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Immuno-Conglutinin in the Detection of Human Blood Group Antibodies

P. L. Mollison and Margaret J. Polley

Medical Research Council's Blood Transfusion Research Unit, Postgraduate Medical School of London, W.12

Summary. The value of sera containing immuno-conglutinin or conglutinin in demonstrating complement-binding by human blood group iso-antibodies was investigated. Provided that a suitable complement was used, positive results were obtained with all human sera which had been shown by other methods to contain complement-binding antibodies.

When human red cells were sensitized with human complement-binding blood group antibody and then treated with horse serum as a source of complement, the strongest reactions were obtained with rabbit immuno-conglutinin; the reactions with bovine serum (containing conglutinin) were distinctly weaker, and the reactions with human sera containing immuno-conglutinin were weaker still. When rabbit complement was used instead of horse complement, stronger reactions were obtained, but it was difficult to avoid 'false positive' reactions. When human complement was used, good reactions were obtained with rabbit immuno-conglutinin, but the reactions with bovine conglutinin and human immuno-conglutinin were completely negative.

INTRODUCTION

Bordet and Streng (1909) showed that normal bovine serum would clump human red cells which had been sensitized with an antibody and had also adsorbed complement; they described the clumping as 'conglutination', and used the term 'conglutinin' to describe the substance in bovine serum that was responsible for this effect. Streng (1930) immunized rabbits with cells or bacteria which had adsorbed human complement. He obtained a serum which would agglutinate complement-coated bacteria or cells, and he described the antibody produced as 'immuno-conglutinin'. Wartiovaara (1932) showed that immuno-conglutinin was also produced when bacteria alone were injected; this was confirmed by Coombs and Coombs (1953), who described this kind of antibody as 'immuno-conglutinin (auto-stimulation)'.

Many human blood group antibodies bind complement. This can be demonstrated by the indirect antiglobulin test (Dacie, Crookston and Christenson, 1957) or by showing that specific lysis occurs either with human complement or with a suitable animal complement (see Mollison and Thomas, 1959).

It seemed worth while to find out whether immuno-conglutinin would be a useful reagent in the detection of human blood group antibodies, and the present paper reports some observations on this subject.

METHODS

HUMAN RED CELLS SENSITIZED WITH HUMAN BLOOD GROUP ANTIBODIES

Red cells were sensitized with several different examples of normal incomplete cold antibody, anti-K and anti-Le^a, and with particular examples of anti-O, anti-Le^b, anti-Fy^a

and anti-Jk^a. All these antibodies were selected for their ability to bind complement as judged by:

1. Their ability to sensitize red cells to an anti-human globulin serum (reacting only with non-gamma globulin) when the red cells were incubated with human complement as well as with antibody.
2. Their ability to lyse enzyme-treated cells in the presence of human complement. (Negative reactions were obtained with anti-K and anti-Fy^a sera, presumably due to destruction of the K and Fy^a antigens by the enzyme.)
3. Their ability to lyse untreated red cells in the presence either of human complement or of rabbit complement.

Red cells were also sensitized with several examples of incomplete anti-Rh, an antibody which never seems to bind complement. Examples of non-complement binding anti-K and anti-Fy^a were available, but examples of anti-Rh were preferred because they were more potent.

Red cells were normally first treated with antibody, then washed and treated with complement. Sera were first inactivated by heating at 56° for 30 minutes. Then four volumes of serum and one volume of a 20 per cent suspension of red cells were warmed to 37°, mixed and incubated together at 37° for 1½ hours. The red cells were then washed three times in saline, resuspended to give an approximate 20 per cent suspension and re-warmed to 37°. Four volumes of fresh serum as a source of complement were now warmed to 37°, then added to the cells and incubated with them for 20 minutes. The red cells were then washed either once (if they were to be tested with immuno-conglutinin) or three times (if they were to be tested with antiglobulin serum).

In sensitizing cells with normal incomplete cold antibody, one volume of group O cells was added to twenty volumes of fresh group AB serum and left at 0° for 1–2 hours. In a few experiments red cells were added to inactivated group AB serum at 0° and left for 1–2 hours, then washed and left at 0° with fresh rabbit serum (as a source of complement) or left at 37° either with fresh rabbit serum or with fresh human serum.

SHEEP RED CELLS SENSITIZED WITH BOVINE SERUM

The method described by Coombs and Coombs (1953) was used. A suspension of sheep cells was sensitized by incubation with inactivated bovine serum. The cells were then incubated with horse serum (as a source of complement) to prepare 'alexinated red cells'.

SERA USED AS A SOURCE OF COMPLEMENT

Human serum was separated from freshly drawn blood and was stored at −45°.

Freshly drawn blood was obtained from several different guinea pigs, horses, pigs and rabbits. The serum was separated within a few hours and absorbed at 0° with six times washed human red cells. Rabbit serum continued to react with human red cells even after three absorptions; that is to say, unsensitized human red cells, after incubation with the absorbed rabbit serum, were agglutinated by an immuno-conglutinin serum. That this reaction was due to adsorption of complement was shown by the fact that unsensitized

human red cells treated with heated rabbit serum were not agglutinated by the rabbit immuno-conglutinin serum, and unsensitized human red cells treated with fresh rabbit serum were not agglutinated by normal rabbit serum. If the rabbit serum was absorbed at 0° more than three times, the content of complement was greatly reduced. Two absorptions at 0°, followed by a single absorption at 37°, removed all complement. The difficulty was partly overcome by absorbing the rabbit serum only twice and then using it diluted 1/2.

SERA CONTAINING IMMUNO-CONGLUTININ ('I-K')

Two sera prepared in rabbits were used. One was kindly provided by Dr. R. R. A. Coombs, and had been prepared by repeated injections of a killed suspension of *Salmonella pullorum*. The other was prepared by ourselves in the same way; three injections of the bacterial suspension were given on days 0, 4 and 7, and blood samples were taken for testing on days 10, 11, 12 and 15. The sample taken on day 12 (5 days after the last injection) gave the best reactions. A further injection of bacterial suspension after an interval of 2 months was followed by a further small increase in the titre of I-K. For routine use, the serum was diluted 1/12 in normal saline.

Human sera containing I-K were obtained by examining the serum of fourteen patients with rheumatoid arthritis (see Marks and Coombs, 1957). The two sera with the highest titre (1/64), when tested against sheep red cells sensitized with bovine antibody and horse complement, were used for further tests.

CONGLUTININ

Fresh normal bovine serum was obtained, inactivated at 56° for 30 minutes, and then absorbed either with human red cells or with sheep red cells.

ANTIGLOBULIN SERA

Several different anti-human globulin sera were used, all of which agglutinated complement-coated cells; that is to say, the sera, if mixed with an equal volume of 1/100 solution of 1 per cent human gamma globulin, would react with cells which had been incubated first with human antibody and then with human complement, but would not react with cells which had been incubated only with inactivated serum containing antibody. All the results with anti-human globulin sera reported in this paper refer to sera to which gamma globulin had been added.

A few tests were made with a single anti-rabbit globulin serum produced in a goat. The serum was absorbed three times with ten times washed red human cells and then used in tests with human red cells treated with a human blood group antibody and with rabbit serum as a source of complement.

TEST FOR HAEMOLYSIS

Human sera were tested for their ability to haemolyse red cells using either enzyme-treated cells and human complement (Haber and Rosenfield, 1957) or untreated red cells and rabbit complement (Mollison and Thomas, 1959).

RESULTS

REACTIONS OF RABBIT IMMUNO-CONGLUTININ WITH HUMAN RED CELLS SENSITIZED WITH HUMAN BLOOD GROUP ANTIBODIES AND TREATED WITH COMPLEMENT

Human Complement

Of the human blood group antibodies tested, those which gave the strongest reactions with anti-human non-gamma globulin serum also sensitized cells to a rabbit I-K serum (see Table 1). Negative reactions were obtained with anti-O and anti-Le^b, but these were

TABLE 1

EXAMPLES OF AGREEMENT BETWEEN RESULTS OF VARIOUS TESTS FOR COMPLEMENT-BINDING, INCLUDING A TEST WITH A RABBIT IMMUNO-CONGLUTININ (I-K) SERUM

Antibody used to sensitize human red cells	*Addition of human complement (undiluted)*		*Addition of rabbit complement (1/2)*	*Enzyme-treated* cells: human complement added*	*Untreated cells:* rabbit complement added*
	Agglutination by anti-human non-gamma globulin serum	*Agglutination by rabbit I-K serum (diluted 1/12)*	*Agglutination by rabbit I-K serum (diluted 1/40)*	*Haemolysis*	*Haemolysis*
Anti-Rh†	−	−	−	−	−
Normal incomplete cold†	+++	++	−‡	−	++
Anti-K†	+++	+	+++	−	+
Anti-Le^a†	+++	+++	+++	++	++
Anti-O	++	−	+++	++	(+)
Anti-Le^b	+	−	++	+	−
Anti-Fy^a	+++	+++	(complete lysis)	−	+++
Anti-Jk^a	+++	++	+++	+	+

* Results in these two columns from Mollison and Thomas (1959).
† Several examples of each of these antibodies were tested, with similar results.
‡ See text.

comparatively weak antibodies. Thus, the only potent antibody which gave completely negative results in tests with I-K serum was anti-Rh, many examples of which were tested.

Red cells could not first be sensitized with normal incomplete cold antibody (without human complement) and then treated with rabbit complement, since red cells become sensitized with normal incomplete cold antibody only when they are mixed in the cold with fresh human serum (containing complement). This latter finding was reported by Dacie (1950) and has frequently been confirmed in this laboratory. By contrast, complement does not seem to enhance the binding of such antibodies as anti-Le^a, anti-Fy^a or anti-Jk^a, since cells were found to react just as strongly with an anti-non-gamma globulin serum or with a rabbit-I-K serum when they were incubated first with inactivated serum containing antibody alone, then washed and incubated with complement, as when they were incubated with antibody and complement simultaneously (cf. Andersen, 1957).

Effect of Varying the Period of Exposure to Complement

Human red cells were sensitized with a complement-binding antibody (an example of anti-Fy^a), then incubated with fresh human serum as a source of complement. After various intervals, a sample of the sensitized red cells was washed three times in saline and then tested with a serum containing I-K and with a serum containing anti-human (non-gamma) globulin. As Table 2 shows, the reactions with I-K became perceptibly weaker

after the cells had been incubated with complement for 40 minutes and became completely negative after 5 hours' incubation. On the other hand, the reactions with anti-non-gamma globulin serum were only slightly weaker after 24 hours.

TABLE 2

EFFECT OF VARYING THE PERIOD OF EXPOSURE TO COMPLEMENT ON THE REACTION OF SENSITIZED CELLS WITH IMMUNO-CONGLUTININ AND ANTIGLOBULIN SERUM

Period of exposure to complement	*Reactions of sensitized cells* with:*	
	Immuno-conglutinin	*Antiglobulin serum*
5 minutes	+++	+++
20 minutes	+++	+++
40 minutes	++	+++
2 hours	+	+++
4 hours	(+)	++
5 hours	−	++
24 hours	−	++

* Fy(a+) red cells sensitized with a complement-binding anti-Fy^a and treated with human complement.

Use of Rabbit Complement

When human red cells are sensitized with a human complement-binding antibody and then incubated with absorbed rabbit serum, haemolysis occurs. In the hope of removing C_3 and thus preventing haemolysis, rabbit serum was treated with zymosan. However, the addition of only 1 mg. of zymosan per ml. of serum completely abolished the ability of the serum to sensitize antibody-coated cells to I-K. (By contrast, positive reactions were obtained with human sera treated with as much as 8 mg. of zymosan per ml.)

Complete lysis was observed only when strongly sensitized cells were incubated with rabbit serum. With lower concentrations of antibody there was little or no lysis, and the red cells were then tested with I-K (*see* Tables 1 and 3). As described in the 'Methods',

TABLE 3

REACTIONS OF FY(a+) RED CELLS SENSITIZED WITH VARIOUS DILUTIONS OF ANTI-FY^a, THEN INCUBATED WITH VARIOUS COMPLEMENTS AND FINALLY TESTED EITHER WITH AN IMMUNO-CONGLUTININ SERUM OR WITH AN ANTIGLOBULIN SERUM

Dilutions of anti-Fy^a	*Serum used as a source of complement*						
	Human (undiluted)		*Rabbit (undiluted)*	*Rabbit (1/2)*	*Horse (undiluted)*	*Guinea pig (undiluted)*	*Pig (1/2)*
	Reactions with I-K	*Reactions with anti-human globulin serum*	*Reactions with I-K**	*Reactions with I-K**	*Reactions with I-K*	*Reactions with I-K*	*Reactions with I-K**
1/1	+++	+++	(lysis)	(lysis)	+++	(lysis)	(lysis)
1/10	(+)	++	+++	+++	++	+	+++
1/20	−	++	+++	+++	++	(+)	+++
1/40	−	(+)	+++	++	(+)	−	++
1/80	−	−	+++	+	−	−	(+)
Control	−	−	++	−	−	−	(+)

I-K = Rabbit immuno-conglutinin serum diluted 1/12.
I-K* = Rabbit immuno-conglutinin serum diluted 1/40.

when unsensitized human red cells were incubated with undiluted rabbit serum they were subsequently agglutinated by I-K. Negative reactions with unsensitized cells could be obtained by using the rabbit serum diluted 1/2 and using a reduced concentration (1/40) of I-K.

As Table 1 shows, positive results were obtained with all the antibodies which could be shown by other tests to bind complement. Table 3 shows the sensitivity of a test involving rabbit complement. Red cells incubated with a 1/80 dilution of a serum containing anti-Fy^a, then treated with rabbit serum diluted 1/2, were agglutinated by a rabbit I-K serum, whereas the greatest dilution of the anti-Fy^a serum which could be detected using human complement and anti-human non-gamma globulin serum was 1/40.

A few tests were also made with a goat anti-rabbit globulin serum to see whether this would prove to be a sensitive method of detecting small amounts of adsorbed rabbit complement. Human red cells which had been sensitized with a complement-binding antibody (anti-Fy^a) were incubated with rabbit serum, then washed and tested with the anti-rabbit globulin serum. Unfortunately the same difficulty was experienced as with rabbit I-K serum; that is to say, unsensitized human red cells, after incubation with absorbed rabbit serum, were agglutinated by the anti-rabbit globulin serum, owing apparently to failure to absorb from the rabbit serum all traces of complement-binding antibody directed against some antigen in human red cells.

Use of Horse Complement

As Table 3 shows, the sensitivity of a test using horse complement and rabbit I-K serum was about the same as a test using human complement and an anti-human globulin serum in detecting low dilutions of complement-binding anti-Fy^a.

Use of Guinea-Pig and Pig Complement

As Table 3 shows, sensitized cells incubated with guinea-pig complement gave only slightly better reactions with I-K than cells incubated with human complement. With pig complement stronger reactions were obtained, but even when the pig serum was diluted 1/2 unsensitized cells were slightly agglutinated; if the pig complement was diluted further or a more dilute I-K serum was used, the results were unsatisfactory.

COMPARISON OF RABBIT IMMUNO-CONGLUTININ, HUMAN IMMUNO-CONGLUTININ AND BOVINE CONGLUTININ SERA (SEE TABLE 4)

TABLE 4

REACTIONS OF RABBIT IMMUNO-CONGLUTININ SERUM, HUMAN IMMUNO-CONGLUTININ SERUM AND BOVINE SERUM (CONTAINING CONGLUTININ) WITH VARIOUS COMPLEMENT-TREATED RED CELLS

Red cells	*Antibody*	*Complement*	*Maximum dilution of I-K† serum or conglutinin serum reacting with cells*		
			Rabbit I-K	*Human I-K*	*Bovine conglutinin*
Sheep	Bovine	Horse	512	64	64
Sheep	Bovine	Human	32	0	8
Human	Human*	Horse	512	8	32
Human	Human*	Human	128	0	0

* An example of anti-Fy^a.
† Expressed as a reciprocal.

As described in the 'Methods', two human sera containing I-K were selected. When tested with sheep red cells sensitized with bovine antibody and then exposed to horse complement (method of Coombs and Coombs, 1953), the sera had a titre of 1/64. The two sera were also tested with sheep red cells treated with bovine antibody and human complement; no reaction was observed. Similarly there was no reaction with human red cells sensitized with a human complement-binding antibody and incubated with human complement, but when the same sensitized cells were incubated with horse complement they were weakly agglutinated by the human I-K sera. The reactions of one of the sera are shown in Table 4 and compared with the reactions of a rabbit I-K serum and a bovine conglutinin serum with the same red cells.

Examination of Antiglobulin Sera for the Presence of Immuno-Conglutinin

In view of the good agglutination produced by rabbit I-K sera with human red cells sensitized with complement-binding antibody and human complement, it seemed possible that the reactions of some antiglobulin sera might be due to their content of I-K. This was investigated by testing the antiglobulin sera before and after the addition of human serum. It was found that whereas the reactions of a rabbit I-K serum were virtually unaffected by the addition of an equal volume of undiluted human serum, the reactions of most of the rabbit antiglobulin sera were completely abolished by the addition of an equal volume of a 1/100 dilution of normal serum. One rabbit antiglobulin serum and one goat antiglobulin serum, after mixture with an equal volume of a 1/10 dilution of normal human serum, gave weak reactions with complement-coated cells, and it was concluded that these sera contained a small amount of conglutinin.

DISCUSSION

A test with an immuno-conglutinin serum is valuable because it is specific for complement, and thus provides further evidence that a particular antibody binds complement. When human serum is used as a source of complement a test with a rabbit I-K serum is not as sensitive as a test with an anti-human non-gamma globulin serum; the use of horse complement makes the test with I-K about as sensitive as the test with antiglobulin serum. Rabbit complement makes a test with rabbit I-K more sensitive, although with rabbit complement 'false positive' results are troublesome.

The reactions of a rabbit I-K serum differ in several ways from those of a rabbit anti-human (non-gamma) globulin serum. Firstly, the I-K serum reacts with sensitized red cells which have been treated with complement derived from many species, whereas anti-human globulin serum reacts only with cells exposed to human complement. Secondly, the I-K serum is not inhibited by mixture with human or other sera, whereas the anti-human globulin serum is inhibited by mixture with even very dilute (1/100) human serum. This latter observation shows that the reactions of anti-non-gamma globulin sera are not due to the presence of I-K (cf. Andersen, 1957). Thirdly, when sensitized red cells are incubated at 37° with complement and subsequently tested with I-K the reactions become progressively weaker, and after 5 hours' incubation the cells are no longer agglutinated by I-K. By contrast, even after 24 hours' incubation the cells are still agglutinated by anti-non-gamma globulin serum. It is known that the complex EAC'_{142} breaks down very rapidly at 37° (Mayer, Levine, Rapp and Marucci, 1954) and it may therefore be concluded that antiglobulin serum, but not I-K serum, can react with decayed

C'_{142}. Work to be reported elsewhere (Jenkins, Polley and Mollison, 1960) has shown that antiglobulin sera will react with red cells which have adsorbed C'_1 and C'_4 with little or no C'_2 whereas, as shown by Coombs, Blomfield and Roberts (1950), I-K serum will react only with cells which have adsorbed C'_1, then C'_4 and C'_2 together.*

In the present work no reactions were obtained between human I-K serum and red cells which had adsorbed antibody and human complement (*see* Table 4). It would be unsafe to deduce from this that human I-K is incapable of reacting with adsorbed human complement, since the sera tested had only a moderate titre of I-K. In any case, it appears that human sera contain a poor 'conglutinating' complement, since red cells treated with human antibody and human complement were not agglutinated by bovine serum containing conglutinin.

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* While this paper was in the press, Gandini (Proceedings of the 7th International Transfusion Congress, *Bibliotheca Haematologica*, 1959, Fasc. 10) reported that sensitized cells, after prolonged incubation with complement were no longer agglutinated by a serum containing conglutinin, whereas they were still agglutinated by an anti-'non-gamma' globulin serum, and he also concluded that C'_2 was not needed for the antiglobulin reaction.