Tissue-specific autoantibodies in haemolytic anaemia

SHEILA WORLLEDGE

From the Haematology Unit, Royal Postgraduate Medical School, Hammersmith Hospital, London

There has been a renewal of interest in recent years in the pathogenesis of antibody-mediated red cell destruction. Most of the experimental work has been done with alloantibodies, but assumedly autoantibodies lead to red cell destruction in a similar manner. This paper attempts to correlate the results of these experiments with the findings in patients with positive antiglobulin tests and autoimmune haemolytic anaemia (AIHA).

Known and speculative methods of red cell destruction

LYSIS BY COMPLEMENT

One molecule of IgM antibody bound to the red cell surface is theoretically able to activate the whole of the complement sequence and lead to lysis of the red cell. Two molecules of IgG close together on the cell membrane are needed to start the same sequence. Most IgM red cell antibodies will bind at least some of the components of complement (although there are important exceptions) while, of the others, only IgG1 and IgG3 bind complement readily. For lysis to occur active C5 must enter the preformed channels on the cell surface formed by C5-7 and damage the membrane. C9 catalyses this process (Müller-Eberhard, 1975).

If membranes lysed by complement are examined with an electron microscope 'holes' will be seen (Humphrey and Dourmashkin, 1965). With human complement these are about 100 nm in diameter regardless of whether the complement was fixed by antibody or not and regardless of the specificity of the antibody. This size is too small for haemoglobin to escape and consequently water and ions are drawn into the red cell until it bursts (Rosse *et al.*, 1966).

Probably all antibodies in the ABO, Lewis, P, or I systems are potentially lytic but the number that cause marked haemolysis *in vitro* is relatively small. Rare antibodies such as anti-Vel are often lytic, and very occasional examples of anti-Jk^a and anti-Di^a will lyse red cells that have not been treated with enzymes. If radiolabelled B or A red cells are given to a volunteer with a potent lytic anti-A or B in the plasma they disappear from the circulation within a few minutes, haemoglobin will be seen in the plasma and, if sufficient, in the urine, and very little radio-

activity will appear in the liver or spleen on scanning (Jandl *et al.*, 1957). This destruction is due to intravascular haemolysis of the red cells by complement and complement 'holes' will be seen if the membranes of the lysed red cells are examined.

MACROPHAGE AND MONOCYTE RECEPTORS FOR COMPLEMENT

There are receptors for complement not only on macrophages and monocytes but also on neutrophils and lymphocytes. Their function on the latter cells is not known. They are found on B lymphocytes and may play some part in the immune response (Rowlands and Daniele, 1975). These cells are not phagocytic and are not thought, as yet, to have any role in red cell destruction. The function of the neutrophil receptor is also not entirely known, and it seems that monocytes and macrophages are the more important cells in red cell destruction.

This complement receptor seems to differ from the IgG receptor (which will be mentioned later) but it is interesting that the latter is specific for IgG1 and IgG3, the two subclasses of IgG that fix complement most easily. When red cells are sensitised with IgG and/or IgG3 antibody and complement the two receptors appear to work together synergistically (Bianco *et al.*, 1975). The complement receptor is specific for active C3, C3b (Huber *et al.*, 1968). Active C42 (C3 convertase) splits the native C3 molecule into C3a and C3b. The C3b can attach to red cell surfaces, and these coated cells can adhere to macrophages and perhaps be phagocytosed by them. The life-span of C3b, however, is short. C3b inactivator is present in the plasma and this, with other enzymes, splits C3b into C3c and C3d (Müller-Eberhard, 1975). The C3d remains attached to the red cell and when this happens the coated cell no longer adheres to the macrophage and can return to the circulation (Brown, 1974). The B lymphocytes that have C3b receptors may also have different receptors that are specific for C3d. Their function is unknown and, again, they do not seem to play any part in red cell destruction (Ross and Polley, 1975).

Macrophages with C3b receptors are distributed throughout the reticuloendothelial system (RES). Native C3 does not compete for these receptors and

therefore the adherence of C3b-coated red cells is not inhibited by plasma. If such cells are labelled and injected into animals they disappear immediately and, on scanning, the liver, because of its greater blood supply, appears to be the major organ of sequestration (Brown *et al.*, 1970). Whether the labelled red cells reappear subsequently in the circulation depends to a large extent on how the C3b was bound.

If only complement is bound to the red cells and precautions are taken to ensure that lysis does not occur the coated cells will reappear in the circulation and their subsequent survival may well be normal (Brown *et al.*, 1970). The adherence of red cells coated with complement without antibody is not a powerful stimulant to phagocytosis, although if the complement coating is heavy enough the membrane of the red cells may be damaged by the macrophage and their subsequent survival may be less than normal. When red cells bind complement because of IgM antibody similar immediate adherence of the cells to macrophages may occur, with subsequent reappearance in the circulation and perhaps normal survival (Brown *et al.*, 1970; Atkinson and Frank, 1974a). Seemingly macrophages have no receptor for IgM, but if the IgM coating on the red cells is heavy direct agglutination will occur. It is difficult to imagine that this will be completely harmless (see later).

Most blood group antibodies bind some of the components of complement but antibodies in the Rh system, even if IgM, are the most important among those that do not usually bind complement at all. This may be explained by the distribution of the antigen sites, which are widely dispersed and tend to be single (Nicolson *et al.*, 1971). Perhaps they are too distant for at least two antigen-binding sites on one IgM molecule to combine with the antigen, which must occur before complement can be activated (Ishizaka *et al.*, 1968). However, Rosse (1968) suggested that when several IgG antibodies with different Rh specificities were all combined at once with the red cell at least C1 could be fixed.

When IgG antibody binds complement the destruction of coated red cells is complicated by the presence of IgG receptors on the macrophage.

MACROPHAGE AND MONOCYTE RECEPTORS FOR IgG

These receptors are also present on neutrophils although there is some evidence that they may be less numerous than on monocytes (Messner and Jelinek, 1970). They appear to react only with the Fc portion of IgG1 and IgG3 (Huber *et al.*, 1971). There is competition between IgG1 and IgG3 in the plasma and IgG1- and IgG3-coated red cells. According to

Huber *et al.* (1971) there is only one receptor for IgG and the adherence of IgG1-coated red cells can be inhibited by pure IgG3 in the surrounding medium and vice versa. In fact this competition by plasma is so great that it is difficult to imagine how this adherence could occur at all *in vivo*: red cells lightly coated with IgG anti-D, which, nevertheless, would disappear rapidly *in vivo*, are inhibited from binding to macrophages by a concentration of IgG one thousandth of that which occurs in normal plasma (Mollison, 1978). When LoBuglio and his colleagues first showed in 1967 that these receptors were present they suggested that this competition with the plasma might be overcome *in vivo* by concentration of red cells and reduction of the plasma in the red pulp of the spleen. Here the sensitised red cells would be forced into close proximity with the macrophage and adherence might occur. In the rest of the RES the macrophages would be bathed in plasma and adherence would be inhibited.

When the adherence of either IgG- or C3b-coated red cells to monocytes is examined in stained films the red cells are seen to form rosettes around the monocytes. If the interface between the monocyte and red cells is looked at with an electron microscope outgrowths of the monocyte distorting and interdigitating with the red cell membrane are seen (Brown and Nelson, 1973). The contact is a powerful local stimulus to pinocytosis and phagocytosis (at least with red cells coated with suitable IgG or heavily coated with complement). Enzymes released from the lysosomes of monocytes probably damage the red cell membrane (Fleer *et al.*, 1978). These damaged red cells try to reseal and their loss of surface without loss of contents leads to spherocytosis. The phagocytic stimulus can be shown to be localised and not generalised because the macrophage membranes will ingest particles coated with whole antibody while failing to ingest other particles adjacent to them and only coated with F(ab)₂ (Griffin and Silverstein, 1974). Cells coated with C3b without IgG do not seem to be readily phagocytosed although they adhere to monocytes rapidly: cells coated with IgG, although they adhere less easily, are rapidly phagocytosed once they have adhered. Brown (1974) has suggested that this difference between IgG and C3b is more apparent than real and may depend on the localisation of the stimulus on the red cell membrane. IgG-coated red cells are likely to have antibody widely distributed over the red cell membrane: C3b-coated red cells are likely to have the C3b deposited in clusters around C42. Certainly Brown and Nelson (1973) showed that cells coated with IgM antibody and complement undergo some phagocytosis.

Most alloantibodies directed against red cell

antigens are at least of the subclass IgG1. Many IgG anti-D antibodies include both IgG1 and IgG3 molecules. Mollison and his coworkers (1965) showed very beautifully that the plot of the percentage survival of radiolabelled red cells coated with small amounts of IgG non-complement-binding antibody was exponential on semi-log paper and relatively long, whereas the plot of the percentage survival of red cells coated with the same quantity of IgG complement-binding antibody was curvilinear and very much shorter. This slow exponential disappearance of red cells coated with IgG non-complement-binding antibody suggested that destruction occurred exclusively in the spleen, and scanning studies showed that this was so when the IgG anti-D coating was of the order of 25 $\mu\text{g/ml}$ or less. With larger amounts of antibody a proportion of the cells were destroyed in the liver. When IgG complement-binding antibody was present the red cells were destroyed in the whole of the RES. This seems to confirm the suggestion from experimental work of the synergistic effect of the two receptors and that activation of both prevents the inhibition of the IgG receptor by plasma.

DIRECT AGGLUTINATION

An IgM antibody coating, if heavy, leads to direct agglutination of the red cells. The experiments of Atkinson and Frank (1974a) seemed to suggest that IgM antibodies do not cause any impairment of red cell survival when they are prevented from binding complement. Nevertheless, they used very small amounts of antibody in their experiments and it is easy to imagine that this minor degree of agglutination would be broken-up by the hurly-burly of the blood stream. It seems logical to assume that strong agglutination hampers the circulation of blood in the capillaries and that such delay would lead to exposure to tissue enzymes and consequent damage. The experiments of Holburn *et al.* (1971) in which radiolabelled Rh-positive blood and purified IgM anti-D were injected into Rh-negative volunteers showed decreased survival of the red cells.

KILLER (K) CELLS AND OTHER CELLS

Results from patients with IgG on their red cell surface will be discussed in the next section. We know, however, that although in any one patient the amount of antibody correlates well with the degree of haemolysis there is little correlation between patients even though the subclass of antibody is taken into consideration. Because of this it has been suggested that cells other than phagocytic cells may play a part in red cell destruction. K cells are lymphocytes that can destroy nucleated cells coated with antibody in the absence of complement

(Perlmann and Holm, 1969). Urbaniak (1976) showed that lymphocytes would lyse Rh-coated red cells in the presence of concentrations of IgG that would inhibit monocyte destruction completely. But other workers do not agree (Poplack *et al.*, 1976) and the results need confirmation. Moreover, the red cells were pretreated with papain and thus were not completely normal.

Whether there is some other method of red cell destruction is not at all clear. It would seem from the evidence of patients that there must be. What it is is unknown.

Evidence from patients with a positive direct anti-globulin test and AIHA

LYSIS BY COMPLEMENT

Haemolytic is perhaps a bad term for the increased red cell destruction of AIHA. The uninitiated might think that this was always due to intravascular haemolysis by complement. Paroxysmal cold haemoglobinuria (PCH), the rarest of the AIHAs (Table 1), is the only one in which intravascular haemolysis

Table 1 *Classification of non-drug-induced AIHAs*

Type of antibody	Disorder	Incidence (%) in our series
'Warm'	Warm AIHA	75
'Cold'	Cold haemagglutinin disease	22
	Paroxysmal cold haemoglobinuria	3

characteristically occurs. It is curious, however, in that the antibody, which is called the Donath-Landsteiner antibody, is IgG. One would expect that a very large amount of IgG antibody would have to be attached to the red cell membrane before the one 'doublet' that would start the complement sequence would be formed. There is evidence, however, that once these doublets have been formed IgG antibody is more effective than IgM in completing the whole sequence (Humphrey and Dourmeshkin, 1969). This antibody has recently been shown by Marcus *et al.* (1976) to be directed against an antigen carried by globoside. Globoside is the most abundant of the glycosphingolipids (GSL), even more abundant than the A or B GSL, and the large quantity of antigen available may perhaps do a little to explain the powerfully lytic qualities of the antibody.

MACROPHAGE AND MONOCYTE RECEPTORS FOR COMPLEMENT

About 8% of routine blood samples from patients in hospital showed a positive result to a direct anti-globulin test due to complement only on the red cell

Table 2 Number of patients with warm AIHA showing different patterns of reaction in the direct antiglobulin test

Antisera	Patterns of reaction										Total	Incidence (%)
IgG	+	−	+	+	+	+	+	+	−	−		
IgA	−	+	+	−	+	−	+	+	−	−		
IgM	−	−	−	+	+	−	−	+	−	−		
C	−	−	−	−	−	+	+	+	+	−		
Idiopathic	64	2	5	0	0	68	2	1	8	17	11	61
Associated with:												
Lymphoma	10	1	0	1	1	26	0	0	1	5	0	16
SLE	0	0	0	0	0	19	0	2	4	0	0	9
Other AID	17	0	0	0	0	12	0	0	1	3	0	11
Miscellaneous	3	0	0	0	0	5	0	0	0	2	0	3
Totals	94	3	5	1	1	130	2	3	14	27	11	291
Incidence (%)	32	5					6.5			9	4	

surface (Dacie and Worlledge, 1969). Most of these patients showed no evidence of increased red cell destruction. When this complement was analysed further it was found to be C3d and/or C4d. What this coating means is not known. Many of the patients were suffering from infections, some had other evidence of 'autoimmune' disorders. It has been shown that cells coated with C3d and/or C4d may survive normally (Mollison, 1965).

A minority of the patients classified as 'warm' AIHA (9% of the total; Table 2) have only complement on their red cells. These patients show evidence of increased red cell destruction, but they do not have significant cold antibodies in the serum and their disorder is unaffected by the ambient temperature. Occasionally some, but not all, of these patients may be found to have small but probably significant amounts of IgG on their red cells by the special technique of Gilliland *et al.* (1970). Most of them have antibody in their serum which is powerfully lytic of enzyme-treated red cells at 37°C. This antibody appears to be IgM but does not give agglutination of normal red cells (Englefriet *et al.*, 1970). We do not know why these patients should show a coating of complement on their own cells which are not enzyme-treated. The destruction of their own and normal red cells may be rapid, they do not usually respond to splenectomy, and many do poorly with prednisolone and immunosuppressive drugs.

The commonest single pattern of the direct antiglobulin reaction with the red cells of patients suffering from warm AIHA is IgG and complement (Table 2). How this complement is bound is not at all clear because it is not usually possible to show that the eluted IgG antibody will bind any complement. The IgG autoantibody of most patients (about two-thirds) with non-drug-induced warm AIHA shows 'Rh' specificity: the remainder show 'En^a' or 'W^r_b' specificity or a 'non-specific' antibody, or a mixture of these with 'Rh' specificity. It is commoner

to find complement as well as IgG on the red cells of patients with autoantibodies in the second category than it is to find it on the red cells of patients with autoantibodies with 'pure' 'Rh' specificity (Table 3). Patients with a positive direct antiglobulin test or AIHA due to methyldopa, who all have autoantibodies with 'Rh' specificity, never show any complement on their red cells.

Table 3 Number of patients with warm AIHA from whose red cells autoantibody was eluted and the specificity of this antibody correlated with the type of protein on the patient's red cells

Protein on red cells	'Rh' specificity (i.e., negative or very weak with Rh null cells)	Not 'Rh' specificity (i.e., reacted equally or more strongly with Rh null cells)	Per cent Rh specificity
Ig only	68	15	82
Ig and complement	36	47	43

Patients suffering from cold haemagglutinin disease (CHAD) show only complement on their red cells if these are washed at 37°C, which they must be in order to get red cells unagglutinated in saline. Further tests show that these cells are coated only with C3d and C4d. Patients with the chronic form of the disease seldom have haemoglobinuria, although the fact that the antibody is potentially lytic can be seen from the constant haemosiderinuria and low total serum complement that is characteristic of the disease. C3d is a non-haemolytic end-stage of the complement sequence and means that further components cannot be bound: this probably protects the red cells from haemolysis. Evans *et al.* (1968) showed that when radiolabelled normal red cells were transfused to such patients about half were destroyed at once, but when the same cells had been pretreated with anti-I and sublytic doses of complement the

Table 4 Number of patients who had IgG on their red cells related to the reactions of their red cells with subclass antisera, to whether they showed overt haemolysis at the time of the tests, and to whether complement was present in addition to IgG

Reactions with anti-IgG*				Overt haemolysis associated with:				Total
				IgG alone or with only a weak reaction with anti-C†		IgG with a strong reaction with anti-C†		
2	3	4		Yes	No	Yes	No	
+	—	—	—	50	36	15	4	105
+	+	—	—	11	2	2	0	15
+	+	+	—	2	0	1	0	3
+	—	+	—	8	1‡	13	0	22
—	—	+	—	0	1‡	0	0	1
+	+	—	+	1	0	0	0	1
+	—	—	+	1	0	1	0	2
—	+	—	—	1	0	1	0	2
—	—	—	—	1	4	5	1	11
48	22	26	3	74	45	38	5	162

*Subclass anti-IgG kindly supplied by Dr C. P. Engelfreit, Dutch Red Cross Blood Transfusion Service, Amsterdam.

†Anti-complement.

‡These patients had had a splenectomy.

destruction was reduced to 0-20%. Haemoglobinuria is common at the onset of the illness in the transient forms of the disease that may follow *Mycoplasma pneumoniae* infection or, rarely, infectious mononucleosis. This, presumably, is due to the rapid increase in complement binding autoantibody in a patient with normal complement levels.

In both the transient and chronic forms of the disorder the antibody is, in our experience, nearly always IgM and has specificity within the I/i system. Cell survival studies using normal red cells show that these are rapidly sequestered, a proportion are destroyed immediately, and the remainder reappear in the circulation with a variable life-span, often nearly normal. Scanning studies show the majority of the radioactivity appears in the liver and thus splenectomy would not be beneficial.

MACROPHAGE AND MONOCYTE RECEPTORS FOR IgG

Most patients with warm AIHA have some IgG on their red cells (Table 2). When this is tested with subclass antisera the majority are found to have at least IgG1 (Table 4). However, Table 4 shows that patients with IgG1 on the red cell surface do not necessarily suffer from overt haemolysis even though the red cells also react strongly with anti-complement. Patients with IgG3 on the red cell surface always show overt haemolysis if the spleen is intact.

Experimental work has shown that the macrophage does not have receptors for IgA, although Abramson and Schur (1972) showed that when IgG was present as well IgA seemed to enhance its adherence to the IgG receptor. Occasionally patients appear to have only IgA on their red cells (Table 2).

These present in a manner similar to those with only IgG on their red cells, and if survival and scanning studies are done the spleen is seen to be the major organ of red cell destruction. They usually respond well to prednisolone and will benefit from splenectomy. How these red cells are destroyed is unknown. If there is a different mechanism for them it may well apply to IgG-coated cells as well.

Spherocytosis is pronounced in the blood films from patients with 'warm' AIHA. It is not characteristic of the blood films from patients with CHAD even though these are made at 37°C to prevent agglutination. It is often seen at the time of haemolysis in PCH. Spherocytosis is also seen when the disorder is caused by alloantibodies, as in transfusion reactions. It is characteristically present in ABO haemolytic disease of the newborn (HDN) but is not usually seen in Rh HDN.

It has been known for some time that erythrophagocytosis can be seen in the peripheral blood after transfusion reactions due to antibodies that bind complement (Hopkins, 1910; Ottenberg, 1911). This is seen mainly in monocytes but some neutrophils may show it too. Erythrophagocytosis, nearly always in monocytes, is occasionally seen in peripheral blood films in warm AIHA. When there is only IgG on the patient's red cells erythrophagocytosis is seen only after splenectomy when the red cells are very heavily coated with IgG.

Splenectomy is known to be often a useful treatment for patients with 'warm' AIHA, particularly when the red cell is coated mainly with IgG. The effect of splenectomy is to allow a greater amount of antibody to accumulate on the red cells without a proportional increase in red cell destruction. Pred-

nisolone probably exerts its immediate effects by inhibiting the reaction between coated red cells and phagocytic cells (Atkinson and Frank, 1974b; Schreiber *et al.*, 1975). In the long term the drug may also lead to a reduction in the amount of auto-antibody produced. Prednisolone may be effective not only in 'warm' AIHA but also in chronic CHAD in an acute crisis. However, it is dangerous to use this drug for prolonged treatment in these elderly patients, so it is not generally advocated.

Most of the observed results fit well with the experimental data but there are some obvious discrepancies. One such discrepancy, the mechanism of destruction of IgA-coated red cells, has already been discussed. Other obvious ones are the patients with strongly positive direct antiglobulin tests due to methyldopa, almost always due to IgG1, who have little or no haemolysis, and the patients suffering from Coombs-negative AIHA who have little or no detectable antibody on their red cells and yet have overt haemolysis. The specificity of the antibody might explain some of these cases. The autoantibody in patients taking methyldopa always has 'Rh' specificity, and when an autoantibody can be demonstrated in Coombs-negative AIHA it never, in our experience, shows 'Rh' specificity.

Summary of mechanisms of destruction in AIHA

The mechanism of red cell destruction in PCH is complement-induced intravascular haemolysis. This disorder is often associated with a virus-like illness and sometimes used to be associated with long-standing syphilis, especially congenital syphilis. The condition usually needs no treatment except perhaps blood transfusion if the haemolysis has been unusually severe. The antibody is generally transient and disappears within three months, although in congenital syphilis it disappears only when penicillin is given.

The mechanism of red cell destruction in CHAD is a balance between complement-induced intravascular haemolysis and complement-induced sequestration and phagocytosis. In chronic cases the intravascular haemolysis is limited by deficiency of complement components and the presence of C3d on the red cell surface. Many of the sequestered cells return to circulation with a nearly normal survival and the haemolytic anaemia is usually mild.

The mechanism of red cell destruction in 'warm' AIHA is not fully known. The red cells of many of the patients show IgG on their surface, and IgG receptors on phagocytic cells may be important. Most of the patients (Table 2) show complement as well as IgG and this may limit the inhibitory effects of IgG in the plasma. Why patients with only large

amounts of complement on the red cell should often show severe destruction is unknown. Also unknown are the mechanism for the destruction of red cells coated only with IgA and the explanations for methyldopa-treated patients with a strongly positive direct antiglobulin test and no haemolysis and patients with a Coombs-negative AIHA. The part played by cells other than phagocytic cells is also unknown.

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