ANTIBODY OF BLOOD-GROUP SPECIFICITY IN SIMPLE ("COLD") HAEMOLYTIC ANAEMIAS

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Two cases of "cold" (simple) haemolytic anaemia are described. The serological investigation showed conclusively that the sera of both patients contained an anti-I antibody and that their cells were I positive. The antibody found is thus a true auto-antibody. This is in keeping with the findings obtained previously in the "warm" (hyperimmune) type of haemolytic anaemia.

The acquired haemolytic anaemias are characterized by the presence of antibodies, and Dameshek (1951) suggested their differentiation into two types, the one with antibodies more active at blood heat ("warm") and the other at lower temperatures ("cold"). One of us (Weiner, 1957a) designated them later as hyperimmune ("warm") and simple ("cold") haemolytic anaemias respectively.

An antibody may be defined as a plasma protein which reacts specifically with its homologous antigen. The blood-group specificity of an antibody can readily be recognized if cells are easily available which are not acted upon by this antibody, but difficult if such cells are scarce. In 1953 (Weiner, Battey, Cleghorn, Marson, and Meynell), it was shown for the first time that the antibodies which occur in "warm" (hyperimmune) haemolytic anaemias may have distinct and definable blood-group specificity. The antibody found in "cold" (simple) haemolytic anaemia was, however, still regarded as "unspecific." Crookston, Dacie, and Rossi (1956) investigated these antibodies and found a marked difference in their reactivity with different cells. Wiener, Unger, Cohen, and Feldman (1956), however, described a case of "cold" (simple) haemolytic anaemia in which the antibody had distinct blood-group specificity. They were able to find amongst many thousands of donors five whose cells did not react with the patient's antibody. They called the antibody anti-I, corresponding to the antigen I on the reacting cells. The non-reacting cells were supposed to lack the I property or to carry the property i.

The great scarcity of i cells prevented further investigation of other cases of "cold" (simple) haemolytic anaemia. Race and Sanger (1958) had in the meantime described a serum, Steg, which seemed to be identical with the serum described by Wiener *et al.* (1956). Later still we learned of the work done by Jenkins, Marsh, Noades, Tippet, Sanger, and Race (1960) and, through the generosity of Drs. Jenkins, Race, and Sanger, were supplied with two samples of i cells and also with anti-I serum of the "natural" type. This material made it possible to investigate the two patients with "cold" (simple) haemolytic anaemia who are the subject of this report.

Case Histories

Mrs. A., then 27 years of age, developed acute haemolytic anaemia 48 hours after the birth of her first child in September, 1956. A week later, her haemoglobin had fallen to 5.9 g. % (40%) with 15% reticulocytes. The liver and spleen were slightly enlarged. The serum bilirubin level was 1.4 mg. %. The haemolysis was controlled by 60 mg. prednisolone daily. Subsequently she remained well, though, whenever steroid treatment was stopped, the haemoglobin fell and the reticulocyte count increased. A daily maintenance dose of 5 mg. prednisolone controlled this. She had a relapse in February, 1959, after a feverish illness due to a urinary infection. Increased dosage of prednisolone brought the haemolysis again under control.

Mr. F. L., aged 77, was admitted to hospital with chronic bronchitis and emphysema, and in the course of a routine blood count in March, 1958, strong auto-agglutination of the red cells was noted. Haemoglobin was 11.8 g. % (80%) with a reticulocyte count of 3%, and the direct Coombs test was positive. He was

again admitted in November, 1958, with herpes zoster. Then he had a haemoglobin of 8.9 g.% (60%), W.B.C. 14,000 with a normal differential count, and reticulocytes 2.7%. Again the direct Coombs test was positive at room temperature and at 37° C. "Cold" agglutinins were reported to be present. An L.E. test and a Schumms test were negative. Haptoglobins were absent. The Wassermann reaction and the Donath-Landsteiner test were negative.

Material and Methods

Venous blood was delivered into six universal containers. Three of these contained acid citrate dextrose which was used in the proportion of 1 part to 9 parts of blood. One container with acid citrate dextrose and one without were immediately dropped into a "thermos" flask filled with water at 37° C., and another pair put into crushed ice; the third pair was kept at room temperature. All the specimens were handled subsequently at the temperatures into which they were first delivered. The cells of the specimens taken into acid citrate dextrose were washed three times with large amounts of saline of the appropriate temperature and eluates were prepared from them, both by the method described previously (Weiner, 1957b) and the heat method (Landsteiner and Miller, 1925). The sera were stored at -25° C. until used. The cells and sera thus collected will be referred to as "37° C.," "22° C.," and "4° C." cells and sera. The i cells were those described by Jenkins *et al.* (1960) (donor M.) and Tippet, Noades, Sanger, Race, Sausais, Holman, and Buttner (1960) (donor R.). The i cells had been kept frozen in glycerol, and to make a valid comparison cells which had been similarly kept were used as controls. Whenever possible the patient's own cells were included among the test cells. When the alcohol method (Weiner, 1957b) was used the eluates were taken into AB serum and with the heat method into saline. Cells were made up as 2% saline suspensions. Both Löw's (1955) papain method and papainized cells were used. The latter were prepared using Löw's activated papain diluted with 19 parts of the buffer solution. This diluted enzyme was used in equal parts with packed red cells. The mixture was incubated for 15 minutes in a water-bath, washed once, and again a 2% suspension was prepared. When Löw's method was used, 1 part of serum or eluate was mixed with 1 part of the papain solution. When the patient's own cells were used as test cells, they were used unmodified.

All tests were read "blind"; the material was coded by an assistant and the code disclosed only after the results had been read.

Results

Mrs. A. was found to be Group A₁ rhesus positive, phenotype CCDee. The specimens were obtained at the beginning of March, 1959, when she was in hospital having suffered the relapse

described above. Serum was also available which had been collected on her first admission to hospital in 1956 and gave identical results. The direct Coombs test on Mrs. A.'s cells gave a distinctly positive result which varied from + to ++. Auto-agglutination was marked in the 4° C. specimen, but the agglutinates could be broken up by shaking in saline. Very small agglutinates were left, but Coombs serum enhanced this agglutination very strongly. No auto-agglutination was seen in the 37° C. specimen, but the positive Coombs test indicated that the cells, even at this temperature, were coated, though slightly.

Mr. L. was Group A₁ rhesus positive, phenotype ccDE. (As the three samples of anti-e available were all of the incomplete variety, it was not possible to establish with certainty whether the patient's cells carried the e antigen.) The result of the Coombs tests was similar to that described above. Auto-agglutination was fairly strong in the 4° C. specimen but was again greatly enhanced by Coombs serum.

The investigation of the sera obtained from the sample from Mrs. A. gave a somewhat intriguing result. When titrated, the three sera which were collected into different temperatures scored differently.

As Table I shows, the highest score was obtained with the 37° C. serum, which seems to imply that at a lower temperature the cells of the patient absorbed some of the antibody. The serum of Mrs. A. was then put up against a random cell from our cell panel, i cells, and her own cells obtained from all her three specimens. Serum M., which contains a "naturally" occurring anti-I, was put up in parallel together with a Group AB serum as a control. The results are shown in Table II.

From this it can be seen that i cells were not agglutinated by any of the sera used but that both the patient's own sera and serum M. agglutinated her cells very strongly indeed. A similar result was obtained when the sera from Mr. L. were investigated (Table III).

TABLE I

TITRATION OF MRS. A.'S THREE SERA AGAINST ONE RANDOM UNMODIFIED CELL AT 4° C.

Sera Separated at								Scores
4° C.	4	4	2	W	—	—	—	34
22° C.	4	4	3	W	—	—	—	38
37° C.	4	4	3	W	1	W	—	53

4 = ++++ 3 = +++ 2 = ++ 1 = + W = weak — = neg.
Scores: 4 = 12 3 = 10 2 = 8 1 = 5 (1) = 3 W = 2.

TABLE II

	Donor M.	Sera			AB
		Mrs. A. Serum Separated at:			
		4° C.	22° C.	37° C.	
Control cell	4	4	4	4	—
Cells donor M. . .	—	—	—	—	—
4° C. } Cells	4	2	3	4	—
22° C. } Mrs. A.	4	3	4	4	—
37° C. }	4	3	4	4	—

Incubated at 4° C.

4 = + + + + 3 = + + + 2 = + + — = neg.

TABLE III

	Sera				
	Donor M.	Mr. L. Sera Separated at:			AB
		4° C.	22° C.	37° C.	
Random group A cells	4	3	3	3	—
Cells Mr. L. separated at 37° C.	4	3	3	3	—
Cells donor M.	—	—	—	—	—

Incubated at 4° C.

4 = + + + + 3 = + + + .

Again i cells (donor M.) were not agglutinated, and the patient's sera and serum M. agglutinated strongly the patient's cells. Tests using cells from donor R. gave identical results.

Jenkins *et al.* (1960) demonstrated that their anti-I serum acted on cord blood cells much less strongly than on cells from adults. We obtained identical results with the sera from our two patients, and Table IV gives the scores obtained when the two 37° C. sera were titrated against cells from adults chosen at random and three cord blood samples.

Jenkins *et al.* (1960) have shown that the i cells of donor M. contain very little H substance. Tippet *et al.* (1960) confirmed this in their donor R., but obtained much higher scores with i cells from three other donors. It is thus obvious that

an anti-H antibody might give reactions similar to anti-I with certain i cells. The following experiments showed that the sera of our patients did not contain anti-H but anti-I. Saliva from secretors did not diminish the titre of the antibody, confirming the findings of Jenkins *et al.* (1960). Further, Drs. Race and Sanger were kind enough to test the sera of our patients against a sample of "Bombay" blood which does not contain any H substance. The cells were strongly agglutinated. If our sera had been anti-H, one would also have expected that Group O and Group A₂ cells would have reacted more strongly than A₁ cells. This, however, was not the case.

Both our patients were Group A₁, the same, therefore, as i cells at our disposal. Their cells were, as shown above, strongly agglutinated by the "naturally" occurring anti-I and by the sera from both patients. We titrated random Group O, A₁, A₂, the two i cells, and the 37° C. cells from our patient L. against *Ulex Europaeus*.

Table V shows the result of this titration. Group O and Group A₂ scored highest, Group A₁ less, and the i cells lowest. Mr. L.'s cells gave a score of 23 showing that they contained the amount of H substance expected of A₁ cells.

The eluates were investigated in a similar way. (No eluate could be prepared from cells taken into room temperature from Mr. L., as unfortunately the container broke in the centrifuge and it was not thought essential to obtain a further sample.) The eluates prepared by the alcohol method reacted more strongly.

It can be seen from Table VI that the eluates also showed blood-group specificity. The i cells were not agglutinated at all, in contrast to the patient's own cells. Eluates prepared from the 37° C. specimen of Mrs. A. did not produce any agglutination when used with unmodified cells. Those prepared from a similar specimen obtained from Mr. L. acted quite well.

Wiener *et al.* (1956) mentioned that ficinized i cells were agglutinated by the serum of

TABLE IV
SCORES OBTAINED AFTER TITRATING MRS. A. AND MR. L. 37° C. SERA AGAINST CELLS FROM THREE RANDOM ADULT DONORS AND FROM THREE RANDOM CORD BLOOD SAMPLES FROM FULL-TERM BABIES

Sample No.	At 22° C.		At 4° C.	
	Mrs. A.	Mr. L.	Mrs. A.	Mr. L.
9735	35	37	90	117
9736	36	51	95	121
9737	61	93	109	132
Baby 1	2	—	39	25
" 2	5	—	51	14
" 3	2	—	42	9

TABLE V
TITRATION OF CELLS WITH *ULEX EUROPAEUS* EXTRACT

											Scores
Gp. O cell . .	4	4	4	4	2	W	—	—	—	—	58
37° C. Mr. L.	2	1	1	(1)	W	—	—	—	—	—	23
Donor R. . .	W	W	—	—	—	—	—	—	—	—	4
" M. . .	W	W	—	—	—	—	—	—	—	—	4
Gp. A ₁ cell	2	2	1	W	W	—	—	—	—	—	25
Gp. A ₂ "	4	4	4	4	3	2	W	—	—	—	68

Incubated at 4° C.

4 = + + + + 3 = + + + 2 = + + 1 = + W = weak.
Scores: 4 = 12 3 = 10 2 = 8 1 = 5 (1) = 3 W = 2

TABLE VI
ELUATES MRS. A.

	Alcohol Eluate			Heat Eluate		
	4° C.	22° C.	37° C.	4° C.	22° C.	37° C.
Random A ₁ cells	4	3	—	3	3	—
Cells donor M.	—	—	—	—	—	—
4° C. } Cells	2	1	—	1	1	—
22° C. } Mrs. A.	3	1	—	1	1	—
37° C. } Cells	3	2	—	2	1	—

ELUATES MR. L. (ALCOHOL ELUATES)

	4° C.	37° C.
Random A ₁ cells	4	3
Cells donor M.	—	—
37° C. cell Mr. L.	3	2

4 = + + + + 3 = + + + 2 = + + .
Scores: 4 = 12 3 = 10 2 = 8.

their patients. We used papain to modify the cells. The activity of our patients' sera was much enhanced by papain both when this enzyme was used to modify the test cells or on its addition to the sera. They behaved in the way described by Wiener *et al.* (1956) and confirmed by Jenkins *et al.* (1960). They also agglutinated i cells, though to a much lower titre. The agglutination was further enhanced when the tests were incubated at low temperatures. The eluates behaved differently; although their activity was also much enhanced when papain was used, i cells were only weakly or not agglutinated at all.

It was of interest to note that the 37° C. eluate of Mrs. A.'s specimen which had shown no activity against unmodified I cells could be made to react when papain was used and when the tests were put up at 4° C. This shows that even at 37° C. the cells of our patients were coated by antibody, though more sensitive methods had to be used to show its presence in the eluate. As Table VII shows, the eluates both from Mrs. A. and Mr. L. agglutinated the 37° C. cells, those of Mr. L. as strongly as the control cells.

TABLE VII
REACTIVITY OF PAPAINIZED CELLS WHEN TESTED WITH SERA AND ELUATES OF MRS. A. AND MR. L.

	Mrs. A.						Mr. L.						Donor M. Serum
	Sera			Eluates			Sera			Eluates			
	37° C.	22° C.	4° C.	37° C.	22° C.	4° C.	37° C.	22° C.	4° C.	37° C.	4° C.		
Random A ₁ cells	4	4	4	2	3	4	4	4	4	4	4	3	
Cells from donor R.	4	4	4	—	W	W	4	4	4	4	1	—	
37° C. cells Mr. L.	4	4	4	3	4	4	4	3	2	3	4	4	

Incubated at 4° C. 4 = + + + + 3 = + + + 2 = + + 1 = + W = weak.

Discussion

Both patients suffered from typical "cold" (simple) haemolytic anaemia. All the symptoms and signs commonly seen were present in both patients, though Mr. L. suffered mainly from emphysema and bronchitis and was found to be anaemic on one occasion only.

The sera of both patients contained a powerful antibody which acted best at a low temperature. The properties of the sera were identical with those described by Wiener *et al.* (1956) and Jenkins *et al.* (1960). The antibody was not anti-H, as it reacted with "Bombay" blood and was not inhibited by secretor saliva. The cells obtained from the specimens kept at 4° C. did not react as well as the cells obtained from specimens collected into 22° C. and 37° C. (see, for instance, Table II). The sera behaved in the opposite way. Those obtained at a higher temperature gave a better titre than sera from the specimen at 4° C. The fact that the cells of both patients were agglutinated by two "naturally" occurring anti-I sera and were also agglutinated by the immune anti-I of their own sera, the sera of the other patient, and the eluates indicates clearly that they are I. Further proof was obtained by the fact that a powerful antibody could be eluted which again had anti-I specificity. This antibody could only have been found on the cells if they contained the homologous antigen, as we were not able to elute any antibody from the cells of donor M. or R. In addition, the cells had a normal complement of the H antigen which, as Jenkins *et al.* (1960) have shown, is low in some i cells. Both our patients were therefore I positive.

There is a definite difference in the behaviour of the "naturally" occurring anti-I and the anti-I in haemolytic anaemia. When i cells were enzyme treated, the sera of our patients agglutinated them to a certain extent though certainly not to as high a titre as I cells. Whereas a low temperature was necessary to bring about a weak agglutination of enzyme-treated cells from donor R. by serum

M., serum R. did not agglutinate cells from donor M. under any conditions (Tippett *et al.*, 1960). (It is interesting to note that the eluates behaved very similarly.) This difference between a "naturally" occurring antibody and an immune antibody is not unique and is seen on occasions in other fields. It is known that when an animal is immunized against an antigen the specificity of the antibody, at first narrow, subsequently widens to include related antigens (Raffel, 1953; Boyd, 1956).

Our results do not help to explain the different serological behaviour of antibodies of the "natural" or "immune" variety. Everyone is exposed to the same antigenic stimuli, but only those who lack the homologous antigen will develop the corresponding antibody, that is, as long as the response to this antigen is "normal." In abnormal conditions, antibodies against antigens carried on the patient's cells may develop.

We cannot agree with the point of view of Wiener *et al.* that *i* people are liable to develop haemolytic anaemia due to their forming an anti-I. In the first instance, we have shown that both our patients were I positive. Wiener *et al.* have not produced any proof at all that their patient was really *i*. The poor reaction of her cells to her own serum could be explained by their being heavily coated with her own antibody. Wiener *et al.* rejected this possibility without apparently carrying out further investigations. If an elution was tried, the result was not published. From the red cells of both our cases, we could elute with ease a powerful antibody. Even the less sensitive heat method yielded a good eluate. Furthermore, it is difficult to imagine that an antibody formed by a patient who is lacking the homologous antigen should be the cause of a severe illness. It is hard to understand their conclusion that: "If further studies show that other similar cases of acquired haemolytic anaemia due to cold auto-antibodies (our italics) behave similarly this could provide a partial explanation for the rareness of this condition since presumably only I-negative individuals, who are quite rare, would be apt to produce such antibodies." "Cold" (simple) haemolytic anaemias are by no means as rare as *i* people. In a short time, we have seen quite a number of "cold" (simple) haemolytic anaemias, but we have not yet managed to find an *i* cell amongst the thousands of specimens which pass through our hands. Antibodies produced by people against an antigen which they themselves

lack are in fact harmless as, e.g., anti-rhesus antibodies in rhesus-negative people. Similarly one would not expect I-negative people, who develop anti-I, to show any signs of illness, and in fact, as Jenkins *et al.* have shown, the presence of anti-I in a truly *i* patient is compatible with full health.

These results further support the assumption of the blood-group specificity of the antibodies in acquired haemolytic anaemias. Even if our present methods and the availability of cells do not allow us as yet to define this specificity in every case, their behaviour is such that specificity is much more likely than an "unspecific" antibody. Crookston *et al.* (1956) explain that by the term "non-specific antibody" they mean that "the receptor for the particular antibody is presumed to be present on the surface of all human red cells." Until really all human cells, an obviously impossible undertaking, have been tested, this assumption is not tenable and liable to lead to wrong conclusions, as the present investigation has shown. Wiener *et al.* (1956) by not assuming "non-specificity" were able to provide donors for their gravely ill patient which would not have been possible had they acted according to the assumption that "all cells" carried the antigen. Up till now, quite a number of I-negative people (or nearly I-negative) have been found (Tippett *et al.*, 1960). Their cells will help to increase our knowledge of blood-group systems in man, but, more important still, they are potential donors for patients with acquired haemolytic anaemia who require transfusion.

This work could not have been done at all had it not been for the help of Drs. Race and Sanger and the cells and sera supplied both by them and by Dr. Jenkins and his colleagues. We should like to express our most sincere thanks to them.

REFERENCES

- Boyd, W. C. (1956). *Fundamentals of Immunology*, 3rd ed., p. 44. Interscience Publishers, New York.
- Crookston, J. H., Dacie, J. V., and Rossi, V. (1956). *Brit. J. Haemat.*, **2**, 321.
- Dameshek, W. (1951). Proc. 3rd Int. Cong. Int. Soc. Hemat., Cambridge, 1950, p. 120. Grune and Stratton, New York.
- Jenkins, W. J., Marsh, W. L., Noades, Jean, Tippett, Patricia, Sanger, Ruth, and Race, R. R. (1960). *Vox Sang.*, **5**, 97.
- Landsteiner, K., and Miller, C. P. (1925). *J. exp. Med.*, **42**, 853.
- Löw, B. (1955). *Vox Sanguinis (Amst.)*, **O.S.**, **5**, 94.
- Race, R. R., and Sanger, Ruth (1958). *Blood Groups in Man*, 3rd ed., p. 328. Blackwell, Oxford.
- Raffel, S. (1953). *Immunity, Hypersensitivity, Serology*, p. 65. Appleton-Century-Crofts, New York.
- Tippett, Patricia, Noades, Jean, Sanger, Ruth, Race, R. R., Sausais, Laima, Holman, C. A., and Buttmer, R. J. (1960). *Vox Sang.*, **5**, 107.
- Weiner, W. (1957a). Trans. 6th Cong. European Soc. Haemat., Copenhagen, 1957. Karger, Basel.
- (1957b). *Brit. J. Haemat.*, **3**, 276.
- Batley, D. A., Cleghorn, T. E., Marson, F. G. W., and Meynell, M. J. (1953). *Brit. med. J.*, **2**, 125.
- Wiener, A. S., Unger, L. J., Cohen, L., and Feldman, J. (1956). *Ann. intern. Med.*, **44**, 221.