dilutions of guinea-pig serum in the presence of the working dilution of antigen and the optimal dilution of conglutinin.

(*d*) *Conglutinin.* Bovine serum, free from antibodies to *R. burneti* as detected by the haemolytic complement-fixation test, after heating at 56° C. for 30 min. to destroy complement activity, was the source of conglutinin.

The serum was titrated with sheep erythrocytes for conglutinin activity using the method described by Hole & Coombs (1947*b*), except that the unit volume for each reagent was 0·1 ml. and that a sheep erythrocyte suspension of 0·4 % was used. The highest dilution of bovine serum that completely conglutinated 0·4 % sheep cells in the presence of 2 units of equine complement was considered to contain one 'apparent minimal conglutinating dose'.

The optimal dilution of bovine serum for the direct conglutination reaction with *R. burneti* was found by chessboard titration to contain 4 'apparent minimal conglutinating doses'.

(*e*) *Sheep red cell suspensions.* These were prepared daily from defibrinated sheep blood. The cells were washed 3 times in 0·9 % saline before use.

(*f*) *Haemolysin.* Commercial haemolysin–horse anti-sheep red cell serum prepared by Burroughs Wellcome and Co. was used for the haemolytic complement-fixation test. The haemolytic system consisted of equal volumes of a 1 % sheep red cell suspension in 0·9 % saline and a dilution of the haemolysin containing approximately 3 M.H.D.

(2) *Technique of the tests*

All tests were done in round-bottomed glass tubes, 50 mm. long, outside diameter 9·25 mm., inside diameter 8 mm. The diluent for all the reagents was 0·9 % saline. The unit volume for every reagent was 0·1 ml.

(*a*) *Haemolytic complement-fixation test.* To doubling dilutions of serum, 2 full units of guinea-pig complement and the working dilution of antigen were added. After shaking the tubes, these mixtures were held at 4° C. overnight before the haemolytic system was added. Controls of serum, antigen and complement were included in each test. After incubation in a water-bath for 30 min. at 37° C., the tubes were centrifuged at approximately 1000 g. for 2 min. and the degree of haemolysis estimated from the supernatant. Sera were considered free from antibodies if there was more than 25 % haemolysis.

(*b*) *Direct conglutination reaction.* Antigen, 2 full units of guinea-pig complement and the optimal dilution of bovine serum were added to doubling dilutions of serum. The tubes containing these reagents were shaken, and, after overnight incubation at 37° C. in a water-bath, were examined for rickettsial aggregation. Examination was carried out with a 'binocular loupe' (magnifying spectacles) and the tubes were held vertically in front of a light obliquely illuminating the tubes

from above. Flicking the tubes dispersed the organisms that had settled, and the results were recorded in the following manner:

+ + + = strong clumping.	tr. = trace of clumping.
+ + = diffuse granular clumping.	0 = no clumping.
+ = weak clumping.	

Sera in which less than + + clumping was observed were considered to be negative. Reading the test was greatly helped by dipping the lower half of each tube in xylol before examination.

(*c*) *Direct agglutination test.* This was the same as for the direct conglutination reaction, with the exception that saline was substituted for complement and conglutinin.

(3) *Demonstration of direct conglutination with* Rickettsia burneti

Two human sera with complement-fixing titres of 1/320 and 1/640 were selected. Both sera when tested by the direct agglutination test showed no agglutination of *R. burneti* at a dilution of 1/5 or higher. Serial dilutions of these antisera were mixed with a suspension of *R. burneti* antigen. Fresh guinea-pig serum, containing 2 full units of complement and heated bovine serum diluted 1/20 were also added to each tube. After overnight incubation at 37° C. in a water-bath, marked clumping of the rickettsiae up to serum dilutions of 1/160 and 1/320 respectively was observed. Since there were apparently no antibodies capable of producing rickettsial agglutination in these sera, and as no clumping of rickettsiae was observed in control tubes devoid of either guinea-pig or bovine serum, this effect was tentatively taken as evidence of conglutination, and the following experiment was done to make certain that this enhanced clumping was true conglutination.

A specimen of serum from a patient convalescent from Q fever was selected which had a complement-fixing antibody titre of 1/320 with Henzerling antigen and an agglutination titre of 1/5.

Increasing, doubling dilutions of this antiserum, which had been inactivated at 60° C. for 30 min., were titrated in chessboard fashion against doubling dilutions of heated bovine serum in the presence of Henzerling antigen. When fresh guinea-pig serum, diluted to contain 2 full units of complement, was added to each tube, marked clumping of sensitized rickettsiae took place after overnight incubation (Fig. 1A). But if saline was substituted for guinea-pig serum or if the guinea-pig serum was heated at 56° C. for 30 min. before addition, clumping of *R. burneti* occurred only in tubes that contained human serum diluted 1/5 but not higher (Fig. 1B, C). The enhanced clumping of sensitized rickettsiae as produced by fresh guinea-pig serum and heated bovine serum (Fig. 1A) did not take place with heated bovine serum from which conglutinin had previously been removed (Fig. 1D) by adsorption with sensitized suspensions of *Salmonella pullorum* which had combined with equine complement. The enhanced clumping of the rickettsiae (Fig. 1A) was dependent on the concentration of conglutinin, because this reaction was not observed in the presence of guinea-pig complement alone or with high dilutions of bovine serum.

These results show that the enhanced clumping was dependent on both the heat-labile factor of guinea-pig serum (complement) and conglutinin of bovine serum. Consequently, it was concluded that the effect was true conglutination.

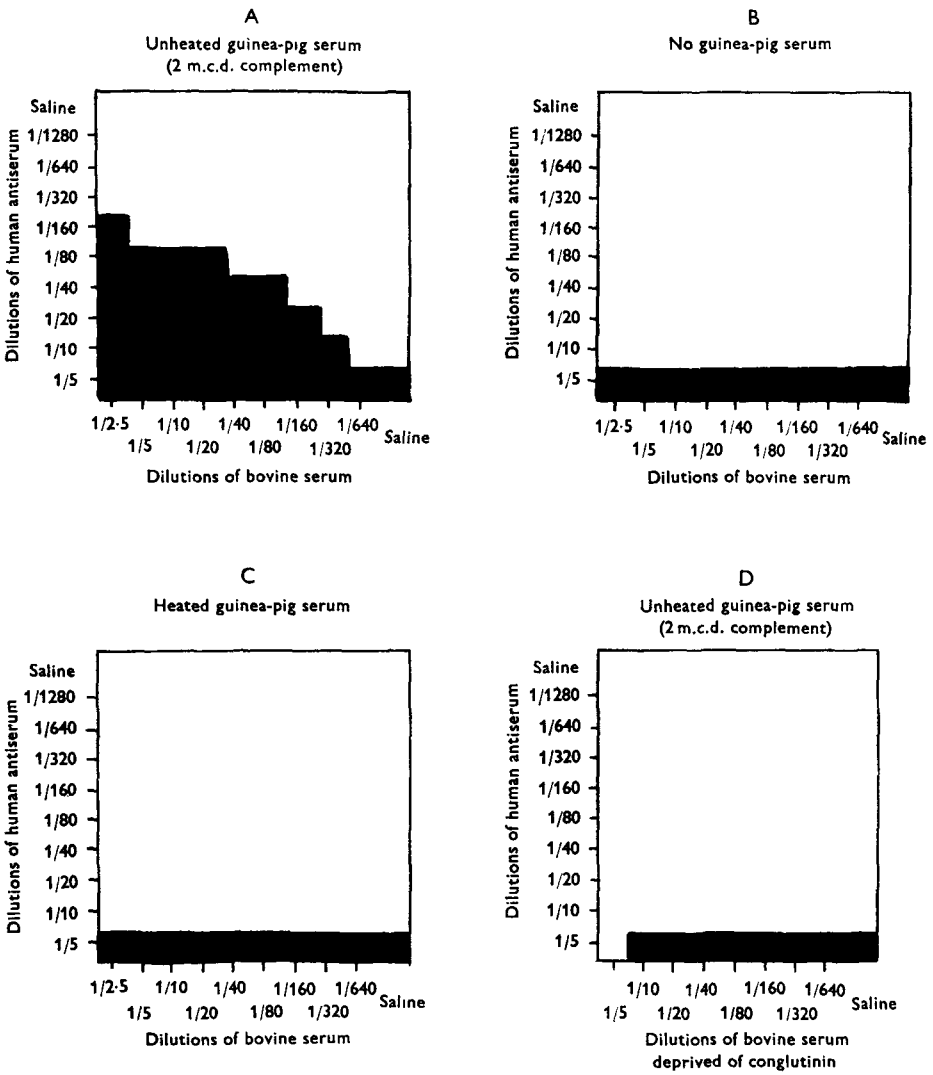

Fig. 1. The effect of guinea-pig complement and of conglutinin on *R. burneti*, showing that enhanced clumping of *R. burneti*, sensitized by antibody, in the presence of heated bovine serum and guinea-pig complement is true conglutination. ■, clumping of rickettsiae; □, no clumping of rickettsiae.

In the experiments recorded in Fig. 1 the human antiserum and the bovine serum were inactivated, but if either of these sera were used fresh in the absence of guinea-pig complement, enhanced rickettsial clumping took place (Fig. 2A, B). In the experiments recorded in Fig. 2A, the human serum was fresh and the bovine serum inactivated, whereas in the test recorded in Fig. 2B the bovine serum was fresh and the human serum heated. This shows that human or bovine

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complement can act as conglutinating complement. Therefore, when examining sera by the direct conglutination reaction it is essential to use a standard dose of complement, and to destroy the complement activity of both the serum under test and of the serum used as a source of conglutinin. If this is not done the effects of both the guinea-pig and the other complements will summate and with variable amounts of the latter the test will not be standardized.

Clumping of the organisms took place in the control tube containing only fresh bovine serum at a dilution of 1/2·5 (Fig. 2B). Aggregation of *R. burneti* had also been observed previously in low dilutions of guinea-pig serum when this was

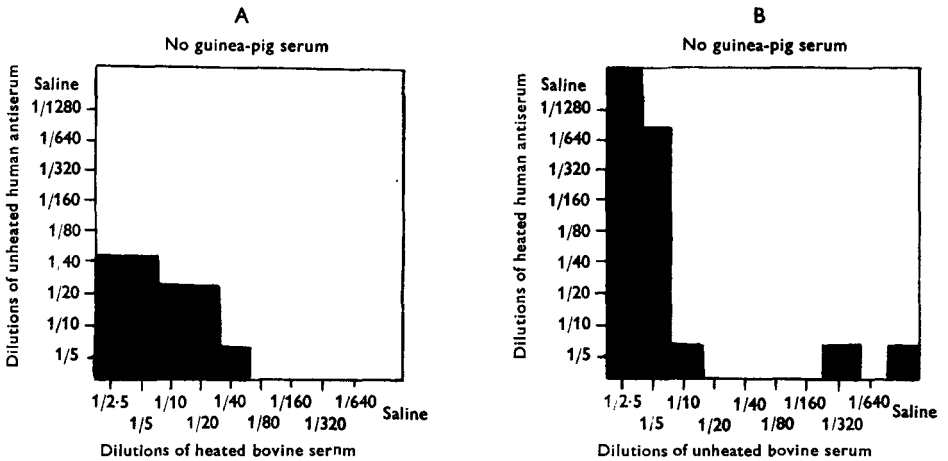

Fig. 2. The effect of unheated human antiserum and of unheated bovine serum on *R. burneti*, showing that both human and bovine complements can act as conglutinating complements on sensitized *R. burneti* in the presence of bovine conglutinin. ■, clumping of rickettsiae; □, no clumping of rickettsiae.

titrated for complement activity in the presence of the optimal dilution of heated bovine serum. In both experiments antibody to *R. burneti* as determined by the haemolytic complement-fixation test was absent in the bovine or guinea-pig sera. This effect may have been due either to non-specific adsorption of complement on to the surface of the rickettsiae which then aggregated in the presence of conglutinin, or, perhaps, to natural agglutinins present in high concentrations of the bovine or guinea-pig serum, which were indetectable by the haemolytic complement-fixation test. In the experiments recorded in Figs. 1D and 2A, B, agglutination of *R. burneti* did not occur in all tubes containing human serum diluted 1/5. As the serum under test contained very little agglutinating antibody, these results are within the limits of experimental error.

(4) *The measurements of antibody to* Rickettsia burneti *by the direct conglutination reaction and comparison of the results obtained by this technique with the haemolytic complement-fixation test and the direct agglutination test*

(*a*) *The serological response to inoculation with Q-fever vaccine.* The sera of eight medical students were examined before and at frequent intervals after injection with Q-fever vaccine. The pre-vaccination specimens of seven when tested by the three techniques were negative at dilutions of 1/2 or greater, but the specimens of the eighth (L.) had a titre of 1/4 by the haemolytic complement-fixation test only.

Five of the eight volunteers received two injections, students R. and Co. on days 0 and 10 and volunteers Ca., S. and N. on days 0 and 84. The student (L.), who already had complement-fixing antibodies to a titre of 1/4, received only one inoculation, and volunteers J. and B. received three injections because they had failed to develop haemolytic complement-fixing antibodies 74 days after the second injection (Fig. 3).

The results of the tests on the sera from these eight subjects, using Nine Mile antigen, are shown in Fig. 3. Of the eight volunteers, five (R., Co., S., N., L.) developed antibodies detected by the haemolytic complement-fixation test and the direct conglutination reaction, including volunteer L. who had a titre of 1/4 by the haemolytic complement-fixation test before vaccination. Only one (Co.) of these five volunteers developed a sustained rise of agglutinins detectable with the direct agglutination test and that was on the 44th day of the experiment. In the others, agglutinins were either sporadically demonstrated much later, or not at all. Of the remaining three volunteers, one (J.) developed antibody detectable at low titre by the direct conglutination reaction 4 days after the second injection of vaccine, but haemolytic complement-fixing antibodies were only demonstrated in a sample taken 50 days after J.'s third inoculation. Volunteers Ca. and B. were considered not to have responded to vaccination as neither showed a sustained rise by any of the three tests. None of the sera which were tested with *R. mooseri* by the haemolytic complement-fixation test and the direct conglutination reaction reacted with this antigen (Fig. 3).

(*b*) *The serological response following infection with* R. burneti. The sera from five people who had had an attack of Q fever, taken at various times during and after their illnesses, were examined. All the five patients developed antibodies, detected by the haemolytic complement-fixation test and by the direct conglutination reaction, and the results obtained with Nine Mile antigen are recorded in Fig. 4. In three patients (W., Mo., F.) antibodies were detected by the direct conglutination reaction before the haemolytic complement-fixation test, but one of these (F.) was the research worker who had received Q-fever vaccine before the onset of his illness and this may have influenced the result with the direct conglutination reaction. Agglutinins were not demonstrated in any of these sera at dilutions of 1/5 or greater with Nine Mile antigen (Fig. 4).

The results of tests with Henzerling antigen were, on the whole, similar to those described with Nine Mile antigen, but there were two exceptions (patients Ht. and Mo.). Patient Ht. developed agglutinins to Henzerling antigen but not to Nine

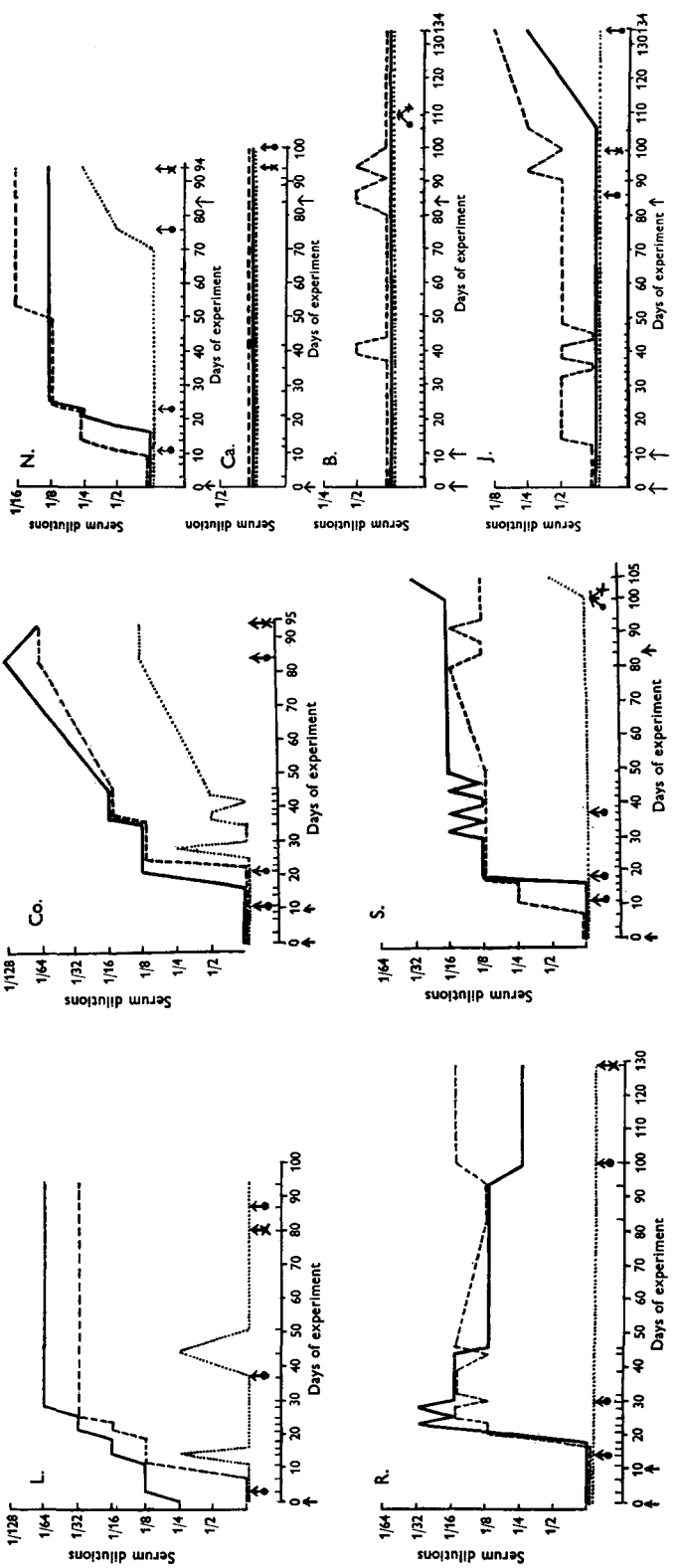

Fig. 3. The serological response to inoculation with Q-fever vaccine. Key: – – –, direct conglutination test; ——, haemolytic complement-fixation test; ……, direct agglutination test; ⊔⊔⊔, blood samples taken. ⬆, day of injection of Q-fever vaccine; ⬆, sample tested with *R. mooseri* by the direct conglutination test; ⬆, sample tested with *R. mooseri* by the haemolytic complement-fixation test.

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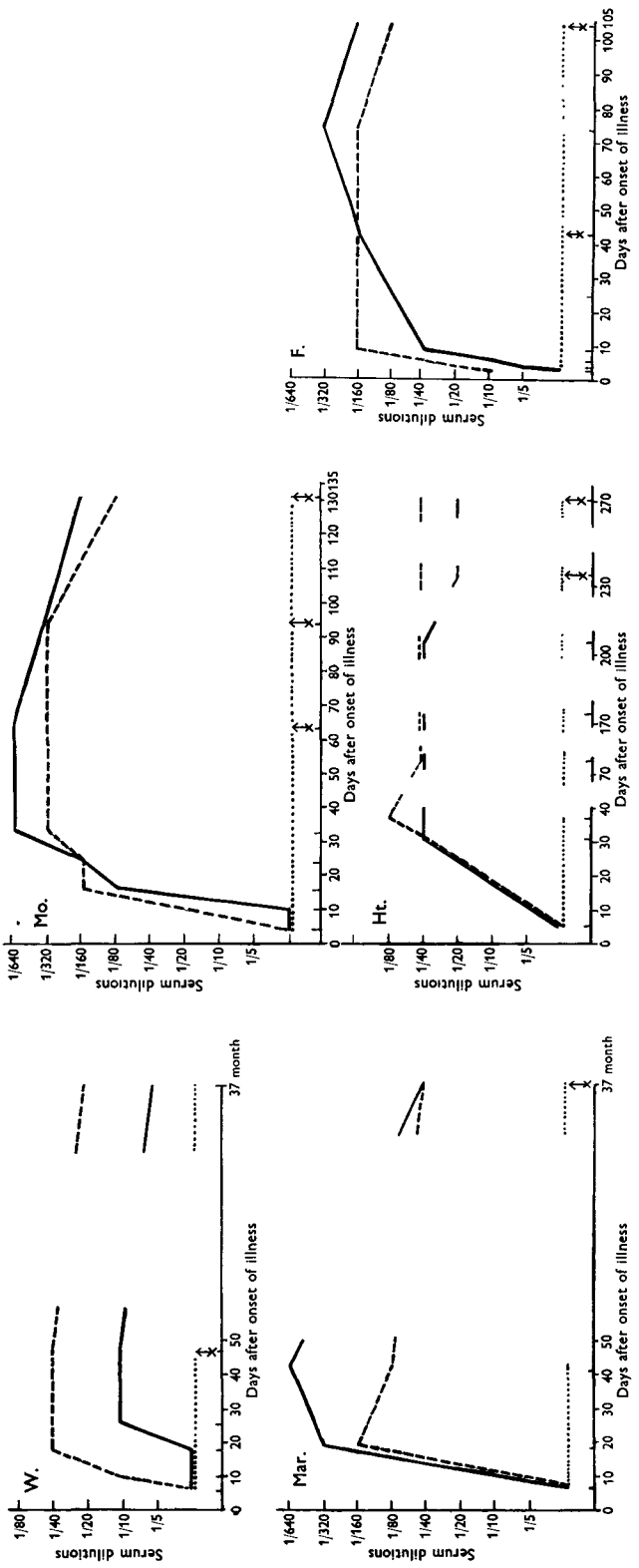

Fig. 4. The serological response to infection with *R. burneti*. Key: – – – –, direct conglutination test; ——, haemolytic complement-fixation test; ....., direct agglutination test. ⊔⊔, blood samples taken. 0 = day of onset of illness; ↕, sample tested with *R. mooseri* by the direct conglutination test.

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Mile antigen 4 weeks after becoming ill. These agglutinins, at a titre of 1/40, were present in another sample taken 6 weeks later but had disappeared from a sample 14 weeks later. The serum samples from patient Mo. taken 64 and 95 days after the beginning of his illness, showed agglutinins to Henzerling antigen alone to a titre of 1/10. None of the samples of patients' sera showed haemolytic complement-fixing antibodies to *R. mooseri*, and the direct conglutination reaction with this antigen was also negative with late specimens from all the patients (Fig. 4).

(*c*) *The examination of nine sera from blood donors.* These sera were negative at dilutions of 1/2 or greater by the haemolytic complement-fixation test and by the direct agglutination test using the Nine Mile strain of *R. burneti* and were intended to serve as controls. However, when they were examined by the direct conglutination reaction with the same antigen, one was positive at a titre of 1/2 and two at titres of 1/4. The remaining six were negative at a dilution of 1/2 or greater. The significance of this finding is not known.

(5) *The use of equine complement for direct conglutination with* Rickettsia burneti.

Equine complement is the non-haemolytic complement usually employed to demonstrate conglutination of sensitized red cells, and for this reason it was tried for direct conglutination with *R. burneti*. Even in the absence of human antiserum, *R. burneti* clumped in the presence of equine and bovine sera unless both reagents had previously been adsorbed with antigen. This adsorption was time-consuming and required large amounts of antigen.

Human sera with a relatively high haemolytic complement-fixing titre gave much lower direct conglutinating-titres using adsorbed equine and bovine sera than when they were examined with unadsorbed guinea-pig and bovine sera. Because of these results further experiments with equine complement were not done.

DISCUSSION

The object of the work reported in this paper was to investigate direct conglutination with *R. burneti*. That the enhanced aggregation of the rickettsiae sensitized by antibody in the presence of fresh guinea-pig serum and heated bovine serum was true conglutination has been demonstrated (Fig. 1).

Slight enhancement of agglutination of sensitized rickettsiae was observed on a few occasions in control tubes containing guinea-pig serum or bovine serum alone. A distinction must be made between this effect and the markedly enhanced clumping due to conglutination. The slight enhancement of clumping of sensitized rickettsiae in the presence of either bovine or guinea-pig serum appears to require a labile factor present in human serum as the phenomenon disappears on storage of the latter at $-20°$ C.

The tests on the human sera show that the results of the direct conglutination reaction were similar to those of the haemolytic complement-fixation test (Figs. 3, 4) and the titres obtained by both tests appeared to be a measure of antibody concentration of the sera. A close correlation of the results of these tests might be expected because both reactions are fundamentally similar, inasmuch as both

depend upon the fixation of complement by an antigen-antibody complex. Therefore, antibodies detected by the haemolytic complement-fixation test should be detected by the direct conglutination reaction. In the main this was found to be so, but in some sera reactions with the direct conglutination reaction were observed when no antibodies were detected by the haemolytic complement-fixation test.

The results with the sera from the vaccinated students and the convalescent subjects suggest that the direct conglutination reaction is as specific as the haemolytic complement-fixation test for the following reasons:

(1) Samples taken before and shortly after vaccination or within the first few days of illness were negative by the direct conglutination reaction with the exception of those from patient F. who had been vaccinated before his illness.

(2) The later samples of serum from five of the vaccinated and all of the convalescent subjects showed rising titres by the direct conglutination reaction as well as the haemolytic complement-fixation test.

(3) None of the sera tested reacted with *R. mooseri* in the direct conglutination reaction or in the haemolytic complement-fixation test.

Satisfactory control sera were difficult to obtain and the nine sera used for this purpose were from blood donors whose medical histories were not known and any of these subjects might possibly have been infected at some time in the past with *R. burneti*. Consequently, the significance of the low titres by the direct conglutination reaction in three of the nine sera from these blood donors who were negative to *R. burneti* by the haemolytic complement-fixation test and the direct agglutination test was doubtful.

The direct conglutination reaction may be a little more sensitive than the haemolytic complement-fixation test, but many more sera will have to be examined before any definite conclusions can be drawn. The results of the direct conglutination reaction are slightly more difficult to read than those of the haemolytic complement-fixation test but no more difficult than those of the direct agglutination test.

Throughout this work the results obtained with the direct agglutination test indicated that this test was not reliable for the demonstration of Q-fever antibodies in human sera. This is contrary to the findings of Lennette, Clark, Jensen & Toomb (1952) who described the appearance of agglutinins in the sera of the majority of patients with Q fever, and at a titre comparable to that obtained in the complement-fixation test. The agglutinins may possibly require different experimental conditions for their detection from those used in these experiments; and experiments carried out in this laboratory suggest that strains of *R. burneti* may differ in their susceptibility to agglutination. The varying results obtained with this organism in the direct agglutination test by workers in different laboratories may be accounted for by this difference in agglutinability and also, perhaps, because not all laboratories inactivate the sera before testing. Fresh human sera contain variable amounts of complement and may also contain variable amounts of a conglutinin-like substance (Coombs, personal communication). Because of this, conglutination, rather than simple agglutination, may take place if *R. burneti* is added to fresh

serum which contains Q-fever antibodies. Under these circumstances, the amount of rickettsial aggregation will depend on the concentration of both complement and conglutinin-like substance as well as on the amount of antibody. The examination of fresh bovine sera by the direct agglutination test as described by Luoto (1953) or the use of fresh bovine serum as a diluent in this test will almost certainly give results which are complicated by the phenomenon of conglutination.

SUMMARY

1. Direct conglutination has been demonstrated with *Rickettsia burneti*.

2. Sera from human volunteers inoculated with killed suspensions of *R. burneti* and from persons who had recovered from an attack of Q fever as well as sera from a small number of healthy blood donors were all examined by the direct conglutination reaction, the haemolytic-complement fixation test and the direct agglutination test. A good correlation was found between the titres of the direct conglutination reaction and the haemolytic complement-fixation test but the results with the direct agglutination test were unreliable.

The author would like to thank Dr M. G. P. Stoker, Dr R. R. A. Coombs and Dr B. P. Marmion for their constant interest, encouragement and valuable advice throughout this work, and Dr J. Wiggin for supplying a method for the removal of conglutinin from bovine serum.

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(*MS. received for publication* 12. viii. 54)