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AUTOIMMUNE HAEMOLYTIC ANAEMIAS

III. PREPARATION AND EXAMINATION OF SPECIFIC ANTISERA AGAINST COMPLEMENT COMPONENTS AND PRODUCTS, AND THEIR USE IN SEROLOGICAL STUDIES

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SUMMARY

The preparation and examination of agglutinating antisera, specific for β1E, β1A, α2D and the /β1C/ determinant of the β1C molecule are described. With these reagents it could be established that anticomplement serum does not contain antibodies against the B determinant of the β1C molecule, while antiserum prepared with β1C globulin does. Further, the complement factors present on red cells, the complement coating of which was effected in various ways, were studied.

Whereas red cells incubated *in vitro* with haemolytic iso- or auto-antibodies are agglutinated by anti-β1E, anti-β1A and anti-α2D sera, the cells of patients with autoimmune haemolytic anaemia of any of the serologically defined groups of this disease, only reacted with anti-α2D. The possible significance of these findings is discussed.

INTRODUCTION

In 1950 Dacie described 'incomplete cold antibodies' with which red cells can be sensitized in chilled fresh serum: the cells are then agglutinated by antiglobulin serum. Later, in 1951 Ferriman and co-workers reported that strong antiglobulin reactions can be obtained using the erythrocytes or serum of patients with cold agglutinin disease, indicating the presence of incomplete cold auto-antibodies. In the same year Dacie (1951) showed that the reaction of the cells and the antiglobulin reagent in these cases could not be neutralized by the addition of γ-globulin to the latter. He concluded that either the antibodies were not γ-globulins or that the reaction was caused by components of fresh serum and factors against these in the antiglobulin serum. It is, of course, generally known now that later Dacie, Crookston & Christenson (1957, 1958) clearly demonstrated that the reaction is not due to the presence

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of antibodies on the cell, but of complement components and anticomplement antibodies. They also believed that C3 is not necessary to give this reaction and Gandini (1959) expressed the opinion that neither is C2.

Rosenfield, Haber & Gilbert (1960) introduced a special anticomplement serum, prepared by immunization of rabbits with antigen–antibody complexes coated with human complement, for use in the 'anticomplement test' on red cells. In such anticomplement reagents or in antisera prepared against single complement components the presence of antibodies against determinants of β1E globulin = C4 and β1C globulin = C3 have been demonstrated (Müller-Eberhard & Nilsson, 1960; Pondman *et al.*, 1960; Jenkins, Polley & Mollison, 1960; Peetoom & Pondman, 1961, 1963; Pondman & Peetoom, 1962; Müller-Eberhard & Biro, 1963). Pondman *et al.* (1960) also showed that sensitized sheep red cells that have reacted with complement are agglutinated by anti-β1E antibodies, thereby confirming that a positive anticomplement test may be obtained without the participation of C2 and C3.

It is quite likely that apart from the above antibodies, antibodies against other complement components are present in anticomplement sera, which may complicate the drawing of conclusions when these antisera are used for investigations. The matter is even further complicated by the fact that several antigenic determinants may be present on one complement component. Peetoom & Pondman (1961) noticed that a reaction product appears in the fluid phase when complement reacts with antigen–antibody aggregates. West *et al.* (1966) called this globulin α2D. These latter authors demonstrated that β1C globulin carries at least three different antigenic determinants: B, A and D. On ageing β1C globulin disappears from fresh serum, and β1A globulin, which only carries the A antigen, appears (Müller-Eberhard & Nilsson, 1960; Peetoom & Pondman, 1961). According to West *et al.*, a second product, appearing in the α2 region, is formed: α2D. It carries the D determinant, but also possesses an antigenic determinant, which is not detectable on the intact β1C molecule (D: determinant). After the conversion, the B determinant cannot be demonstrated by immuno-precipitation. Pondman & Peetoom (1964) found also that when β1C reacts with EAC $\overline{142}$, β1A appears in the fluid phase. Later they saw that a part of the β1C molecule is not demonstrable any more after this reaction. They designated this part /β1C/. It is probably identical with the B determinant described by West. In earlier work Peetoom & Pondman (1961) had shown that β1C is first bound to the immune complex, after which, conversion leading to the formation of β1A takes place. West *et al.* assume that when β1C reacts with EAC $\overline{142}$, the B determinant is fixed on the cell membrane, while β1A and α2D go into the fluid phase.

The purpose of this paper is to report on the preparation of agglutinating antisera, specific for β1E, β1A, α2D, or /β1C/ and the difficulties encountered in their preparation. Further, to give some results obtained with these sera and red cells coated with complement fixing iso- or auto-antibodies against red cells, and red cells from patients with different kinds of autoimmune haemolytic anaemia. In a following paper, some special results that we obtained with the red cells and serum of patients with cold agglutinin disease will be discussed.

MATERIALS AND METHODS

For the examination of antisera use was made of

1. Tanned red cells coated with immunoglobulins G, M, A or D, albumin, fibrinogen, transferrin, α2 macroglobulins or lipoproteins.

2. Tanned red cells coated with C1q, β1E, β1C, β1A and α2D. The purified components C1r, C1s, C2 and C5–9 are not yet available.

Serological methods

The direct and indirect antiglobulin (or anticomplement) tests were applied as described elsewhere (Engelfriet *et al.*, 1968). All reactions were read microscopically.

Preparation of complement components or products of complement components

β1E preparation was obtained with the method of Pondman, Wolters & Bleumers (1967). β1C was separated from fresh serum with a slight modification (Pondman *et al.*, to be published) of Steinbuch's method (1963). β1A and α2D preparations were made as described by Pondman *et al.* (1967) and as to be described by Wolters *et al.* (to be published).

C1q was prepared from EDTA serum as described by Lepow *et al.* (1963).

Preparation of Le^a or Le^b positive red cells sensitized with incomplete antibodies anti-Le^a or Le^b and complement

One part of anti-Le^a or -Le^b serum, containing only non-agglutinating antibodies was mixed with one part of a 5% suspension of twice washed 0 Le^a or Le^b positive red cells and incubated for 1 hr at 16°C and thereafter washed three times at room temperature. The cells were then resuspended in one part of fresh AB serum and incubated for a further ½ hr at either room temperature or 37°C, depending on the antiserum used. The cells were finally washed thrice at room temperature.

Incubation of red cells with cold auto-agglutinins in vitro

One part of patient's serum containing strong cold auto-agglutinins, one part of fresh AB serum and two parts of a 5% suspension of twice washed 0 red cells were incubated for 1 hr at 16°C and washed three times in saline at 37°C.

Incubation of red cells with biphasic haemolysins in vitro

One part of patient's serum containing biphasic haemolysins, one part of fresh human complement and two parts of a 5% suspension of twice washed red cells were incubated for 1 hr in melting ice (serum and cells were precooled for 15 min.) The cells were then washed three times at 0°C. Part of the cells were then tested with the anticomplement reagents at 37°C, part of the cells were resuspended in one part of fresh AB serum and incubated for 20 min at 30°C and then washed three times before use in the anticomplement reactions.

Because human and not guinea-pig complement was added for the incubation at 37°C, hardly any haemolysis took place.

Patient's blood

Blood samples were obtained from several patients with cold agglutinin disease or autoimmune haemolytic anaemia with incomplete warm auto-antibodies or biphasic haemolysins.

Preparation and examination of the anticomplement serum

This was prepared by injecting a horse with an optimal complex of egg-albumin/rabbit

anti-egg albumin incubated with fresh human AB serum and washed six times before injection. After immunization the serum was tested by the Ouchterlony technique and immuno-electrophoresis.

Precipitating antibodies against C1q, β1E, β1C, β1A and α2D were demonstrable but also anti-IgG, anti-IgM, anti-albumin and anti-lipoprotein. The serum was absorbed with IgG, IgM, albumin and lipoprotein preparations till it only precipitated β1E, β1C, β1A and α2D. The antiserum was then tested for the presence of agglutinating antibodies with tanned red cells coated with different plasma-proteins or complement fractions or products. The results are shown in Table 1.

From Table 1 it may be seen that the antiserum, although it only precipitated complement factors, still contained strong agglutinating antibodies against lipoproteins and α2 macroglobulins. However, no agglutinating antibodies against immunoglobulins were demonstrable and as there is no reason to assume that the above antibodies will interfere with our studies on red cells, no further absorption with lipoproteins or α2 macroglobulins was carried out.

TABLE 1. Agglutinating antibodies detectable in the anticomplement serum

Titres with tanned red cells coated with:			
IgG	0	α2 macroglobulins	128
IgM	0	lipoprotein	256
IgA	0	C1q	64
IgD	0	β1E	256
transferrin	0	β1C	256
fibrinogen	0	β1A	128
albumin	0	α2D	128

From the reactions with coated tanned cells it may be concluded that the agglutinating antibodies against the following complement components and products were present: C1q, β1E, β1C, β1A and α2D. The presence or absence of antibodies against C1r, C1s, C2 and C5-9 could not be established as these antigens were not available.

Preparation and examination of anti-β1C serum

Rabbits were injected with a 'pure' β1C preparation separated from fresh serum by a modification of Steinbuch's method (Pondman *et al.*, to be published). The serum of immunized rabbits, after examination by the Ouchterlony and immuno-electrophoresis methods, was first absorbed with immunoglobulins G, A and M. It then only precipitated β1C and β1A. The antiserum was tested for the presence of agglutinating antibodies. Results are given in Table 2. Again it may be seen that several strong agglutinating antibodies were demonstrable that gave no precipitation in agar: anti-α2 macroglobulins, anti-C1q, anti-β1E and anti-α2D. The serum was absorbed first with β1E and α2D preparation. It is not possible to state with how much of the antigenic preparations the antisera were absorbed, as the specific antigen concentration of these was very variable. Trial absorptions, based on the optical density at 280 mμ of the antigen preparations, were first

done, adding one drop of preparation to 2, 3, 5, 8 or 10 drops of antiserum. The samples were tested and further absorptions carried out with the appropriate amount of antigen.

After absorption with β1E and α2D the following results were seen (Table 2): the antiserum now only agglutinated red cells on which β1C or β1A was present. The anti-C1q antibodies had been removed as specifically either due to dilution or to impurity of the antigen preparations (used for the absorptions).

Preparation and examination of anti-β1E serum

Rabbits were injected with an optimal complex of egg-albumin/rabbit-anti-egg-albumin incubated with fresh human AB serum and washed six times before injection (Pondman *et al.*, 1960). After immunization the serum was examined by the Ouchterlony and immunoelectrophoresis techniques. Precipitation was seen with IgG, IgM, α2 macroglobulins, lipo-

TABLE 2. Agglutinating antibodies present in anti-β1C serum

Titres obtained with tanned cells coated with:			
before absorption		after absorption	
IgG	0		
IgM	0		
IgA	0		
IgD	0		
transferrin	0		
fibrinogen	0		
albumin	0		
α2 macroglob.	128		
lipoprotein	64		
C1q	16	C1q	0
β1E	256	β1E	0
β1C	512	β1C	256
β1A	64	β1A	32
α2D	128	α2D	0

proteins, β1E, β1C, β1A and α2D. It was absorbed with IgG, IgM, α2 macroglobulins, lipoproteins, β1A and α2D until it only precipitated β1E globulin. The serum was then examined for the presence of agglutinating antibodies: although the antiserum only precipitated β1E, quite strong agglutinating antibodies, anti-β1C and anti-α2D, were present. However, by further absorption with these antigens the antiserum could be made specific for β1E globulin (Table 3).

Preparation and examination of anti-α2D serum

The immunization was carried out as for anti-β1E serum. The serum of immunized rabbits was absorbed in the following two ways:

1. It was absorbed with IgG, IgM, α2 macroglobulins, lipoproteins, β1E and β1A till it only precipitated α2D. It then still contained quite strong agglutinating antibodies anti- β1E and β1A. By further absorption with these antigens the antiserum could be made specific for α2D.

2. The antiserum was absorbed with heated (1 hr at 56°C) hydrazine-treated normal AB serum, in which all proteins are present except α2D which is destroyed by this treatment. The antiserum was then found to specifically precipitate α2D and to only agglutinate α2D coated tanned red cells (Table 4).

TABLE 3. Agglutinating antibodies present in anti-β1E serum

Titres with tanned red cells coated with:			
before absorption		after absorption	
IgG	0		
IgM	0		
IgA	0		
IgD	0		
transferrin	0		
albumin	0		
α2 macroglob.	0		
fibrinogen	0		
C1q	0		
β1E	64	β1E	32
β1C	128	β1C	0
β1A	32	β1A	0
α2D	512	α2D	0

TABLE 4. Agglutinating antibodies present in anti-α2D serum

Titres with tanned red cells coated with:			
IgG	0	lipoprotein	0
IgM	0	fibrinogen	0
IgA	0	C1q	0
IgD	0	β1E	0
transferrin	0	β1A	0
albumin	0	α2D	64
α2 macroglob.	0		

RESULTS

The above specific antisera against single components or products of complement were used to detect which of these are present on red cells, the complement-coating of which had been effected in various ways, or on red cells from patients with various kinds of autoimmune haemolytic anaemia.

1. It was first examined if the results obtained with absorption studies in earlier work in this laboratory (Peetoom, van der Hart & Pondman, 1964) could be confirmed by direct

agglutination by the specific anticomplement-component sera. The red cells used in these studies were: red cells sensitized at 0°C in fresh human serum with the so-called incomplete cold antibodies (*RC-ICA*), red cells from patients with cold agglutinin disease (*RC-CAD*), and red cells sensitized with complement fixing incomplete antibodies, anti-Le^a or -Le^b (*RC-Lewis sens.*). The results are given in Table 5.

Had it been shown that RC-ICA only removed anti-β1E (then called β1E2) from the anticomplement serum leaving the precipitation with β1C globulin and α2D intact, it could now be directly confirmed that such cells are strongly agglutinated by anti-β1E, but not by anti-β1C or anti-α2D serum.

In the same way it was confirmed that only α2D can be demonstrated on the red cells of patients with cold agglutinin disease. Finally, as could be expected from the absorption studies, red cells sensitized with complement fixing incomplete anti-Lewis antibodies were strongly agglutinated by anti-β1E, anti-β1C and anti-α2D serum.

TABLE 5. Complement factors demonstrable on complement coated red cells

Absorption studies		Agglutination reactions	
Anti-complement serum absorbed with:			
RC-ICA	→ anti-β1E removed	anti-β1E	++
	anti-β1A, anti-α2D } intact	anti-β1C	−
		anti-α2D	−
RC-CAD	→ anti-α2D removed	anti-α2D	++
	anti-β1E, anti-β1A } intact	anti-β1E	−
		anti-β1C	−
RC-Lewis sens.	→ anti-β1E, anti-β1A, anti-α2D } removed	anti-β1E	++
		anti-β1C	++
		anti-α2D	++

2. As was mentioned in the introduction West *et al.* (1966) showed that β1C, when it reacts with EAC $\overline{142}$ or immune-aggregates is split in three parts: β1A carrying the A antigenic determinant, α2D carrying the D and D^d determinants of which the latter could not be demonstrated on the intact β1C molecule by immune precipitation in agar and a part which carries the B determinant and which is probably identical with our /β1C/. When working with anticomplement and anti-β1C serum it is therefore necessary to investigate which of the theoretically possible antibodies against β1C antigenic determinants are present. Both antisera were therefore tested with tanned red cells coated with the intact β1C globulin, β1A or α2D and with RC-CAD and *RC-Lewis sens.* The /β1C/ is not available (Table 6).

From the results in the table the following may be learned:

A. The anticomplement serum contains anti-β1A and anti-α2D, but not anti-/β1C/ as the reaction of this serum versus β1C coated cell could be neutralized by the addition of β1A and α2D alone. The anti-β1C serum, however, does contain anti-/β1C/ as the same absorptions left a positive reaction with β1C coated tanned cells. Unless specifically stated the anti-β1C serum therefore contains anti-β1A and anti-/β1C/.

B. The anticomplement serum contains anti-α2D antibodies that cannot be absorbed with the intact β1C molecule (see the positive reactions that are left with RC-CAD and α2D coated tanned cells).

C. The /β1C/ is not present on *RC-Lewis sens.* that have reacted with β1C globulin, as the reaction of anti-β1C with these cells could be completely neutralized with β1A and α2D alone.

3. (a) As was already mentioned, the red cells of patients suffering from cold agglutinin disease, were only agglutinated by anti-α2D serum and not by anti-β1C or -β1E serum, which means that α2D, but not β1A, /β1C/ or β1E is present on these cells.

When the serum of patients with cold agglutinin disease is incubated with normal red cells in the presence of human complement, these cells are, after proper washing, strongly agglutinated by anti-β1E, anti-β1C and anti-α2D serum, which means that by this *in vitro* incubation the cells must at least have reached the state EAC $\overline{1423}$.

(b) The red cells from patients suffering from autoimmune haemolytic anaemia with warm haemolysins were also only agglutinated by anti-α2D and not by anti-β1C or -β1E serum and showed therefore identical reactions to the RC-CAD.

TABLE 6. Antibodies against antigenic determinants of β1C and anti-C and anti-β1C serum

	Tanned cells coated	
	β1C	β1A
anti-C serum	++	++
absorbed × β1C+α2D	−	−
absorbed × β1A+α2D	−	−

	Tanned cells coated	
	β1C	β1A
anti-β1C serum	++	++
absorbed × β1C+α2D	−	−
absorbed × β1A+α2D	++	−

	α2D coated tann. cells	RC-CAD	*RC-Lewis sens.*
anti-C serum	++	++	++
absorbed × β1C	++	++	++
absorbed × β1A	++	++	++
absorbed × α2D	−	−	++

	α2D coated tann. cells	RC-CAD	*RC-Lewis sens.*
anti-β1C serum	−	−	++
absorbed × β1C	−	−	−
absorbed × β1A	−	−	−
absorbed × α2D	−	−	++

(c) The red cells of patients suffering from autoimmune haemolytic anaemia with biphasic haemolysins were also examined with our anticomplement reagents. It was found that when the blood of these patients was kept at 37°C after withdrawal, the cells were again only agglutinated by anti-α2D and not by anti-β1C or -β1E serum.

Normal red cells were incubated with biphasic haemolysins, either at 0°C alone or at 0°C and subsequently at 37°C. The following results were obtained:

After incubation at 0°C alone or after incubation at 0°C and subsequent incubation at 37°C, red cells sensitized by three sera, containing biphasic haemolysins, were agglutinated strongly by anti-β1E, anti-β1C and anti-α2D serum. The complement fixation reaction goes therefore, also when kept strictly at 0°C, at least as far as C3.

(d) As was mentioned in another publication (Engelfriet *et al.*, 1968), complement *may* be detected on the red cells of patients suffering from autoimmune haemolytic anaemia with IgG, IgM, or IgA incomplete warm auto-antibodies.

Cells of some of these patients were examined with the anticomplement reagents and again found to be agglutinated only by anti-α2D. After incubation of normal red cells with either the serum of these patients or an eluate prepared from their red cells, *no* complement could be detected on these cells. The results obtained with the blood of the patients with autoimmune haemolytic anaemia are summarized in Table 7.

TABLE 7. Complement factors detectable on the red cells of patients with autoimmune haemolytic anaemia or red cells incubated with their serum

RC patients	anti-β1E	anti-β1A	anti-α2D
CAD	−	−	++
Warm haemolysins	−	−	++
Bipphasic haemolysins	−	−	++
Incomplete warm antibodies	−	−	++
RC incubated serum patients			
CAD	++	++	++
Biphasic haemolysins 0°C or 0°C+37°C	++	++	++
Incomplete warm antibodies	−	−	−

DISCUSSION

When preparing antisera specific for one class of immunoglobulins for use in the antiglobulin reaction, we found that such antisera, when made monospecific in agar-precipitation, may still contain strong *agglutinating* antibodies of other specificity (Engelfriet *et al.*, 1968).

The same was found in the preparation of antisera against single complement components or products. Before application in the anticomplement reaction, such antisera must therefore be carefully examined for their content of *agglutinating* antibodies. This could be done by coating tanned red cells with antigen preparations. In this way it was possible to further characterize our anticomplement serum, in which anti-C1q, anti-β1E, anti-β1A and anti-α2D could be demonstrated and to prepare antisera, that as far as we can test with the presently available antigens, are specific for β1E, β1A, /β1C/ and α2D.

It should be stressed again here that we *cannot* exclude in these antisera, the presence of anti-C1r, anti-C1s, anti-C2, and anti-C5-9, as these antigens were not yet available. We can, therefore, only conclude to the presence or absence of *those* complement components against which antibodies could be specifically demonstrated, first by absorption studies and later by direct agglutination, i.e. β1E, β1A, α2D and /β1C/.

The results obtained with the above specific antisera will be discussed now.

1. The results of absorption studies performed earlier in this laboratory could be confirmed by direct agglutination (Table 5): (a) Red cells sensitized at 0°C in fresh serum with the so-called incomplete cold antibodies were only agglutinated by anti-β1E serum and *not* by the antisera against the antigenic determinants of the β1C molecule. It means that the complement fixation reaction in this case has not gone as far as C3. (b) Red cells from

patients with cold agglutinin disease were only agglutinated by anti-α2D serum. As these cells also only removed antibodies against anti-α2D from anticomplement serum and not anti-β1E or anti-β1A, the conclusion seems warranted that no β1E is present on the cells and only the α2D part of the β1C molecule. This will be further discussed below. (c) The complement factors β1E, β1A, and α2D could be demonstrated on red cells sensitized with incomplete complement binding anti-Lewis antibodies and complement, confirming the absorption results.

2. It could be shown that anticomplement serum, prepared by immunization with an immune-aggregate, coated with complement, contains anti-β1A and anti-α2D but *not* antibodies against that part of the β1C molecule that we call /β1C/ and which is probably identical with the B determinant of West *et al.* However, antiserum produced by immunization with β1C globulin *does contain* anti-/β1C/. This is in complete agreement with the results of Pondman *et al.* obtained in agar-precipitation (Pondman *et al.*, to be published). It also agrees with the finding that with specific antiserum /β1C/ cannot be demonstrated on EAC $\overline{1423}$. This is in contrast to the conclusion of West *et al.* who assume that this B determinant does fix on the cell membrane when EAC $\overline{142}$ reacts with β1C globulin.

Their assumption that β1A and α2D only go into the fluid phase cannot be true either, as these complement factors can be demonstrated on any cell that has reacted *in vitro* with complement fixing antibodies and complement.

From our results it is clear that β1A and α2D are fixed on the cell-membrane, while /β1C/ is lost into the fluid phase. Therefore, when either β1A or α2D or both are demonstrated on red cells, it means that the complement-fixation reaction has gone as far as C3.

We could confirm West's finding, that α2D contains an antigenic determinant (D^d) which is not detectable on the intact β1C molecule.

3. It appeared that when red cells of patients suffering from *any* of the serologically defined groups of autoimmune haemolytic anaemia (Engelfriet *et al.*, 1968) are agglutinated by anticomplement serum, only α2D and not β1E or β1A was detectable on such cells with our specific reagents. Where the cells of patients with cold agglutinin disease are concerned, this is in contrast to data in the literature. For example Evans *et al.* (1967) concluded to the presence on such cells of β1E and β1C globulin, using ^{131}I-labelled antisera. The presence of anti-α2D antibodies in these reagents was not excluded and when present these would presumably also be labelled with ^{131}I. It is clear from the *in vitro* incubation of red cells with biphasic haemolysins that also when kept strictly at 0°C, activation of β1C takes place. This is in contrast to the results of the above absorption studies (Peetoom, van der Hart & Pondman, 1964) in which it was found that red cells sensitized *in vitro* with biphasic haemolysins and complement did not absorb anti-β1A from anticomplement serum. This discrepancy can probably be explained on quantitative grounds.

The finding that on red cells from patients with haemolytic, complement binding autoantibodies in their blood (i.e. cold agglutinin/haemolysins, warm haemolysins or biphasic haemolysins) only the split product α2D of the β1C molecule is present, while red cells sensitized *in vitro* by these antibodies are strongly coated with β1E and β1A also, seems very interesting. The presence of α2D on the patient's cells means that they, through the activity of the haemolytic antibodies *in vivo*, reached at least the state EAC1423. The mere fact that such cells are present in the patient's blood means that the reaction of the cell with the haemolysins does not always lead to haemolysis. This is also true *in vitro* where, through incubation with haemolysins and complement under proper circumstances, intact cells

may be produced that have at least reached the state EAC 1423. The reason why this does not always lead to haemolysis is unknown. Apparently when this occurs *in vivo*, β1E and β1A are subsequently removed from the cell, leaving only α2D. The mechanism of this is not known either. *In vitro* β1A may easily be removed from such cells by incubation for 2 hr at 37°C. However, β1E cannot be removed *in vitro*.

As will be shown in a following paper, we obtained evidence that red cells that have reacted with haemolytic antibody and complement without being haemolysed, thereafter behave differently when again confronted with the haemolytic antibodies and complement.

Biphasic haemolysins when incubated with normal red cells, strictly at 0°C, cause these to be agglutinated by anti-β1A and anti-α2D.

Much controversy exists in the literature about the fact if the complement reaction caused by biphasic haemolysins at 0°C reaches C3. Our results with three sera indicate, that C3 is bound.

Red cells from some of the patients with autoimmune haemolytic anaemia with incomplete warm autoantibodies are also agglutinated by anti-α2D serum. The reason of the presence of complement on these cells is not known as the antibodies, when detectable in serum or eluate, do not seem to be able to fix complement *in vitro*.

The preparation of specific agglutinating antisera against single complement components or products thus enabled us to study some aspects of the complement fixation reaction. Specific antisera against other complement components are needed for further study.

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