AUTO-ANTIBODIES IN ACQUIRED HAEMOLYTIC ANAEMIA

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It is now widely recognized that cases of haemolytic anaemia of spontaneous or endogenous origin may be divided into two broad groups: those determined by a genetical cause resulting in the production of defective red cells, and those not so determined. Among the latter group of acquired haemolytic anaemias are cases in which there is evidence for the presence of antibodies active against the patient's own red cells. That abnormal auto-antibodies might be formed in some cases was suggested by the reports of French authors in the early years of this century. Widal, Abrami, and Brulé (1908a and b; 1909) drew attention to the frequency of autohaemagglutination in cases of *l'ictère hémolytique acquis*, and stressed that this was a distinguishing and characteristic finding. At about the same time Chauffard and Troisier (1908) and Chauffard and Vincent (1909) described as suffering from *ictère hémolysinique* and *hémoglobinurie hémolysinique* patients whose sera were found to contain haemolysins. Although it is true that their reports are somewhat marred by the fact that no account was taken of the existence of the normal isohaemolysins, it seems very probable that the patients' sera did in fact contain abnormal iso-antibodies.

The literature between the pioneer writings of the French haematologists and more recent times is described by Dameshek and Schwartz (1940) in a comprehensive review. They found a number of references to the occurrence of marked autohaemagglutination in acquired haemolytic anaemia, but very few reports of the presence of abnormal haemolysins. They themselves, however, had previously described three patients whose sera appeared to contain abnormal haemolysins (Dameshek and Schwartz, 1938), and a few additional cases have been reported since (Farrar, Burnett, and Steigman, 1940; David and Minot, 1944; Neber and Dameshek, 1947; Dacie, 1949).

The discovery by Coombs, Mourant, and Race (1945) that red cells sensitized with "incomplete" forms of iso-antibodies might be agglutinated by antiglobulin rabbit sera has provided a new tool for the investigation of cases of haemolytic anaemia. Boorman, Dodd, and Loutit (1946) and Loutit and Mollison (1946) reported that the direct Coombs (antiglobulin) test was positive in a number of cases fulfilling the diagnostic criteria of idiopathic acquired haemolytic anaemia, whilst the test was negative in a series of cases of haemolytic anaemia of varying pathogenesis, including several examples of the familial type. This work has since been confirmed.

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Thus auto-antibodies have been observed to affect the red cells of patients with acquired haemolytic anaemia in three different ways, i.e., they may cause auto-haemagglutination, autohaemolysis, and/or sensitization permitting agglutination by antiglobulin sera. Whether or not these different effects are caused by qualitatively different antibody molecules or are dependent on environmental or other factors is uncertain. Recent work suggests that with Rh antibodies differences in serological behaviour may be correlated with differences in the physico-chemical nature of the antibodies (Hill, Haberman, and Guy, 1949). It is certainly convenient to refer to antibodies as agglutinins or haemolysins, as if these were separate entities, and, while admitting that there may be some doubt about the justification for this, the convention will be followed in this paper.

It is the purpose of this paper to stress the value of trypsinized normal red cells and the red cells of patients with paroxysmal nocturnal haemoglobinuria (P.N.H. red cells) in the detection of abnormal antibodies, to record the antibody patterns that have been observed in various types of haemolytic anaemia, and in particular to present evidence for the quite frequent existence in patients' sera of antibodies capable of causing haemolysis *in vitro*.

The patients have been grouped into three clinico-pathological syndromes: Group I, idiopathic acquired haemolytic anaemia; Group II, haemolytic anaemia following virus pneumonia; and Group III, chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena. A control group of patients suffering from other types of haemolytic anaemia or blood disorder and a series of normal subjects has also been studied (Group IV).

Twelve patients were considered to belong to Group I. Four patients have died, three despite splenectomy; several were in partial or almost complete spontaneous or post-splenectomy remission when examined. The four patients of Group II each gave a history of a transient haemolytic anaemia following a pulmonary infection considered to be probably a virus pneumonia. The anaemia of the three patients belonging to Group III was worse in winter time, and each patient gave a history of haemoglobinuria following exposure to severe cold.

A further consideration of the clinical histories of these patients is beyond the scope of this report; an account of the three patients of Group III is to be reported elsewhere (Ferriman, Dacie, Keele, and Fullerton, 1951). A brief note on the histories of most of the patients studied and certain relevant haematological data are, however, given in Tables I and II.

Three other patients suffering from acquired haemolytic anaemia of unknown origin and type have also been investigated. These cases (Nos. 20-22) have, however, been excluded from Group I because of a complete lack of any evidence for the development of abnormal auto-antibodies; in particular, Coombs tests were negative and the cold agglutinin titres were normal. They have been placed with some other distinct types of haemolytic anaemia in Group IV.

Materials

Patients' Sera.—Sera were obtained by defibrinating blood at room temperature or by allowing blood to clot at 37° C. and then centrifuging the expressed serum to rid it of suspended red cells whilst still warm. The sera were used fresh or stored frozen at -20° C. for varying periods of time.

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Patients' Red Cells.—These were obtained from blood allowed to clot at 37° C., and then washed at least three times in a large volume of warmed saline.

Normal Red Cells.—Cell suspensions were made from the fresh defibrinated blood of a healthy subject of blood group O Rh+ (O Rh— in certain cases). The corpuscles were repeatedly washed in large volumes of saline and used at final concentrations varying from 0.5 to 5%. Group O corpuscles of known Rh, MN.S., *Kell*, *Lutheran*, *Lewis*, and *Duffy* types have also been used for detecting the possible presence of immune iso-antibodies.

Complement.—Fresh normal compatible human serum at a final concentration of 1 in 2 was used as a source of complement.

Haemolysis.—This was estimated approximately, after comparing the colour of the supernatant serum with that of a control tube, according to a scale varying from c (complete) +++ (almost complete, deep red supernatant) to + (definite, slight haemolysis).

Agglutination.—The presence or absence of agglutination was determined macroscopically with a concave mirror after gently resuspending the deposited corpuscles and comparing the degree of visible agglutination with that of a control tube. The reciprocal of the highest serum dilution giving detectable agglutination was recorded as the titre of the serum.

pH.—Sera were in some instances made more alkaline by the addition of 10% by volume of N/5 or N/10 NaOH or more acid by the addition of 10% by volume of N/10 to N/3 HCl. pH was estimated approximately by using thymol blue, phenol red, or bromthymol blue as an indicator, and was checked, when sufficient serum was available, by means of a glass electrode.

Technical Methods

Titration of Cold Agglutinins.—Sera were separated off at 37° C. Serial dilutions of the serum were made in saline using a glass automatic pipette, and equal volumes of a 1% suspension of group O red cells added. A series of 10 tubes giving final concentrations of from 1 in 4 to 1 in 1,024 in addition to a saline control has generally been used. If necessary, the range of serum dilutions has been extended by six or more tubes. Volumes of 0.25 ml. were used as a rule, and the tests carried out in tubes of 10 mm. internal diameter.

The intensity of agglutination was assessed after allowing the suspension to remain undisturbed for at least two hours at 2–5° C. in a refrigerator. In some cases readings were taken at room or higher temperatures before the tubes were chilled. Finally, the racks of tubes were placed in the 37° C. water bath to confirm (or refute) the expected disappearance of agglutination at this temperature.

Detection and Titration of Cold Haemolysins.—Acid-sensitive cold haemolysins were demonstrated by the methods used by Dacie (1950c). In its simplest form the test consists of adding an equal volume of fresh normal serum to the patient's serum, acidifying the mixture with a 1/10 volume of N/3.5 HCl and then adding a 1/10 volume of a 30% suspension of normal red cells. The pH of the cell-serum suspension should be about 6.5. After allowing the tubes to stand for one hour at about 15° C., the suspensions are placed in a water bath at 30° C., the temperature of which is then gradually raised to 37° C. The tubes are centrifuged and inspected for haemolysis after one hour at 37° C.

Paroxysmal Nocturnal Haemoglobinuria Red Cells and Trypsinized Normal Red Cells.—There also were serial dilutions of the patient's serum made in fresh normal compatible serum and an equal volume of a 2% saline suspension of red cells added.

After the tubes have been allowed to stand for one hour at about 15° C., they were warmed for a further hour at 30–37° C.

Detection and Titration of Warm Haemolysins.—Tests using normal corpuscles, normal “trypsinized” corpuscles, and P.N.H. red cells were employed.

Tests with Normal Corpuscles.—The patient's serum was first acidified to a pH of approximately 6.5 by the addition of a 1/10 volume of N/3.5 HCl. It was then placed in a water bath at 37° C. and a 1/10 volume of a warmed 30% saline suspension of normal red cells added. The suspension was centrifuged after one hour. If no haemolysis had resulted, the supernatant serum was removed and the corpuscles resuspended in a volume of fresh undiluted normal serum acidified to pH 6.8–7.0 by the addition to it of a 1/10 volume of N/5 HCl. The tube was again inspected for haemolysis after a further hour at 37° C.

Tests with Normal Corpuscles Modified by Trypsin (Morton and Pickles, 1947).—Normal corpuscles were “trypsinized” by the addition of 1 volume of washed packed red cells to 5 volumes of diluted trypsin solution (1 volume of 1.0% crystalline trypsin (Armour) in N/20 HCl added to 9 volumes of isotonic phosphate buffer at pH 7.7). The suspension of corpuscles in the enzyme solution was warmed at 37° C. for 60 minutes, then centrifuged, and the red cells washed in two changes of saline. The corpuscles were generally used at a final concentration of 1%.

Tests for the haemolysis of trypsinized normal red cells were carried out by adding equal volumes of a 2% suspension of enzyme-treated red cells to two- or four-fold dilutions of the patient's serum in fresh normal unacidified compatible serum. The tubes were centrifuged after one hour at 37° C. and then inspected for haemolysis. If the serum is acidified, as for tests with normal unmodified corpuscles, haemolysis is slightly increased. Acidified serum was not, however, employed as a routine.

Tests with P.N.H. Red Cells.—Three patients were available; two were of blood group ORh+ and one was of group ARh+. The red cells from these patients were used unmodified or trypsinized as described above, except that the time of the exposure to the enzyme was reduced to 15 minutes. Undiluted unacidified fresh normal serum has been used as the diluent for the patients' sera.

Paroxysmal nocturnal haemoglobinuria red cells, even if exposed to trypsin for as little as 15 minutes, undergo a small but variable amount of haemolysis in some normal unacidified human sera. It is necessary, therefore, to select a serum for use as diluent which produces no haemolysis or at the most a trace of haemolysis on its own account.

Detection and Titration of Incomplete Warm Antibodies.—Several methods were used, including the direct and indirect Coombs tests.

Direct Coombs Test.—The method employed by Mollison and Cutbush (1949) was used. The patient's corpuscles were collected directly into isotonic citrate-saline or obtained from blood allowed to clot at 37° C. They were washed in at least three changes of a large volume of saline warmed to 37° C. A 10% suspension in saline was then mixed upon a white translucent tile with an equal volume of well-absorbed antiglobulin rabbit serum diluted to its point of maximum avidity. Antiglobulin sera were prepared according to the methods of Proom (1943) and Wootton (1950).

Indirect Coombs Test.—A 5% suspension of normal group O corpuscles in the patient's serum was made by adding to it a 1/10 part of a 50% suspension of washed red cells. The suspension was warmed to 37° C. for two hours and the corpuscles then washed three times in a large volume of warmed saline. Tests for agglutination by antiglobulin serum were carried out as detailed above. Acidified serum was also used, as suggested by Gardner (1949); a 1/10 volume of N/3.5 HCl was added to a volume of serum before adding the suspension of corpuscles. Tests for the presence of immune

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iso-antibodies were carried out on unacidified sera using corpuscles of known Rh, MN.S., Kell, Lutheran, Lewis, and Duffy types.

Preparation of Antibody-containing Eluates of Red Cells.—Kidd's (1949) method was followed, except that the time of elution at pH 3.2–3.4 was extended from 10 to 15 minutes to 30 to 45 minutes.

The eluates were tested for antibody content in three ways: (1) by making serial dilutions in saline and adding equal volumes of a 2% suspension of washed normal red cells, warming to 37° C. for two hours, and then carrying out tests for adsorbed incomplete antibody using an antiglobulin serum; (2) by making serial dilutions in saline and adding equal volumes of a 2% suspension of trypsinized normal red cells, warming to 37° C. for two hours, and then inspecting for agglutination; and (3) by making a series of dilutions of the eluate in fresh normal compatible serum and adding equal volumes of 2% suspensions of trypsinized normal red cells, P.N.H. red cells, and trypsinized P.N.H. red cells respectively and inspecting for haemolysis after one hour at 37° C.

The tests for haemolysis were also carried out in the following way. The cell-eluate suspensions were lightly centrifuged after warming for one hour at 37° C. and the supernatants then replaced by volumes of fresh normal serum. The tubes were finally inspected for haemolysis after a further hour at 37° C.

Agglutination Tests with Serum-bovine Albumin Diluent.—The method was slightly modified from that described by Neber and Dameshek (1947). Serial dilutions of the patient's serum were made in fresh compatible normal serum and a 2% suspension of normal corpuscles in 30% bovine albumin (Armour) added to each tube. The tubes were kept at 37° C. for two hours and agglutination then read off macroscopically after gently resuspending the deposited corpuscles.

Agglutination Tests with Trypsinized Normal Corpuscles.—Tests for the agglutination of trypsinized normal red cells were carried out by adding equal volumes of a 2% suspension of enzyme-treated red cells to two- or four-fold dilutions of the patient's serum in saline. The presence or absence of agglutination was ascertained after two hours incubation at 37° C.

Titration of Complement.—Serial dilutions of freshly obtained sera were made in saline so as to give a range of serum dilutions of 1 in 2 to 1 in 64. Equal volumes of a 2½% suspension of sensitized sheep cells were then added to each tube. The tubes were centrifuged after 30 minutes at 37° C., and the amount of the haemolysis determined photoelectrically. The titre of complement was recorded as the reciprocal of the final concentration of serum causing 50% haemolysis (Mayer, Eaton, and Heidelberger, 1946).

The sheep cells used were sensitized with a rabbit anti-sheep cell serum diluted to 1 in 1,000 in saline (approximately 1½ M.H.D.).

Other Haematological Estimations.—Haemoglobin estimations, reticulocyte counts, and osmotic fragility determinations were carried out by methods described by Dacie (1950a).

Results

The results of applying the methods described above for the detection and titration of auto-antibodies are summarized in Tables I, II, and III.

Cases of Acquired Haemolytic Anaemia (Groups I, II, and III).—The results for these groups are discussed in detail.

Cold Agglutinins.—In seven out of the 12 patients of Group I the titre of cold agglutinins was abnormal, ranging from 256 to 1024; in one other case it was at the upper limit of the normal range (64). The agglutinin titres and thermal ranges

TABLE I
RESULTS OF SEROLOGICAL AND OTHER INVESTIGATIONS IN IDIOPATHIC ACQUIRED HAEMOLYTIC ANAEMIA
(GROUP I)

Case No., Sex, Age, Date	Non-specific Cold Agglutinins	Non-specific Warm Haemolysins				Non-specific Incomplete Warm Antibodies		
	Normal Red Cells in Saline (titre 2-5° C.)	Normal Red Cells in Acidified Serum (titre 37° C.)	Trypsinized Normal Red Cells in Serum (titre 37° C.)	P.N.H. Red Cells in Serum (titre 37° C.)	Trypsinized P.N.H. Red Cells in Serum (titre 37° C.)	Direct Coombs Test (37° C.)	Indirect Coombs Test	
							Unacidified Serum (37° C.)	Acidified Serum (37° C.)
1. F., 18 March, 1947	512	8	256	64	1,024	++	+	+
2. M., 35 6.12.48	512	0	0	0	0	+	0	+
3. F., 27 .. 18.4.49	512	2 (trace haemolysis)	32	0	64	+	0	+
4. F., 22 .. 18.10.49	256	0	64	32	512	+	+	++
5. F., 56 .. (a) 23.10.49 (b) 27.10.50	1,024 256	2 (trace haemolysis) 0	32 4	32 16	512 256	++ +	0 0	++ 0
6. F., 50 .. 30.10.50	4	0	0	0	0	+++	±	+
7. F., 72 .. 12.10.50	64	0	0	0	0	+	0	+
8. M., 55 .. 31.10.50	4	0	0	0	0	+++	0	0
9. M., 42 .. 1.11.50	2	0	0	0	0	+	0	0
10. M., 39 .. 21.10.50	4	0	0	0	0	+	0	0
11. F., 54 .. (a) 19.10.50 (b) 1.2.51	1,024 1,024	2 (trace haemolysis) 0	128 256	512 1,024	8,000 8,000	++ +	0 0	++ +
12. F., 73 .. (a) 26.10.50 (b) 31.1.51	512 1,024	2 (trace haemolysis) 2 (trace haemolysis)	16 32	4 32	256 512	++ +	0 0	++ +

TABLE I (continued)

Non-specific Incomplete Warm Antibodies				Specific Immune Iso-antibodies	Serum Complement	Other Data
Agglutination of Normal Red Cells in Serum-albumin (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)	Red Cell Eluates (Kidd)		Indirect Coombs Test (Unacidified Serum)	Units	
		Indirect Coombs Test (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)			
16	4	—	—	Trace (unidentified)	70	Splenectomy 5 years previously. Severe relapse. Hb 5.0 g. Reticulocytes 75%. Died
16	64	—	—	0	100	Very severely ill; terminal haemoglobinuria. Hb 6.6 g. Reticulocytes 28%. Died
8	4	—	—	0	—	Severely ill; died despite splenectomy
8	16	—	—	0	—	Severely ill; Hb 2.5 g. Reticulocytes 38%. Died despite splenectomy
0	2	—	—	—	—	Severely ill; haemoglobinuria. Hb 4.1 g. Reticulocytes 0.5%
0	0	0	4	0	100+	1 year after splenectomy. Hb 14.8 g. Reticulocytes 0.6%
16	16	128	128	0	100+	In remission. Splenectomy 3 years previously. Hb 14.5 g. Reticulocytes 2.2%
0	0	—	—	0	—	In spontaneous remission. Hb 14.5 g. Reticulocytes 1%
0	4	64	512	0	100+	In remission. Splenectomy 6 months previously. Hb. 14.7 g. Reticulocytes 7%
2	1024	8	32	++ (anti-c)	100+	In remission. Splenectomy 9 months previously. Hb 15.4 g. Reticulocytes 1%
0	0	—	—	0	—	In remission. Splenectomy 3 years previously. Hb 16.3 g. Reticulocytes 1%
16	8	8	16	0	10-32	Moderately active haemolysis. Hb 9.5 g. Reticulocytes 6%
8	4	—	—	0	27	9 weeks after splenectomy. Hb 14.5 g. Reticulocytes 3.5%
0	0	16	16	0	10	Moderately active haemolysis. Hb 5.0 g. Reticulocytes 11%
4	0	—	—	+ (? anti-E)	30	10 weeks after splenectomy. Hb 13.2 g. Reticulocytes 1%

— signifies no observation.

0 signifies a negative result.

TABLE II

RESULTS OF SEROLOGICAL AND OTHER INVESTIGATIONS IN HAEMOLYTIC ANAEMIA FOLLOWING VIRUS PNEUMONIA, CHRONIC HAEMOLYTIC ANAEMIA WITH HAEMOGLOBINURIA AND RAYNAUD'S PHENOMENA, AND A CONTROL GROUP OF OTHER TYPES OF HAEMOLYTIC ANAEMIA OR BLOOD DISORDER AND NORMAL SUBJECTS

Case No., Sex, Age, Date	Non-specific Cold Agglutinins	Non-specific Warm Haemolysins				Non-specific Incomplete Warm Antibodies		
	Normal Red Cells in Saline (titre 2-5° C.)	Normal Red Cells in Acidified Serum (titre 37° C.)	Trypsinized Normal Red Cells in Serum (titre 37° C.)	P.N.H. Red Cells in Serum (titre 37° C.)	Trypsinized P.N.H. Red Cells in Serum (titre 37° C.)	Direct Coombs Test (37° C.)	Indirect Coombs Test	
							Unacidified Serum (37° C.)	Acidified Serum (37° C.)
GROUP II								
13. F., 22 .. 8.9.50 ..	16,000	0	0	16	512	+	0	0
14. F., 73 .. 14.11.50	4,000	0	0	16	1,024	+	0	+
15. F., 33 .. 28.11.50	4,000	0	64	0	256	+	0	0
16. M., 35 .. 12.12.50	2,000	0	0	16	64	+	0	0
GROUP III								
17. F., 67 .. 10.5.50	64,000	0	16	0	16	+	0	0
18. M., 79 .. 16.12.49	128,000	0	0	0	64	+	0	0
19. F., 64 .. 23.5.50	64,000	0	0	64	512	+	0	+
GROUP IV								
20-22 ..	8	—	0	0	0	0	—	—
23-29 ..	16	—	0	0	0	0	—	—
30. F., 27 .. 20.11.50	8	—	0	16	16	0	—	—
31. F., 46 .. 2.10.50	8	0	0	0	0	0	0	—
32-33 ..	4-8	0	0	0	0	0	0	—
34. F., 52 .. 12.10.50	4	—	0	0	0	+	—	—
35-36 ..	4-8	—	0	0	0	+	—	—
37-54 ..	—	—	0	0	—	—	—	—
55. F., 72 .. 20.10.50	4	—	16	0	64	0	—	—
56-161 ..	—	—	0	0	—	—	—	—

TABLE II (continued)

Non-specific Incomplete Warm Antibodies				Specific Immune Iso-antibodies	Serum Complement	Other Data
Agglutination of Normal Red Cells in Serum-albumin (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)	Red Cell Eluates (Kidd)		Indirect Coombs Test (Unacidified Serum)	Units	
		Indirect Coombs Test (titre 37° C.)	Agglutination of Trypsinized Normal Red Cells in Saline (titre 37° C.)			
GROUP II						
—	0	—	—	—	—	Presented as puerperal anaemia
—	0	—	—	—	110	In a severe haemolytic episode with jaundice
—	0	—	—	—	160	Recovering from an acute episode of haemolysis. Hb 10·5 g. Reticulocytes 18%
—	0	—	—	—	70	In an acute episode of haemolysis
GROUP III						
—	0	—	—	—	90	In remission (warm weather). Hb 14·8 g. Reticulocytes 1%
—	0	—	—	—	—	Cyanosis of extremities, pallor, and jaundice. Hb 8·9 g. Reticulocytes 20%
—	0	—	—	—	10	In partial remission (warm weather). Hb 8·3 g. Reticulocytes 14%
GROUP IV						
—	0	—	—	—	—	Adults affected with haemolytic anaemia of unknown origin and type. Osmotic fragility normal
—	0	—	—	—	—	Congenital haemolytic anaemias (hereditary spherocytosis, etc.)
—	0	—	—	0	110	Atypical congenital haemolytic anaemia (relapse after splenectomy). Hb 7·5 g. Reticulocytes 50%
—	0	0	0	+ (anti-Kell)	70	Paroxysmal nocturnal haemoglobinuria. Transfused many times
—	0	—	—	0	—	Paroxysmal nocturnal haemoglobinuria. Transfused many times
—	0	—	—	—	<4	Haemolytic anaemia with lymphadenoma. Hb 7 g. Reticulocytes 4%
—	0	—	—	—	—	Disseminated lupus erythematosus. Slight to moderate anaemia
—	0	—	—	—	—	Miscellaneous anaemias and leukemias
—	0	—	—	—	100+	Pernicious anaemia. In remission
—	0	—	—	—	—	Non-anaemic hospital patients and normal subjects

FIG. 1.—The effect of temperature on the titre of non-specific cold agglutinins.

were higher in the patients with haemolytic anaemia following virus pneumonia (Group II) and higher still in those patients (Group III) suffering from chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena (Fig. 1).

Incomplete forms of cold antibodies were demonstrated in all the sera that were tested (Dacie, 1950b). In some cases the titres were greater than are found in health.

Cold Haemolysins.—Using the acidified serum technique, haemolysis of normal red cells in the presence of fresh serum was readily observed with the sera of all the patients of Groups II and III, and a cold antibody in weak concentration was similarly detected in the serum of one patient of Group I (Case 5). Titration of cold haemolysins using P.N.H. red cells gives very high titres, as already recorded (Dacie, 1950c). The sera which gave the highest titres of the present series belonged to patients from Group III. Details of the results of titration at 37° C. and at 15° C. followed by a period at 37° C. are given in Table III.

The titres observed when trypsinized normal red cells were used in the place of P.N.H. red cells were less high. In Case 15 it is interesting to note that a temperature effect was observed when P.N.H. red cells were used but not when trypsinized normal red cells were substituted (Table III); thus, in this case there appears to be

evidence for the existence of a fraction of antibody indifferent to temperature in addition to the cold antibody.

The effect of temperature was tried with most of the sera of the patients of Group I; preliminary cooling to 15° C., or, below, before warming to 37° C. did not increase the amount of haemolysis that was observed. The results obtained with the sera of Cases 11 and 12 are included in Table III.

Warm Haemolysins.—Using normal corpuscles, haemolysis at 37° C. was easily demonstrated only in the first patient of this series, fuller details of whom have already been reported (Dacie, 1949). Using the same technique, small amounts of haemolysis were produced by the sera of Cases 3, 5, 11, and 12, but only when the sera were acidified.

By using trypsinized normal red cells, definite indications of the presence of a haemolytic antibody active at 37° C. and requiring the presence of fresh serum complement were obtained with the sera of six out of the 12 patients of Group I. These patients (three after splenectomy) were suffering from negligible to extremely serious haemolysis *in vivo* at the time the serum samples were obtained. The serum from the other seriously ill patient of this series (Case 2) gave a negative result. Of the patients belonging to Groups II and III, the serum of one (Case 15) haemolysed trypsinized normal red cells at 37° C.

Paroxysmal nocturnal haemoglobinuria red cells similarly underwent haemolysis at 37° C. in the sera of five of the 12 patients of Group I, in the sera of three of the four patients of Group II, and in one of the three sera of Group III. When trypsinized, P.N.H. red cells become extremely sensitive reagents. They were haemolysed at 37° C. by six of the 12 sera of Group I, in most instances to high titres, and by all the sera of Groups II and III.

It is interesting to note the lack of parallelism between the behaviour of trypsinized normal red cells and P.N.H. red cells with different sera; e.g., the increased sensitivity of P.N.H. red cells compared with trypsinized normal red cells in Case 5b and the opposite relationship in Case 12a, both titrations being carried out side by side at the same time.

Direct Coombs Tests.—In all of the 19 cases of acquired haemolytic anaemia of Groups I, II, and III, antiglobulin tests carried out on the patients' corpuscles were found to be positive. The apparent strength of the test varied from very strong reactions (agglutination within a few seconds) to quite weak reactions as in Case 5a. The strength of the reactions recorded in Table I have been transcribed from the records made at the time the patients were examined, a period extending well over two years. Of necessity various samples of antiglobulin sera were used, and the recorded strength of the reactions can be taken as only roughly quantitative.

The tests were weakly positive in the case of three patients of Group IV (Cases 34–36); in these patients all other tests for the presence of abnormal anti-red cell antibodies were, however, negative. In three patients with acquired haemolytic anaemia of idiopathic type, Cases 20–22, the reactions were negative; in two of the patients the tests were carried out repeatedly, with varying dilutions of anti-globulin sera, so as to overcome possible prozone inhibition phenomena (van Loghem, 1950; van Loghem, Kresner, Coombs, and Fulton Roberts, 1950).

The significance of the positive tests obtained with the corpuscles of the patients of Groups II and III is possibly different from that pertaining to Group I. In

Group I it seems certain that a warm antibody (i.e., an antibody whose activity is not significantly increased by reduction of temperature below 37° C.) is involved. In Groups II and III the direct antiglobulin reaction probably results from the presence of incomplete cold antibody irreversibly adsorbed due to minor degrees of cooling *in vivo*. The corpuscles were in all cases kept at 37° C. before being exposed to the action of the antiglobulin serum so as to avoid the possibility of the adsorption *in vitro* of incomplete cold antibody.

Indirect Coombs Tests.—Positive indirect antiglobulin tests apparently due to sensitization by non-specific antibodies were obtained with corpuscles exposed to the sera of all the patients of Group I in whom haemolysis was proceeding actively *in vivo* (Cases 1, 2, 3, 4, 5, 11, and 12); in five of these patients a positive result was, however, obtained only when their sera were acidified. In two patients in clinical remission (Cases 6 and 7) the reactions were also positive. In three instances definite reactions in acidified sera were associated with weaker sensitizations in unacidified serum; in one of these patients traces of unidentified specific iso-antibodies were probably present in her serum in addition to non-specific antibodies (Case 1).

The indirect antiglobulin test was positive with the acidified serum of one out of the four patients of Group II and with that of one out of the three patients of Group III.

Activity of Red Cell Eluates.—Eluates were made by Kidd's method from the corpuscles of six patients of Group I. Four of the patients were in remission. In each case antibodies could be detected by the indirect antiglobulin test, and the eluates were also capable of agglutinating trypsinized normal red cells. The eluates had, however, no haemolytic activity, even for trypsinized P.N.H. red cells.

Agglutination of Normal Red Cells in Serum-albumin at 37° C.—In none of the patients of this series was a non-specific agglutinin demonstrated to be active at 37° C. in saline dilutions of patients' sera. In eight patients of Group I there was, however, definite evidence for the presence of non-specific incomplete antibodies detectable at 37° C. by titration with normal corpuscles in a serum-albumin medium.

In several patients quite strong auto-agglutination of whole blood was observed to take place at 37° C. This phenomenon is probably due to agglutination of strongly sensitized corpuscles in whole plasma or serum. This was particularly striking with the blood of Case 8. Normal corpuscles were not, however, agglutinated by this patient's serum either at 37° C. or at room temperature. In other instances where cold panagglutinins were present at high concentrations (titres > 256), auto-agglutination was obvious at room temperature, and in some cases it did not disappear completely until the temperature was raised to 30–32° C. (Fig. 1).

Agglutination of Trypsinized Normal Red Cells at 37° C.—The sera of nine out of the 12 patients of Group I caused the agglutination of trypsinized normal red cells at 37° C. This technique was found to be in general about as sensitive as titration in serum-albumin using normal red cells; with trypsinized normal red cells the end points are, however, much more easily read (at 37° C.). The serum from Case 9 was unusual; in addition to anti-c it appeared to contain a non-specific antibody which agglutinated trypsinized normal red cells to a far higher titre than it agglutinated normal red cells in serum-albumin.

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Agglutination of trypsinized red cells at 37° C. was not caused by any of the sera of the patients of Groups II, III, and IV.

Titration of Complement.—In three patients (Cases 11, 12, and 19) the content of complement in the sera was found to be significantly lowered; it was markedly reduced in the serum of Case 34 (haemolytic anaemia with lymphadenoma).

Control Cases (Group IV)

Tests for haemolysins using trypsinized normal red cells and unmodified P.N.H. red cells were carried out with the sera from a mixed series of cases of haemolytic anaemia not considered to belong to Groups I, II, and III, as well as with the sera of a group of patients suffering from various anaemias and leukaemias and with sera from normal healthy subjects and from hospital patients not considered to be suffering from blood diseases. The tests were negative with two exceptions, Cases 30 and 55 (Table II).

Case 30 was a patient with an atypical non-spherocytic congenital haemolytic anaemia which persisted after splenectomy. Her serum contained a thermostable haemolytic factor which caused the haemolysis of P.N.H. red cells at 37° C., even

TABLE III
EFFECT OF TEMPERATURE ON THE HAEMOLYSIS OF VARIOUS TYPES OF RED CELLS BY THE
SERA OF PATIENTS SUFFERING FROM ACQUIRED HAEMOLYTIC ANAEMIA

Case No.		Normal Red Cells in Acidified Serum (pH 6.5)	Trypsinized Normal Red Cells (titre)	P.N.H. Red Cells (titre)	Trypsinized P.N.H. Red Cells (titre)
11	37° C. 15° C., then 37° C.	Trace ,,	128 128	256 256	8,000 —
12	37° C. 15° C., then 37° C.	,, ,,	64 64	16 16	512 —
13	37° C. 15° C., then 37° C.	0 +	0 32	16 1,024	512 —
14	37° C. 15° C., then 37° C.	0 +	0 16	16 1,024	1,024 —
15	37° C. 15° C., then 37° C.	0 +	64 64	0 1,024	256 —
16	37° C. 15° C., then 37° C.	0 +	0 0	16 512	64 —
17	37° C. 15° C., then 37° C.	0 ++	16 256	0 16,000	16 —
18	37° C. 15° C., then 37° C.	0 ++	0 1,024	0 32,000+	64 —
19	37° C. 15° C., then 37° C.	0 ++	0 1,024	64 32,000	512 —

Cases 11 and 12, idiopathic acquired haemolytic anaemia; Cases 13–16, haemolytic anaemia following virus pneumonia; Cases 17–19, chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena.

when the serum was unacidified. Trypsinized normal red cells were not affected, nor were trypsinized P.N.H. red cells haemolysed to a higher titre than were the unmodified P.N.H. red cells. There were no other evidences of abnormal auto-antibody formation; the Coombs test was negative, the cold agglutinin titre normal, and the survival of transfused normal red cells unimpaired. Case 55 had suffered from pernicious anaemia but was in complete remission following vitamin B₁₂ therapy when investigated. Her serum contained a thermolabile factor which haemolysed at 37° C. trypsinized normal red cells but not unmodified P.N.H. red cells. The significance of these observations is obscure; the haemolytic factors present in the sera of these two cases differed in their behaviour from each other and from the haemolytic antibodies demonstrated in the sera of the patients with acquired haemolytic anaemia.

Discussion

The methods outlined in this paper have provided useful information concerning the pathogenesis of some types of haemolytic anaemia. In particular, the existence of abnormal non-specific haemolytic antibodies in the sera of some of the patients can scarcely be doubted. Trypsinized normal red cells and P.N.H. cells are clearly valuable laboratory reagents, the former the more so because they are readily available.

The use of unusually sensitive red cells in the detection of antibodies in cases of haemolytic anaemia is relatively new. Trypsinized red cells have been observed to be agglutinated by the sera of patients with haemolytic anaemia (Wright, Dodd, and Bouroncle, 1949; Wheeler, Luhby, and Scholl, 1950) and have been widely used in the detection of incomplete iso-antibodies, particularly anti-Rh. That haemolysis may be observed with sera from cases of haemolytic anaemia does not seem, however, to have been reported. Similarly, although the very great sensitivity of P.N.H. red cells to haemolysis by cold haemolysins has already been reported (Dacie, 1950c), the fact that they may undergo haemolysis at 37° C. in the diluted unacidified serum of certain patients with acquired haemolytic anaemia does not seem to have been recognized. There appear to be two possible reasons for the haemolysis that has been observed; either that trypsinized normal red cells and P.N.H. red cells are sufficiently sensitive to demonstrate antibodies existing in concentrations too low to affect normal corpuscles or, alternatively, that they are acted upon by higher-titre antibodies to which normal corpuscles are almost completely insensitive. It can be only a matter of speculation at the moment which of these possibilities is correct. The remarkably high titres of warm antibodies in some of the patients' sera, Case 11, for example, certainly suggest that the patients' red cells (and normal unmodified red cells) may be remarkably insensitive to these antibodies. Acidification of serum, as used by Dacie (1949, 1950c) and Gardner and Harris (1950), seems capable of increasing the sensitivity of normal red cells to both warm and cold non-specific antibodies. However, the apparent haemolytic titres are far less than those which may be observed using trypsinized normal red cells or P.N.H. red cells. Acidification of sera slightly increases the amounts of haemolysis which result when trypsinized red cells are used, but this seems an unnecessary refinement for diagnostic purposes, and will lead to complications if P.N.H. red cells are used. The acidification of serum when carrying out indirect Coombs tests (Gardner, 1949) is, however, clearly an important practical advance.

The Pattern of Antibodies in Haemolytic Anaemia Sera.—An interesting feature of the behaviour of the sensitive cells used in this study has been the different patterns of results which have been obtained with different sera, suggesting perhaps individual variations in the molecules of antibody globulin. The reciprocal relationship, already referred to, between the sensitivity of trypsinized normal red cells and P.N.H. red cells to haemolysis at 37° C. by two sera (Nos. 5b and 12a), tested side by side, illustrates this. Case 2 illustrates a further variation. This patient died of his disease and could never be made fit enough for splenectomy to be attempted; although his serum strongly agglutinated trypsinized normal red cells, haemolysis *in vitro* in the presence of complement did not take place. Case 11 is in complete contrast; her serum was powerfully haemolytic at 37° C., but only weakly agglutinated trypsinized normal red cells.

The individuality of serum antibodies is also demonstrated by the results obtained with the sera of Group II. The antibodies present in the sera of the patients of this group were essentially cold ones, but with some of the sera, but not with all, haemolysis took place at 37° C. This appears to be due generally to cold antibodies being present at such high concentrations that their thermal range extends to 37° C. when tested for by sufficiently sensitive red cells. In Case 15, however, the haemolysis of trypsinized normal red cells was not increased by preliminary cooling; presumably, therefore, there was in this serum a warm component of antibody, detectable by means of trypsinized normal red cells, in addition to cold haemolysin detectable by P.N.H. red cells (Table III). In contrast, the antibody detectable by trypsinized normal red cells in the serum of Case 14 appeared to be a cold one. In Case 16 the cold antibody was easily demonstrated with P.N.H. red cells, but not at all with trypsinized normal red cells. Similar subtle differences can be noted if the results obtained with the sera from the patients of Group III are studied (Table III).

Significance of Different Types of Non-specific Antibody.—The role of haemolysins as an important cause of haemolysis *in vivo* in cases of acute haemolytic anaemia has been vigorously propounded by Dameshek and his collaborators (Dameshek and Schwartz, 1938, 1940; Dameshek and Miller, 1943). A difficulty in accepting this hypothesis has been the rarity with which the presence of an abnormal autohaemolysin has been unequivocally demonstrated in the laboratory. Ham and Castle (1940) and Wagley, Shen, Gardner, and Castle (1948) have emphasized that auto-agglutination occurring *in vivo* might itself be a cause of haemolysis, and the spleen might be particularly important in this respect owing to the slow circulation through it and the opportunities for auto-agglutination and trapping of agglutinates which might result from this.

The discovery by the application of the Coombs test that incomplete forms of auto-antibodies were frequently present in cases of acquired haemolytic anaemia suggested that this type of antibody might be the elusive haemolytic factor. It was soon realized, however, that, although the direct Coombs test was usually strongly positive during periods of intense haemolysis, this was not necessarily true, and that patients might be observed in complete or almost complete clinical remission, in whom the test was markedly positive (Loutit and Mollison, 1946; Evans and Duane, 1949; our Cases 6 and 8). It was also not easy to understand how cells sensitized by an antibody only capable of causing agglutination under artificial conditions *in vitro* could be the cause of haemolysis *in vivo*. Wagley *et al.* (1948) observed

that the red cells obtained from the spleens removed from three patients suffering from acquired haemolytic anaemia were more strongly agglutinated by an antiglobulin serum than those obtained from samples of peripheral blood. They considered that this higher concentration of antibody might result in local auto-agglutination with stagnation of blood within the spleen and subsequent haemolysis.

The potent antibody-containing eluates which have been obtained from the red cells of several patients of the present series emphasize that incomplete antibodies are by themselves of minor importance in causing haemolysis *in vivo*, at least in patients whose spleens have been removed. The most potent eluates were obtained from two patients in almost complete remission (Cases 6 and 8), whose corpuscles gave strongly positive direct Coombs tests. It is noteworthy perhaps that antibodies capable of causing haemolysis could not be demonstrated in the eluates of these cases or in eluates from the corpuscles of other patients in whom haemolysis was proceeding rapidly, and in whose sera haemolytic antibodies could be demonstrated. The reason for this awaits an explanation. Possibly the haemolytic antibodies are hardly adsorbed at all by normal corpuscles under physiological conditions, or, if they are, the adsorption of a very few molecules is sufficient to cause haemolysis to take place rapidly; both explanations, if correct, would reduce the chance of obtaining in an eluate of washed red cells antibodies of haemolytic potency. The fact that these eluated antibodies failed to cause haemolysis under what appeared to be optimal conditions, although capable of producing agglutination, seems in favour of the conception that the incomplete and haemolytic antibodies are separate entities.

The observations recorded in this paper thus appear to confirm the relative unimportance of incomplete antibodies. By demonstrating the relative frequency of haemolytic antibodies, they reopen the question of the importance of haemolysins. The findings certainly indicate the existence of haemolytic antibodies in the sera of certain patients affected with acquired haemolytic anaemia if sensitive methods are used to detect the antibodies. It must be conceded that these observations do not necessarily prove that the antibodies play an important, or even any, part in causing haemolysis *in vivo*; for one thing there seems to be no exact correlation between antibody content and the clinical evidence of haemolysis. On the other hand, it is difficult to believe that the ability of the antibodies to cause haemolysis *in vitro* is entirely without significance. However, it is certainly not claimed, nor is it thought probable, that the haemolytic antibodies are the sole cause of haemolysis in the types of case under review. The possibility that auto-agglutination *in vivo* may be of some importance or that the presence of adsorbed incomplete antibody may predispose a red cell to phagocytosis or shorten its life in some other way cannot be denied, and it is quite clear, judging from the frequent (but not invariable) beneficial results of splenectomy (Welch and Dameshek, 1950; our Cases 5, 11, and 12) that the spleen may be of considerable importance also. Whether this is solely due to the organ exerting a haemolytic function depending upon circulatory stagnation or the phagocytic action of its reticulo-endothelial cells acting on corpuscles damaged by antibodies or any other mechanism, is not quite clear.

Effect of Splenectomy on Antibody Titres.—There seem to be few studies available of antibody concentrations following splenectomy in man for acquired haemo-

lytic anaemia. Evans and Duane (1949) for instance, studied two patients before and after splenectomy; in each case a diminution in the rate of haemolysis was associated with a striking reduction in the agglutinability of the patients' red cells by antiglobulin sera. Three of the patients in this series were studied in some detail before and after splenectomy. Haemolytic antibodies could still be demonstrated in the serum of Case 5 a year after splenectomy although she appeared to be in almost complete remission. There was a fall in titre to between one-quarter and one-half of its original value. The cold agglutinin titre also had fallen to one-quarter of its former value. In Cases 11 and 12 little or no change in the antibody titres could be demonstrated in the first 10 or 11 weeks after splenectomy.* Both patients were, however, greatly improved clinically; a red cell survival study showed that transfused blood was surviving in Case 11 at least twice as long as it was before splenectomy. In these patients, therefore, it appears that the spleen was an important haemolytic organ, but a minor source of antibody. That the spleen is, however, capable of being an important source of antibody both in animals and man is shown by the recent studies of Rowley (1950a and b) and Wolfe, Norton, Springer, Goodman, and Herrick (1950). Further studies are necessary before the relative importance of these two aspects of splenic activity can be reliably assessed; probably both play a part in cases of human haemolytic anaemia, the relative importance of each varying from case to case.

Importance of Osmotic Fragility.—Another factor that must play some part in the production of haemolysis *in vivo* is abnormal spherocytosis. The spleen is to some extent involved in this phenomenon too. An increase in osmotic fragility is generally, but not invariably, found in acquired haemolytic anaemia. Moderate to marked increases were found in all the patients of Group I of this series in whom the estimation was carried out and in whom haemolysis *in vivo* was markedly increased (Cases 1, 2, 4, and 5). On the other hand the fragility of the red cells of Cases 11 and 12 in whom haemolysis *in vivo* was moderately severe was only slightly increased, and when Case 6 was first seen (in 1947 before splenectomy), fragility was within the normal range, although haemolysis was proceeding quite actively. Thus it is not possible to correlate the fragility changes absolutely with the rate of haemolysis *in vivo*. Indeed, it does not seem likely on the basis of studies *in vitro* that increased osmotic fragility can be due to the direct effect of antibodies on the cell surfaces. Nevertheless, there is significant experimental evidence that the administration of anti-red cell antibodies *in vivo* results in spherocytosis and a marked increase in fragility when the blood is subsequently tested *in vitro* (Dameshek and Schwartz, 1938; Young, Ervin, and Yuile, 1949). In man, too, the transfusion of anti-A (or anti-B) iso-antibodies to group A (or B) recipients results in an increase in osmotic fragility (Ebert and Emerson, 1946; Ervin and Young, 1950). Possibly the change towards spherocytosis is brought about as the result of stasis of blood in small vessels due to auto-agglutination (Ham and Castle, 1940). Spherocytes certainly have a greatly reduced life span, at least when the spleen is present, and marked changes always seem to be associated with a serious rate of haemolysis *in vivo*, as in Case 2 of this series who suffered from a terminal haemoglobinuria. This patient's blood underwent rapid haemolysis *in vitro* when incubated at 37° C. This was

* Both these patients are in good clinical remission eight months after splenectomy. The antibody titres have remained unaltered.

apparently due to the spontaneous lysis of extremely spherocytic red cells, for no haemolytic antibodies could be demonstrated in his serum.

Amongst other possible significant factors affecting the rate of haemolysis *in vivo* are variations in the sensitivity of the patient's corpuscles to the auto-antibodies he develops—at present of quite unknown importance—and variations in the activity of serum complement. The potential importance of complement activity in complement-haemolysin haemolytic systems like those under discussion is obvious; indeed, the results of tests for haemolysis *in vitro* were negative with the sera of Cases 11 and 17 unless additional fresh serum was first added to the patients' serum. (In these cases the complement content of the patients' fresh sera was notably diminished, as it was also in the serum of Case 12.) It is possible that these observations *in vitro* may be relevant to haemolysis *in vivo*.

Antibody Patterns in Relation to the Haemolytic Syndromes.—Although it is convenient and in most cases quite easy to classify patients suffering from haemolytic anaemias associated with the formation of auto-antibodies into separate groups on clinical grounds, as has been done with the patients separated into Groups I, II, and III of the present series, classification based on pathology is far less clear. The types of antibody present are to some extent shared amongst the different groups, and whilst cold antibodies predominate in Groups II and III, and warm antibodies in Group I, there is a considerable overlap.

It seems likely that the clinical pattern of the disease is determined at least in part by the nature of the antibodies which are being produced, whether they are of the warm or cold variety and whether cold agglutinins are present at very high titres. The minor variations within the class of antibodies which are revealed by their differing ability to act on different types of sensitive cells appear to be additional reflections of the individuality of the patients, who all react slightly differently to the usually unknown stimulus which results in the development of non-specific antibodies active against their red blood cells and the clinical syndromes of acquired haemolytic anaemia.

Summary

A series of patients suffering from acquired haemolytic anaemia has been investigated with particular reference to the presence in their sera of abnormal antibodies active against red blood cells. Abnormal non-specific antibodies were demonstrated in 19 patients suffering from idiopathic acquired haemolytic anaemia, haemolytic anaemia following virus pneumonia, or chronic haemolytic anaemia with haemoglobinuria and Raynaud's phenomena.

Trypsinized normal red cells and red cells from patients with paroxysmal nocturnal haemoglobinuria (P.N.H. red cells) were found to be valuable reagents for the detection of haemolytic antibodies; by their use non-specific haemolysins of the "warm" or "cold" variety were demonstrated in the sera of 13 out of the 19 patients.

The variations which were observed in the antibody patterns in the patients' sera are emphasized, the differences being thought to be reflections of the individuality of the responses of the patients to the stimuli causing the formation of auto-antibodies.

Haemolytic antibodies are believed to play a part in the production of haemolysis *in vivo*, but it is not claimed that they are the only cause. Other types of antibody.

are probably important in this respect, and the spleen certainly contributes to the haemolytic process in many cases. In three patients belonging to Group I of the present series in whom splenectomy was carried out, a marked immediate clinical improvement was not accompanied by significant reductions in antibody titre. In these patients, therefore, the spleen seems to have been acting by removing altered or damaged red cells, rather than by being an important source of antibody.

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