AGGLUTINATION AND COMPLEMENT FIXATION IN HUMAN AND RABBIT TYPHOID AND DYSENTERY SERA AND THE SPECIFICITY OF COMPLEMENT

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(With 2 Figures in the Text)

INTRODUCTION

In the past quite a few examples of a quantitative parallelism between the various serum reactions have been made public as support for the unitarian view on the nature of antibodies. Bail & Tsuda (1909) dissociated bacteriolytic cholera antibodies from precipitates formed by the interaction of normal bovine serum and extracts of cholera cultures. In their work on anaphylaxis Doerr & Russ (1909) showed that precipitin content and sensitizing titre cover each other. Dean (1912) found that the lack of parallelism between precipitin reactions and complement-fixation test was due to the fact that the two secondary reactions are conditioned by physical factors of different descriptions. The parallelism between the agglutination and complement fixation has also been used as evidence to support the unitarian view. Altman (1910), who believed in the diversity of antibodies, demonstrated a divergency between the titres of complement-fixation test and agglutination in typhoid patients' sera. The importance of the difference between O and H agglutination was then not known. As a matter of fact this result lost its value when Weil & Felix (1920, 1921) launched their opinion in the case of *B. proteus* and the typhoid and paratyphoid groups, to the effect that complement fixation is occasioned principally by the heat-stable antigenic constituents together with the corresponding antibody. They demonstrated that the complement-fixing power of sera is in agreement with their O-antibody content, and further, that the complement-fixing power of bacteria coincides with their O-antibody content. As to these results Singer (1924) examined the sera of a number of typhoid patients. He found a parallelism between the complement-fixation test and the O agglutination, and concluded that the complement-fixation test was superfluous. Generally speaking this is not in agreement with our experience. A patient's serum showing an agglutination for *B. Bang* may or may not show a positive complement-fixation test. The same holds true in *Morbus* Weil (Pot & Dornickx, 1936). For this reason I went over the question again in respect of dysentery and typhoid sera. Dysentery bacteria are non-motile and have neither flagella nor H antigen. Hence the titre of the agglutination of the smooth form should correspond to the titre of the complement-fixation test in case O antibody and complement-fixing antibody are identical. The typhoid strain O 901, isolated by Felix (containing the O antigen, but not H or Vi antigen), afforded an antigen for anti-typhoid sera meeting the same conditions. The intention of the experiments described below is to inquire, in these two cases, if a relation exists between the titres of the O agglutination and the complement-fixation test in rabbit sera, and if an identical relation exists in human sera.

TECHNIQUE

I. *Strains.* For the experiments with typhoid sera only strain O 901 was used. This strain contained the O antigen, but not H or Vi antigen. The Flexner strains V 6354, W 1109 and Z 1435 were isolated at the Rijks Instituut voor de Volksgezondheid; the strains X Toner and Y Oxford were obtained from Oxford.

II. *Preparation of the bacterial suspensions.* Agar cultures on Roux bottles were dispersed in salt solution and resuspended in about 60 ml. saline with phenol (0·5 %). Eliminating the influence of the phenol a small quantity of this stock suspension was diluted for each experiment. The agglutination tests were performed with a density corresponding to Brown's comparator no. 2, the complement-fixation tests with a slightly higher dilution, which showed no anticomplementary effect.

III. *Serum reactions.* (*a*) *Serum dilutions.* The titres of the complement fixation and the agglutination were determined by a series of dilutions of the immune serum in duplicate. In order to avoid irregularities larger quantities of each dilution were prepared, from which $\frac{1}{2}$ ml. for each complement-fixation test and agglutination test was taken.

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(*b*) *Agglutination tests.* Readings were obtained after immersion for 24 hr. at 47° C. in a water-bath. (In experiments with human dysentery sera the readings were made after 4½ hr.) The titres were based on Dreyer's agglutination technique. The degree of flocculation (t = total, t^- = total minus, st^+ = standard plus, st = standard, st^- = standard minus, tr^+ = trace plus, tr = trace, tr^- = trace minus) in the last tube showing agglutination was estimated, and the titre was obtained by multiplying the dilution denominator with a corresponding factor, respectively 1·5, 1·3, 1·1, 1·0, 0·9, 0·8, 0·7 and 0·6 (Dreyer & Inman, 1917; Gardner, 1930).

(*c*) *Complement-fixation tests.* Equal amounts of all reagents were used. A method analogous to Dreyer's method for the agglutination test was devised in order to determine the titres of the complement-fixation test. An estimate was made of the lysis in the first tube showing laking of the blood, and the dilution denominator of the tube showing incomplete laking of the blood was multiplied by a corresponding factor. If in the last tube showing incomplete lysis three-quarters or more of the total amount was lysed, the titre was calculated with the factor 1·8 from the dilution denominator of the serum added to the second last tube showing incomplete lysis. Table 1 includes these factors. The haemolytic system consisted of fresh guinea-pig serum and a suspension of 2·5% sheep blood corpuscles. These were sensitized by adding an equal volume of saline solution containing six times the dose of rabbit haemolysin, giving complete lysis with an excess of complement. The titration of the complement was done in three tubes containing ½ ml. of a 1, 2 and 3% dilution of fresh guinea-pig serum respectively (0·1, 0·2, and 0·3 ml. of a 5% dilution made up to 0·5 ml. with saline). In the complement-fixation test 2·2% more complement was taken than the minimum dose of complement giving no lysis. If no lysis was observed in a tube containing 1% complement, and in the tube containing 2% complement three-tenths of the blood corpuscles were not lysed, it was assumed that 1·3% complement figured as the minimum dose giving no lysis.

Table 1. *Factors used to establish the titre of the complement-fixation test*

In order to obtain the titre of the complement-fixation test, the amount of lysis (between complete lysis (+ + + +) and no lysis (−)) in the last tube showing incomplete laking of the blood is estimated, and the dilution denominator of the ½ ml. of serum added to this tube is multiplied by the corresponding factor.

Sign	Degree of lysis	Factor
−	No lysis	1·8
tr	Trace of lysis	1·7
±	Slight lysis	1·6
+	¼ lysed	1·4
+ ±	⅜ lysed	1·2
+ +	Half lysed	1·0
+ + ±	Somewhat more than half lysed	0·9
+ + +	¾ lysed	—
+ + + ±	Almost entirely lysed	—
+ + + +	Entirely lysed	—

(1) *Comparison of the titres of agglutination and complement-fixation test in rabbit immune sera*

For the purpose of an extensive comparison I determined with an antigen obtained from Ty O 901 the titres of the O-agglutination test and the complement-fixation test of thirteen sera in quadruplicate. Seven of these rabbit sera were obtained with *S. typhi*, two with *S. enteritidis* (Gaertner), two with *S. paratyphi* B (Schottmüller) and two with *S. paratyphi* A. *S. enteritidis* has the IX (XII) antigen in common with *S. typhi*, *S. paratyphoid* A and B the (XII) antigen only.

A linear relation between the titres of the two tests appeared to exist. In a graph the logarithms of the titres of the complement-fixation test are plotted graphically as ordinates, taking the logarithms of the titres of the agglutination tests as abscissae and a straight line is drawn through the points obtained. The tangent of the angle this line made with the abscissa was arrived at by the Gauss method. Its value was 1·01, the value of the distance of intersection with the abscissa from the origin, 0·25. (The distance was measured in the logarithm of the titre.) The sides of the rectangles surrounding the points indicate the standard deviation of complement fixation and agglutination respectively. It may be noted that eleven of these points are placed at a distance from the line smaller than the standard deviation, and that two points lie somewhat away. In a repeated experiment with these two and four other sera the titres of agglutination and complement-fixation test appeared to be directly proportional within the limits of error. It

was assumed, therefore, that this deviation was caused by a technical error. From this it may be concluded that a close parallelism exists between complement fixation and agglutination for the strain O 901, not only in homologous but also in heterologous rabbit sera.

experiment with a V serum as an example to show the parallelism found in these tests.

As the strain X Toner showed positive reactions with sera of all types, a comparative test of ten sera of different types was made using this antigen. The titres of the complement-fixation test and the

Table 2. *Agglutination and complement fixation in a Flexner rabbit serum type V*

Antigens type	Serum dilutions 1/250	1/500	1/1000	1/2000	1/4000	1/8000
	Agglutination test					
V	*t*	*t*	*t*	*t*	*n*	*n*
W	*n*	*n*	*n*	*n*	*n*	*n*
X	*t*	*t*	*t*	*tr*	*n*	*n*
Y	*t*	*n*	*n*	*n*	*n*	*n*
Z	*t*	*t*	*t*	*t*	*t*	*n*

	Serum dilutions 1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400
	Complement fixation test						
V	−	−	−	+	++++	++++	++++
W	++++	++++	++++	++++	++++	++++	++++
X	−	−	−	+++	++++	++++	++++
Y	±	+++±	++++	++++	++++	++++	++++
Z	−	−	−	−	−	±	++++

t = total flocculation. *tr* = trace of flocculation. *n* = no flocculation.

Table 3. *Comparison of the titres of the agglutination test and the complement-fixation test in human sera*

The lowest serum dilution examined in the complement-fixation test was 1/10; after adding the bacterial suspension in the agglutination test a lowest dilution of 1/25 was obtained. With the suspension factors the reduced titres can be ascertained. Serum dilutions (1/10, 1/25, 1/50 and 1/125) were prepared for both reactions. In the agglutination test final dilutions of 1/25, 1/62·5, 1/125 and 1/312·5 were obtained by adding ¾ ml. bacterial suspension to ½ ml. serum dilution. The titres were obtained from the dilution denominator by multiplication with factors corresponding to the degree of agglutination observed.

Sera	Titre agglutination with suspension V	W	X	Y	Z	Titre complement-fixation test with suspension V	W	X	Y	Z
1	90	85	340	95	187·5	*n*	*n*	*n*	*n*	*n*
2	85	*n*	90	70	*n*	*n*	*n*	*n*	*n*	*n*
3	35	*n*	30	*n*	90	*n*	*n*	*tr*?	*n*	*tr*?
4	375	*n*	185	55	310	*n*	*n*	*n*	*n*	18
5	94	*n*	>470	90	>470	*n*	*n*	10	*n*	10
6	*n*	*n*	375	35	75	*n*	*n*	*n*	*n*	*n*
7	*n*	*n*	185	15	70	*n*	*n*	*n*	*n*	*n*
8	75	*n*	375	125	35	*n*	*n*	*n*	*n*	*n*
Suspension factors	4	3	20	4	2					

n = no agglutination, no complement fixation in the lowest dilution. *tr*? = doubtful complement fixation in the lowest dilution.

Similar results were obtained when using Flexner sera and Flexner antigens. In a series of tests I examined twelve Flexner rabbit sera (four of type V and two of the other types), using antigens of all types for each serum. Table 2 gives the result of an

agglutination test were determined sixfold, and plotted on a graph. A straight line fitted all points.

In the case of sera obtained from other micro-organisms which agglutinized Flexner bacteria in a low titre, corresponding complement-fixation re-

https://doi.org/10.1017/S0022172400013656 Published online by Cambridge University Press

actions were found. For instance, a typhoid serum agglutinizing Flexner bacteria up to a titre of +75 gave a positive complement-fixation test of +35.

The accuracy reached in these experiments was such that the result of the complement-fixation test in a particular serum, as predicted from the result of the agglutination test and from the ratio

$$\frac{\text{titre agglutination test}}{\text{titre complement-fixation test}}$$

obtained from other sera with the haemolytic system used, differed not more than one tube of the series of dilutions from the result obtained in reality. These results strongly suggest, if they do not prove, that complement fixation and O agglutination are, in rabbit sera, two reactions on an identical fraction.

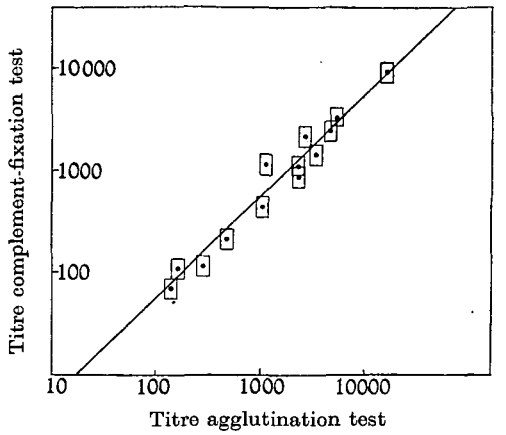

Fig. 1. Graph showing the linear relation between the titres of the complement-fixation test and the O-agglutination test with strain O 901 in rabbit sera. The logarithms of the titres of the complement-fixation test are plotted graphically, taking the logarithms of the O-agglutination test as abscissae.

(2) *Agglutination and complement fixation in dysentery patients' sera*

Proceeding from the relation between the titres of agglutination and complement-fixation tests in rabbit sera, it is natural to inquire whether, from the titre of the agglutination test in a patient's serum, the titre of the complement-fixation test can be foretold. Before starting with the examination of the dysentery patients' serum the antigens were tested with twenty human sera received for the Wassermann reaction. Non-specific reactions, observed with types X and Y, consisting of incomplete lysis in the lower dilutions but never giving rise to complete inhibition of lysis in any dilution, could be eliminated by using a twofold higher dilution of the antigen. This dilution fixed the guinea-pig's complement up to an almost equally high titre with rabbit-immune sera. Checking the binding power of the antigens for the particular haemolytic system used in the tests if charged with rabbit antibodies, I examined eight sera obtained from dysentery patients. The results are given in Table 3. It may be noted that only two sera gave positive results, the titres of the complement-fixation test remaining far below the values that could be expected from the agglutination test and the data obtained from rabbit sera. In the human sera the relation

$$\frac{\text{titre agglutination}}{\text{titre complement fixation}}$$

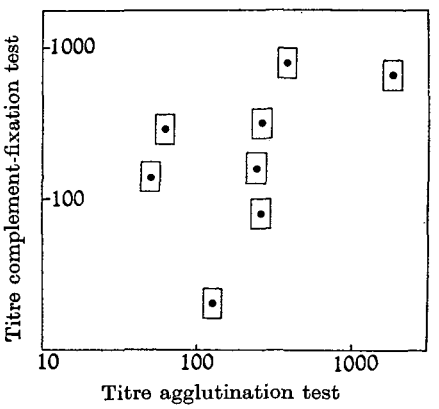

Fig. 2. Graph showing that there is no relation between the titres of the O-agglutination test and the complement-fixation test in human typhoid sera. The logarithms of the titres of the complement-fixation test are plotted graphically, taking the logarithms of the agglutination test as abscissae.

was 17, >47, >47. In rabbit sera, however, usually it was about 4, often as low as 2, but never higher than 10. This shows that the relation

$$\frac{\text{titre agglutination}}{\text{titre complement fixation}}$$

is, if constant in human sera, considerably larger than in rabbit sera. It must be kept in mind that these experiments were performed with antigens prepared by only one method using one single antigen dilution. But it is unlikely that antigens prepared by another method or in other concentrations would give more favourable results in the complement-fixation test in human sera.

(3) *Agglutination and complement fixation in typhoid patients' sera*

Bordet & Gengou (1901) showed in their first report on the complement-fixation test that positive

https://doi.org/10.1017/S0022172400013656 Published online by Cambridge University Press

results with typhoid bacteria can be obtained not only with sera from immunized animals, but also with sera from typhoid patients. Felke (1915), Hage & Korff Petersen (1915), Garbat (1914, 1915, 1916) and Pyper (1923) also studied the diagnostic value of the reaction with sera of inoculated persons.

The question which interested me most was whether the titres of the complement-fixation test and the O-agglutination test, as determined with strain O 901, run parallel. In a first experiment the titres of the agglutination and complement fixation determined in five sera did not run parallel (Table 4). For instance, no complement fixation was found parallel to the agglutination of serum 4 in Table 4. Further I examined eight sera, determining the titres in quadruplicate. The titres found were plotted on a graph as described previously (Fig. 2). It was impossible to indicate a straight line fitting all points. Two other sets of patients' sera gave similar results.

From these experiments it may be concluded that the complement-fixation test and the O agglutination test do not depend on identical factors in human sera.

Table 4. *Comparison of agglutination test and complement-fixation test in sera from typhoid patients*

Patient's serum	Agglutination test 1/25	1/62	1/125	1/250	1/500	1/1000	1/2000	1/4000
No. 1	*t*	*t*	*t*	*t*−	*n*	*n*	*n*	*n*
No. 2	*t*	*t*	*t*	*t*	*t*	*t*	*t*−	*tr*−
No. 3	*t*	*t*	*t*	*t*	*tr*−	*n*	*n*	*n*
No. 4	*t*	*t*	*t*	*t*	*t*	*n*	*n*	
No. 5	*t*	*t*	*tr*−	*m*	*n*	*n*		

	Complement-fixation test 1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
No. 1	−	−	−		+++	++++	++++	
No. 2	−	−	−	−	−	+	++++	++++
No. 3	−	−	++	++++	++++	++++	++++	++++
No. 4	+±	++++	++++	++++	++++	++++	++++	++++
No. 5	++++	++++	++++	++++	++++	++++	++++	++++

t = total flocculation. *t*− = nearly total flocculation. *tr*− = trace of flocculation.

DISCUSSION

The fact that dysentery bacteria eagerly adsorb the guinea-pig's complement when sensitized with rabbit antibody, but hardly or not at all when sensitized with human antibody, made me think of a group of phenomena referred to as the specificity of complement. Muir (1912) described among others the following example: 'Guinea-pig's and rabbit's complement have apparently the same combining power, when tested on ox corpuscles sensitized with immunebody of the rabbit... that it is practically immaterial, whether a combined haemolytic dose of immunebody is saturated by the guinea pig's complement so far as lysis is concerned. If, however, we take the case of the immunebody of the duck for ox corpuscles, we find, that the rabbit's complement combines readily and produces lysis in a comparatively small dose, in fact as small a dose with the duck's immunebody as with the mammalian immunebodies; on the other hand the guinea-pig's complement does not lyse the sensitized corpuscles at all and does not even combine with them.'

The possibility that similar phenomena play a role in the complement-fixation test with bacterial antigens has not yet attracted attention. A priori it cannot be excluded, that bacterial antigens exist, behaving like the antigens of ox blood corpuscles and fixing the guinea-pig's complement if sensitized with the antibodies of a certain species, but not if sensitized with antibodies of a different species.

The phenomena observed in dysentery and typhoid sera can be explained as follows from this viewpoint:

All O antigens of typhoid and dysentery bacteria fix the guinea-pig's complement when saturated with the corresponding rabbit antibodies, hence the parallelism between agglutination and complement-fixation test in rabbit sera.

When comparing complement fixation and O agglutination in human sera the O antigens appear to be of diverse characters. Only part of the O antigens of typhoid bacteria and part of the O antigens of dysentery bacteria fix the guinea-

https://doi.org/10.1017/S0022172400013656 Published online by Cambridge University Press

pig's complement when saturated with human antibodies. This part of the O antigen gives rise to the few positive complement-fixation tests in dysentery cases and to many positive tests in typhoid fever. The other part of the O antigen of typhoid or dysentery bacteria (though capable of fixing the guinea-pig's complement, when saturated with rabbit antibodies) does not fix the guinea-pig's complement when saturated with human antibodies. Giving rise to agglutination only, they cause the divergencies in titres between the agglutination and complement-fixation tests in both typhoid and dysentery cases. Similarly, divergencies in titre between complement-fixation tests and agglutination tests in all infectious diseases may be explained. The greater specificity of the complement-fixation test in human sera is caused by its being a reaction for a more restricted group of antibodies than the O agglutination.

Positive serum reactions in 'normal' human sera (mostly in low concentrations) are far more frequent in the agglutination test than in the complement-fixation test. This may be explained in terms of specificity, as follows. The antigens, to which the normal antibodies correspond, are for the greater part of such description, for reasons of specificity, as described above, that they do not fix the guinea-pig's complement if sensitized with human antibodies. Rarely, if ever, are they of such a class as to fix the guinea-pig's complement with the corresponding human antibody.

SUMMARY AND CONCLUSION

The titres of the complement-fixation test and the agglutination test run parallel in rabbit sera, but not in human sera. These facts are explained in connexion with the specificity of complement.

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(*MS. received for publication* 11. III. 1946.—Ed.)