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AUTOIMMUNE HAEMOLYTIC ANAEMIAS

I. SEROLOGICAL STUDIES WITH PURE ANTI-IMMUNOGLOBULIN REAGENTS

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SUMMARY

In this paper the preparation and examination of pure antisera against immunoglobulins is described. The results of serological studies obtained with pure anti-IgG, anti-IgM and anti-IgA sera as well as an anti-complement serum and the blood of four patients with acquired haemolytic anaemia are discussed. The presence of incomplete warm autoantibodies belonging to the G, M and A classes of immunoglobulins was demonstrated. Further, evidence was obtained that warm haemolysins are a separate antibody category independent of the presence of incomplete warm autoantibodies.

A new serological classification of the autoimmune haemolytic anaemias based on these findings is given.

INTRODUCTION

For many years autoimmune haemolytic anaemias have been divided into three types according to the serological characteristics of the autoantibodies that could be demonstrated: (1) biphasic haemolysins, (2) cold agglutinins and haemolysins, and (3) incomplete warm antibodies. Further, many cases have been described in which the serum of the patient was capable of haemolysing red cells *in vitro* with an optimal activity at 37°C. It is difficult to know from previous publications if this warm haemolysis is caused by a separate antibody (Schubothe, 1958; Dacie, 1962).

Several types of immunoglobulins are now recognized: IgG, IgM, IgA, IgD and IgE. Consequently, in the serological classification of red cell autoantibodies, the type of immunoglobulin to which the antibody belongs is now taken into account. Weiner (1967) distinguished two groups:

I. A group in which the autoantibodies are immunoglobulin G, subdivided into: (a) incomplete warm autoantibodies, (b) 'incomplete' warm haemolysins (i.e. antibodies that

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only lyse enzyme-treated cells), (c) biphasic haemolysins (Donath–Landsteiner antibodies), and (d) 'acid' warm agglutinins.

Weiner is of the opinion that 'incomplete' haemolysins are the same antibodies as incomplete warm antibodies.

II. A group in which the autoantibodies are immunoglobulin M, i.e. cold agglutinins and haemolysins.

Gerbal *et al.* (1967) divide the autoimmune haemolytic anaemias in groups according to the results of the direct anti-human-globulin test (AHGT): in some patients the direct AHGT was positive with anti-IgM and anti-complement serum, when the cells were washed at 4°C. In these cases cold autoagglutinins anti-I were always demonstrable in the patient's serum or in an eluate prepared from the patient's red cells. In other patients the direct AHGT was found positive with anti-IgG serum only. Finally a combination of these types was also seen. In our hands it is not possible to use red cells coated with cold autoagglutinins anti-I washed at 4°C in an antiglobulin test as these cells are already agglutinated. In our studies these cells were washed at 37°C; they then do not react with anti-IgM serum as will be shown later in Table 6. Furthermore, Jeannet (1965) drew attention to the fact that so-called 'pure' anti-IgM sera may still contain strong antibodies against complement factors, which may lead to erroneous conclusions.

Apart from an anti-IgG and an anti-complement serum, we have, for some time used a pure anti-IgM and IgA serum for the serological examination of the blood of patients with acquired haemolytic anaemia. The purpose of this paper is to give a detailed description of the preparation and examination of these reagents and of the results obtained in the serological examination of the blood of some patients with autoimmune haemolytic anaemia.

Evidence is given that warm haemolysins are a separate antibody category. A more detailed division of autoimmune haemolytic anaemia on serological grounds is proposed.

MATERIALS

Patients' blood

The blood of the patients was sent to our laboratory for serological examination because their clinical and haemotological data were suggestive of the diagnosis acquired haemolytic anaemia.

Antiglobulin reagents

These were prepared by one of us (M.v.d.G.).

(1) *Anti-IgG serum.* This was prepared by injecting rabbits with their own red cells incubated for 1 hr at 37°C with an IgG preparation isolated by a double Cohn fractionation from a pool of human plasma. The cells were washed four times before injection.

The serum of immunized rabbits was tested in the Ouchterlony and immunoelectrophoresis techniques against a pool of normal human sera. The antiserum was absorbed, when needed, with the necessary antigens until only one precipitation line was seen in the above mentioned techniques. The anti-IgG serum was also absorbed with human red cells to remove all heteroagglutinins as were the other antiglobulin reagents. The antiglobulin reagent was then tested with serological methods against red cells senstitized with different kinds of red cell antibodies and tanned cells coated with different pure protein frac-

tions. The results of these tests together with those obtained with the anti-IgM and anti-IgA sera, are given in Table 1.

The anti-IgG serum was also absorbed with IgG, IgM and IgA globulin and then retested against IgG coated tanned cells. The reaction with these cells could only be prevented by absorption with immunoglobulin G (see Table 2).

TABLE 1. Reactions of pure anti-IgG, anti-IgM and anti-IgA serum with red cells sensitized with different red cell antibodies and tanned red cells coated with different protein fractions

	Anti-IgG	Anti-IgM	Anti-IgA
Red cells sensitized with:			
Inc. ab anti-D	++	−	−
Inc. ab anti-K	++	−	−
Inc. ab anti-Fy^a	++	−	−
C′ fixing inc. ab anti-Jk^a	++	−	−
C′ fixing inc. ab anti-Le^a	−	++	−
Nat. occ. inc. cold ab	−	−	−
Red cells from patients with the cold agglutinin syndrome	−	−	−
Tanned cells coated with:			
IgG	++	−	−
IgM	−	++	−
IgA	−	−	++
Albumin	−	−	−
Fibrinogen	−	−	−

TABLE 2. Absorption of pure anti-IgG, anti-IgM and anti-IgA serum with IgG, IgM and IgA globulin

	IgG coated tanned E		IgM coated tanned E		IgA coated tanned E
Anti-IgG	++	Anti-IgM	++	Anti-IgA	++
Abs. with IgG	−		++		++
Abs. with IgM	++		−		++
Abs. with IgA	++		++		−

From these results it may be concluded that the anti-IgG serum only reacts with cells sensitized or coated with IgG globulin and that it does not contain anti-complement antibodies as it gave no reactions with red cells sensitized with complement fixing antibodies and red cells from patients with the cold agglutinin syndrome. It further appeared that the activity of the anti-IgG serum could only be neutralized by IgG.

(2) *Anti-IgM serum.* This was prepared by injecting rabbits with IgM globulin isolated from the serum of patients with Waldenström's disease in the following way. The euglobulins were precipitated and twice reprecipitated by dilution with distilled water (1:10) and were then fractionated by gel filtration on Sephadex G-200 equilibrated with 0·1 M-Tris buffer, pH 8·2, containing 1 M-NaCl.

The serum of immunized animals was examined in a similar fashion to the anti-IgM serum. It appeared that the antiserum after suitable absorption gave only one line in the precipitation techniques, but still contained strong agglutinating anti-complement antibodies. A further absorption with sheep red cell stroma sensitized with rabbit anti-sheep red cell antibodies and human complement was, therefore, necessary. The antiserum then only reacted with IgM-sensitized or IgM-coated cells (Table 1). The anti-complement antibodies had been removed and the antiserum's activity *versus* IgM coated cells could only be specifically absorbed with IgM (see Table 2).

(3) *Anti-IgA serum.* This was prepared by injecting rabbits with defatted human milk. The serum of immunized animals was examined as mentioned above and absorbed with human cord serum in which no IgA globulin could be detected. The anti-IgA serum which gave only one line in the precipitation techniques appeared to still contain agglutinating anti-IgG antibodies. A further absorption with IgG was, therefore, carried out. From the results in Table 1 it may be concluded that the final product only reacted with IgA coated cells. The activity *versus* IgA coated cells could only be absorbed with immunoglobulin A.

(4) *Anti-complement serum.* A horse was immunized with a complex of ovalbumen–rabbit anti-ovalbumen which had been washed six times and then incubated with fresh human serum and washed a further four times. The anti-complement serum thus obtained contained, apart from anti-complement antibodies, antibodies against immunoglobulin G and M. It was, therefore, absorbed with these protein fractions till no precipitation was seen. It was also absorbed with human O red cells.

SEROLOGICAL METHODS

Direct antiglobulin test

Two-fold serial dilutions of the antiglobulin sera were prepared. To one drop of antiglobulin reagent one drop of a 4% suspension of the patient's red cells, which had been washed three times at room temperature or at 37°C was added. After incubation for 1 hr at 37°C the results were read microscopically.

Indirect antiglobulin test

One drop of serum and dilutions thereof were incubated for 1 hr at 37°C with one drop of a washed 4% suspension of panel red cells. The red cells were then washed three times (at room temperature or at 37°C) and one drop of antiglobulin serum was added. After a further incubation of $\frac{1}{2}$ hr results were read macroscopically.

Agglutination techniques

(1) *Saline agglutination at 16°C and 37°C.* One drop of the serum and dilutions thereof were incubated with one drop of a once washed 4% suspension of panel red cells for 1 hr. The serum and the cells were pre-incubated at the desired temperature for 15 min. Results were read microscopically.

(2) *Two-stage bromelin method.* Bromelin-treated cells were prepared by incubating one part of a thrice washed 4% panel red cell suspension with nine parts of a 0·5% bromelin solution in EDTA-buffer. After incubation for 30 min at 37°C the cells were washed three times. The agglutination test was as described above.

Techniques for the demonstration of monophasic haemolysins

Technique at pH 7·5. The serum of the patient was diluted 1:2 in fresh AB serum. From this a two-fold serial dilution was made in fresh AB serum. To five of these dilutions one drop of a 20% suspension of panel red cells or bromelin treated panel red cells was added. The mixtures were incubated for 1 hr at 16° and 37°C. *All reagents must be pre-incubated at the desired temperature for* 15 *min before mixing*. Reactions were read after centrifugation for 1 min at 1000 rev/min.

Technique at pH 6·5. Identical to the above mentioned technique except that the patient's serum and the fresh AB serum must be brought to pH 6·5 by mixing thirteen drops of serum with one drop of a 0·254 N solution of HCl.

Technique for the demonstration of biphasic haemolysins

From the serum, diluted 1:2 in fresh AB serum, two-fold dilutions were made in fresh AB serum. Five drops of these dilutions, pre-incubated at 0°C were mixed with one drop of an equally pre-cooled, thrice washed 20% suspension of panel red cells or bromelinized panel red cells. After incubation for 1 hr at 0°C the cells were washed three times at 0°C. These cells and commercial guinea-pig complement (supplied by the Rijksinstituut voor de Volksgezondheid, National Institute of Health, Utrecht, the Netherlands) diluted 1 : 10 were then pre-warmed at 37°C for 15 min. The cells were then resuspended in five drops of guinea-pig complement and incubated for 20 min at 37°C. Reactions were read after a very careful centrifugation for 1 min at 1000 rev/min.

RESULTS

The results of the serological examination of the following patients will be given in detail.

I. A patient with incomplete IgM warm autoantibodies.
II. A patient with incomplete IgA warm autoantibodies.
III. A patient with incomplete IgG, IgM *and* IgA warm autoantibodies.
IV. A patient with warm autohaemolysins.

Patient 1

Brief history

This 78-year-old female patient was admitted to hospital because of extreme lassitude and dypsnoea of 4 months duration, anaemia and slight jaundice.

Physical examination

This revealed no abnormalities except slight jaundice of skin and mucosae.

Laboratory findings

Haemoglobin 9·3 g/100 ml, indirect biluribin 1·75 mg/100 ml, increased osmotic fragility, strongly increased erythropoiesis in the bone marrow. No free haptoglobin was detectable in the serum. There was no autoagglutination of the red cells.

Diagnosis

Haemolytic anaemia of unknown origin.

Serological examination

The direct anti-human-globulin test. The results of the direct AHGT on the patient's red cells with the different antiglobulin reagents and anti-complement serum are given in Table 3. The antiglobulin reagents

that gave positive results were also absorbed once more with the different pure immunoglobulin preparations to demonstrate further the specificity of the reaction. The results of these absorption experiments are also given in Table 3. The antiglobulin reagents were absorbed in the following way for all the experiments: two parts of antiglobulin reagent were absorbed with one part of a 0·1% solution of IgG, IgM or IgA preparation and as a control mixed with one part of saline solution. The anti-complement serum was absorbed with red cells sensitized with so-called incomplete cold antibodies, incomplete complement-fixing anti-Le^a antibodies and red cells from patients with cold agglutinin disease (two parts of anti-complement serum with one part of packed cells). The absorption was continued until the anti-complement serum did not react anymore with the cells used for the absorption.

TABLE 3. Results of the direct anti-human-globulin test in a patient with incomplete warm IgM antibodies

Titration of antiglobulin reagents					
Before absorption		After absorption			
Anti-IgG	—	Anti-IgM × saline	256	Anti-C′ × saline	128
Anti-IgM	512	Anti-IgM × IgG	256	Anti-C′ × IgM	128
Anti-IgA	—	Anti-IgM × IgM	—	Anti-C′ × E coated with C′	—
Anti-C′	256				

From the results shown in Table 3, it may be concluded that the cells of this patient were not sensitized with IgG or IgA antibodies, but that IgM molecules and complement were present on their surface. Similar results were described by us in 1966 (Engelfriet *et al.*, 1968). The reaction of the anti-IgM serum with the patient's cells could be specifically absorbed with IgM globulin and the reaction of the anti-complement serum with complement-coated cells.

Examination of serum and eluate. No antibodies of any kind were demonstrable in the patient's serum. In an eluate prepared from the patient's red cells by the ether method (Rubin, 1963) incomplete warm autoantibodies could be detected with the indirect anti-IgM test.

The indirect anti-complement reaction gave negative results.

No blood group specificity of the antibodies could be demonstrated, e.g. no specificity connected with the Ii system (Gerbal *et al.*, 1967). However, the eluate could not be tested with cells totally deleted for the Rh antigens.

Summary

From the results of the serological examination of the blood of this patient it may be concluded that she suffered from autoimmune haemolytic anaemia with *incomplete IgM warm autoantibodies.* It is important to note that these antibodies caused *no* autoagglutination of the patient's cells and did not react in the saline agglutination technique. Apart from the incomplete IgM antibodies, complement was also demonstrable on the patient's cells. However, the antibodies that could be eluted from the patient's red cells did not bind complement *in vitro.* As will be discussed in a later paper similar results were obtained in cases with incomplete IgG warm antibodies.

Patient 2

Brief history

This 60-year-old male patient was admitted to hospital in 1964 because of severe haemolytic anaemia of unknown origin.

Physical examination

Apart from jaundice no abnormalities were found.

Laboratory findings, 1964

Haemoglobin 8·3 g/100 ml, indirect biluribin 1·75 mg/100 ml, reticulocytes 96‰. No free haptoglobin was detectable in the serum. There was no autoagglutination of the red cells. The osmotic fragility was increased.

Diagnosis

Haemolytic anaemia of unknown origin.

Serological examination

The direct anti-human-globulin test was positive with anticomplement serum and negative with anti-IgG serum. No red cell antibodies were demonstrable in the serum. The survival time of the patient's own red cells and of normal donor cells was greatly decreased. An increased osmotic fragility of the red cells is found both in congenital spherocytosis and in cases of autoimmune haemolytic anaemia with incomplete warm autoantibodies. However, only complement could be demonstrated on the red cells. When this is the only detectable serological finding, the survival time of the red cells is always normal (to be published). The cause of the increased haemolysis and the increased osmotic fragility was therefore not clear. However, as a strong sequestration of radioactivity in the spleen was found by surface counting, a splenectomy was performed with very good results.

A further sample of blood was recently received for serological follow-up. The results of the direct AHGT performed on the red cells of this sample are given in Table 4.

TABLE 4. Results of the direct anti-human-globulin test in a patient with incomplete warm IgA antibodies

Titration of the antiglobulin reagents					
Before absorption		After absorption			
Anti-IgG	4	Anti-IGA × saline	512	Anti-IgG × saline	2
Anti-IgM	—	Anti-IgA × IgG	512	Anti-IgG × IgG	2
Anti-IgA	2048	Anti-IgA × IgM	512	Anti-IgG × IgA	—
Anti-C′	128	Anti-IgA × IgA	—		

It is clear from Table 4, that the red cells of this patient were strongly coated with IgA and that the reaction of the anti-IgA serum with the patient's red cells could only be neutralized by IgA. Complement was also demonstrated on the red cells. The weak reaction of the anti-IgG serum with the patient's erythrocytes could be neutralized by IgA, but not by IgG. This weak reaction was probably caused by the presence of some anti-IgA antibodies left in the anti-IgG serum, the presence of these antibodies becoming apparent because of the very strong sensitization of the patient's red cells. The reactions of the anti-complement serum could only be neutralized by absorption with complement-coated cells.

Examination of serum and eluate. In the patient's serum no red cell antibodies were demonstrable. In an eluate prepared from the patient's red cells by the ether method incomplete IgA warm autoantibodies were demonstrable with the indirect anti-IgA technique. The antibodies had the specificity anti-e. Here again the indirect anti-complement test was negative.

Summary

Because of the application of a pure anti-IgA serum it was demonstrated that this patient suffered from autoimmune haemolytic anaemia with IgA incomplete warm antibodies, a type of autoantibody not so far described. In view of the previous findings it is very probable that the patient already suffered from this disease in 1964.

Patient 3

Brief history

This 58-year-old male patient was admitted to hospital because of haemolytic jaundice of unknown origin. In the period preceding the admission he had felt very tired. Apart from splenomegaly, no abnormalities were found on physical examination.

Laboratory findings

The haemoglobin content of the blood was 8·3 g/100 ml, reticulocyte count 300‰, indirect bilirubin content 3·2 mg/100 ml. There was no autoagglutination of the red cells and the haptoglobin content of the serum was strongly decreased.

Diagnosis

Idiopathic acquired haemolytic anaemia.

Serological examination

Direct anti-human-globulin test. The results of the direct AHGT on the patient's red cells are given in Table 5.

TABLE 5. Results of the direct AHGT and absorption of antiglobulin reagents with immunoglobulins in a patient with incomplete IgG, IgM and IgA warm antibodies

Anti-IgG	1024	Anti-IgG × saline	1024	Anti-IgM × saline	512	Anti-IgA × saline	1024
Anti-IgM	1024	Anti-IgG × IgG	2	Anti-IgM × IgG	512	Anti-IgA × IgG	1024
Anti-IgA	1024	Anti-IgG × IgM	1024	Anti-IgM × IgM	–	Anti-IgA × IgM	1024
Anti-C′	64	Anti-IgG × IgA	1024	Anti-IgM × IgA	512	Anti-IgA × IgA	–

From the results in Table 5 it is clear that IgG, IgM and IgA incomplete warm autoantibodies were demonstrable on the red cells of this patient. This may be concluded from the reaction of the patient's erythrocytes with the anti-IgG, anti-IgM and anti-IgA sera and because the reaction of each of these reagents could only be neutralized by the corresponding immunoglobulins.

Examination of serum and eluate. No antibodies were demonstrable in the patient's serum. In an eluate prepared from the patient's red cells incomplete IgG and IgA warm autoantibodies were demonstrable with the indirect anti-IgG and anti-IgA test. The indirect anti-IgM test was negative, as was the indirect anti-complement reaction. No blood group specificity of the antibodies could be found; the antibodies also reacted with cells totally deleted of the Rhesus antigens.

Summary

This patient suffered from autoimmune haemolytic anaemia with incomplete IgG, IgM and IgA warm autoantibodies.

Patient 4

Brief history

This 78-year-old female patient was admitted to hospital because of a myocardial infarction.

Laboratory findings

The haemoglobin level of the blood was 7 g/100 ml. There were 330‰ reticulocytes and the indirect bilirubin content was slightly increased. There was no autoaglutination of the red cells. No free haptoglobin was found in the serum.

Diagnosis

Myocardial infarction and haemolytic anaemia of unknown origin.

Serological examination

Direct anti-human-globulin test. The patient's red cells gave a negative reaction with the antisera against immunoglobulin G, A and M. However, a very strong reaction (titre 256) was seen with the anti-complement serum i.e. no IgG, IgM or IgA antibodies were detectable on the red cells, but these were found to be strongly coated with complement factors.

Examination of serum and eluate. No agglutinating or incomplete antibodies were demonstrable in the patient's serum or in an eluate prepared from the patient's red cells. However, the serum strongly haemolysed bromelin-treated red cells. The reactions for the demonstration of monophasic haemolysins gave the following titres using the patient's serum and bromelin-treated red cells.

Incubation at 16°C		Incubation at 37°C	
pH 6·5	pH 7·5	pH 6·5	pH 7·5
2	—	128	16

The haemolytic activity of the patient's serum was weak—negative at 16°C and very strong at 37°C, total haemolysis having taken place in the first four dilutions. Haemolysis was strongest at pH 6·5 and complement dependent. By means of Sephadex column chromatography it was established that the activity was found in the fraction containing the immunoglobulins M. It is clear that the haemolytic activity of the serum is independent of the presence of IgG, IgM or IgA incomplete warm autoantibodies.

Because of these findings the warm haemolysins found in this patient's serum and in the serum of others were submitted to intensive serological, immunochemical and *in vivo* studies. The results of these will be reported in a following paper.

Summary

In summary it may be concluded that the patient suffered from autoimmune haemolytic anaemia with warm haemolysins.

DISCUSSION

From the results of the serological examination of the blood of these four patients, it is evident that incomplete warm autoantibodies against red cells may be found in the G, M and A classes of immunoglobulins. Of these three types, incomplete IgG warm antibodies are by far the most frequent while incomplete IgA warm antibodies are rare. IgA antibodies in isolation have been found in only one patient so far. Details concerning the relative frequency of the different kinds of autoimmune haemolytic anaemia will be given in a later paper. Further studies should reveal whether they also may be immunoglobulins D or E.

Distinction between these different kinds of incomplete warm autoantibodies was made possible by the application of pure antisera against single immunoglobulins and was confirmed by absorption of the antiglobulin reagents with pure immunoglobulins and complement-coated cells.

It became apparent that incomplete IgG, IgM and IgA warm autoantibodies may occur separately or in combination. It also appeared that *IgM* warm autoantibodies behave like typical incomplete antibodies, i.e. they do not cause autoagglutination of the patient's red cells nor are they demonstrable in a saline agglutination technique.

This suggests that these antibodies could be 7S immunoglobulins M as described by Rothfield, Blas & Franklin (1965) and Stobo & Tomassi (1967). Studies will be undertaken to investigate this point.

It is interesting that the incomplete IgA warm autoantibodies in one patient had anti-e specificity. This suggests that, like incomplete IgG warm autoantibodies, the IgA variety are closely connected with the Rhesus-system. No such specificity could be demonstrated for the incomplete IgM warm antibodies found so far.

Finally, evidence was obtained that warm haemolysins are a separate antibody category, independent of the presence of incomplete IgG, IgM and IgA warm autoantibodies. The red cells of patients with warm haemolysins are always coated with complement factors.

Because of these findings the serological classification of autoimmune haemolytic anaemia given in Table 6 is proposed.

TABLE 6. Serological classification and serological findings in patients with autoimmune haemolytic anaemia

	Direct AHGT with:						
	Anti-IgG	Anti-IgM	Anti-IgA	Anti-C′	Serum	Globulin fraction	Eluate
I. AIHA with incomplete warm autoantibodies							
1. IgG inc. warm antibodies	+	−	−	−	Inc. warm ab	IgG	Inc. warm ab
	+	−	−	+	not C′ fixing	IgG	not C′ fixing
2. IgM inc. warm antibodies	−	+	−	−	Inc. warm ab	IgM	Inc. warm ab
	−	+	−	+	not C′ fixing	IgM	not C′ fixing
3. IgA inc. warm antibodies	−	−	+	+	Inc. warm ab not C′ fixing	IgA	Inc. warm ab not C′ fixing
II. AIHA with warm haemolysins	−	−	−	+	Warm haemolysins	IgM	No antibodies
III. AIHA with cold agglutinins/haemolysins	−	−	−	+	Cold agglutinins/ cold haemolysins	IgM	No antibodies
IV. AIHA with biphasic haemolysins	(+)	−	−	+	Biphasic haemolysins	IgG	Sometimes weak biphasic haemolysins

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