REVIEW ARTICLE

The James Blundell Award Lecture 2007: Do we really understand immune red cell destruction?

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SUMMARY. We have learned a great deal about immune red blood cell (RBC) destruction since the elaboration of biochemical/immunological interactions of antibodies, complement and macrophages during the past 50 years. We first learned about the direct lysis of RBCs involving complement. We then learned of the role of the macrophage (particularly in the spleen and the liver) in initiating phagocytosis and antibody-dependent cytotoxicity of antibody-coated RBCs. Later, as the complexities of the human complement system were unravelled, we learned that complement-coated RBCs that were not directly haemolysed could interact with macrophages and that specific complement molecules on the RBC membrane could lead to a phagocytic event or the RBC (although heavily coated with complement) could survive normally. The application of isotope-labelling procedures (e.g. ^{51}Cr) for RBC survival (starting in the 1950s) advanced our knowledge considerably. Advances in knowledge in immunology helped us understand the complexity of the immunoglobulins (e.g. subclasses) and the specific receptors on macrophages and their role in immune haemolysis. Nevertheless, after more than 30 years researching this area, I am sometimes embarrassed to realize how much I cannot explain. Why do some patients have severe haemolytic transfusion reactions because of antibodies that are only detectable by one technique or not detectable by any? How do we explain autoimmune haemolytic anaemia with negative direct antiglobulin tests (DATs)? Why do RBCs strongly coated with immunoglobulin (Ig)G1 or IgG3 sometimes have normal survival? Are cells, other than macrophages, involved in immune RBC destruction? Could the relative amount of cytotoxicity vs. phagocytosis explain different clinical findings and response to treatment? How do we explain 'hyperhaemolysis' in sickle cell disease? Could novel mechanisms involving IgG glycosylation, CD47, 'armed' macrophages, bystander lysis, antibody activated reactive oxygen species, natural killer cells or antibody perturbation of RBC membrane be involved? Why do RBCs die after circulating for 100–120 days in healthy individuals? How should we define a 'clinically significant' antibody; how do we evaluate this? So many questions, so little time!

Key words: immunoglobulins, phagocytosis, RBC destruction.

I started my career with tuition and inspiration from two 'Kings' of this field, Professors Sir John Dacie (Garratty, 2007) and Patrick Mollison, at the Royal Postgraduate School of London. I have been involved in research associated with immune red blood cell (RBC) destruction/haemolytic anaemia (HA) for about 40 years and so have no excuse for not being able to tell you all you need to know about immune haemolytic anaemia (IHA), yet I find that we really do not have answers for many of the basic questions.

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Table 1 shows examples of some of these questions. In the Blundell Lecture, I attempted to review and discuss some of these and suggest some novel mechanisms that may be involved. My hope is that young investigators will not think, as many textbooks suggest, that we totally understand IHA. There is a great need for further research in this area.

MECHANISMS OF IMMUNE RBC DESTRUCTION (HAEMOLYTIC ANAEMIA)

IHA can be caused by autoantibodies or alloantibodies; the RBC destruction may be mediated through complement activation which, if conditions are

Table 1. Questions that still require answers

Can T (cytotoxic) lymphocytes, NK cells, granulocytes and dendritic cells participate in immune haemolysis?

Do differences in clinical severity and response to treatment relate to relative efficiency of macrophage-induced phagocytosis vs. cytotoxicity?

Why do RBCs strongly coated with IgG1 or IgG3 sometimes survive normally?

Why do some autoantibodies and alloantibodies cause severe IHA not detected by routine techniques?

Why does 'hyperhaemolysis' occur in SCD?

Can antibodies cause IHA without activating complement or interaction with macrophage Fc/CR receptors?

Why do circulating RBCs die after 110–120 days?

How should we define a clinically significant antibody?

optimal, can lead to membrane damage and breakdown of the RBCs in the circulation (so-called intravascular lysis) or the sensitized RBCs may react with macrophages. Extravascular lysis occurs when RBCs become coated with IgG (IgG1, IgG2 or IgG3), IgA or complement (C3b, iC3b), and these proteins react with receptors specific for these proteins present on macrophages in the spleen and liver (Kupffer cells). Figure 1 is typical of cartoons used to show students that macrophages can shorten RBC survival by phagocytosing the RBC completely, or partially (with the release of immune spherocytes), or part or all the destruction may occur external to the macrophage by antibody-dependent cellular cytotoxicity (ADCC). Some RBCs may escape immediate destruction but have a shortened life span because of macrophage-
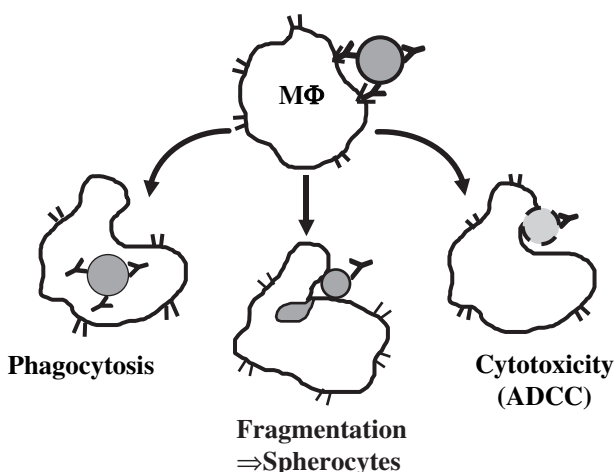

Fig. 1. RBCs sensitized with IgG1, IgG3, Ig2 (sometimes), or C3b and iC3b, can interact with Fc (illustrated here) or complement receptors on macrophages (Φ), leading to complete or partial phagocytosis or cytotoxicity (ADCC).

enzyme-induced membrane defects. Such cartoons are commonly used to make an extremely complex set of interactions appear quite simple. Although macrophages are undoubtedly the most important cell line involved, it is still unclear how important the role of other cells [e.g. lymphocytes ('killer' T-cytotoxic cells; natural killer (NK) cells); dendritic cells; granulocytes] can be in individual patients. All these cells can be shown to interact with sensitized RBCs (e.g. direct lysis) *in vitro*, when conditions are optimal, but are thought by most investigators to generally play a minor role.

There are multiple factors that influence the interactions between sensitized RBCs and macrophages. Table 2 lists some of the more important factors. Most of these have been discussed previously by me in detail elsewhere (Garratty, 1989, 1991; Petz & Garratty, 2004, pp 133–161). Two of these factors, which are not mentioned as much as the others, are discussed below.

Glycosylation of IgG affects macrophage interactions

A series of papers were published in 1989 and 1990, showing that there was an important association of the carbohydrate attached to the Fc domain of Ig and the interaction with macrophages (Walker *et al.*, 1989; Malaise *et al.*, 1989, 1990). In particular: (a) aglycosylation of human IgG1 and IgG3 monoclonal antibodies eliminated recognition by FcRI and FcRII receptors; (b) the ability of normal human monocytes to phagocytose IgG-coated RBCs was related to the number of accessible galactosyl and mannosyl residues of the Fc domain of IgG; (c) accessible galactosyl and mannosyl residues on the Fc domain of IgG-affected clearance of IgG-coated RBCs in the rat.

Kumpel *et al.* (1994) showed that monoclonal IgG anti-D produced by Epstein-Barr virus (EBV)-transformed B cells, using two different culture methods, differed in their efficiency in participating in phagocytosis and ADCC with monocytes because of

Table 2. Factors that influence the pathogenicity of antibodies

Characteristics of antibody (class, subclass, specificity, thermal amplitude, complement-activating efficiency, affinity and amount of galactose on the Fc carbohydrate)

Quantity of RBC bound

Type of complement on RBCs

Activity of macrophage system

? CD47/inhibitor balance

differences in IgG Fc-containing N-glycans. Malhotra *et al.* (1995) showed that glycosylation changes of the IgG Fc domain were common in rheumatoid arthritis and that these IgG isoforms could activate complement through the mannose-binding protein pathway; no one has shown that this can occur with RBCs, but it is an interesting suggestion that might add to a growing list of novel mechanisms that may explain some of the findings in individual patients that we cannot explain at present.

There are two publications studying whether glycosylation changes in the Fc domain of Ig shows any association with IHA. Hadley *et al.* (1995) eluted IgG from the RBCs of 27 haemolysing or non-haemolysing patients. The ability of the autoantibodies to promote K-cell-mediated RBC lysis in an *in vitro* assay correlated inversely with agalactosyl IgG. Barker *et al.* (1999) studied a patient with autoimmune haemolytic anaemia (AIHA) over 21 months. They found that there were wide fluctuations in galactosylation of the IgG autoantibody, but unfortunately, these seemed to be unrelated to the severity of the haemolytic process, thus it may take a total, or near total, loss of carbohydrate before immune RBC destruction is affected. Nevertheless, I hope someone tries to correlate *in vitro* findings of seemingly strong IgG RBC sensitization with minimal *in vivo* haemolytic anaemia (HA) (see later) with the amount of Fc carbohydrate.

Role of CD47 in cell death

CD47 is present on almost all cells; it has been known for some time to be associated with the Rh complex. In 2000 and 2001, Oldenborg *et al.* showed that RBCs from mice lacking CD47 were rapidly destroyed by normal mice but not CD47-negative mice. The presence of CD47 appeared to be a 'don't eat me' signal. A signal regulatory protein (SIRPα) was described as an inhibitory receptor for CD47 (e.g. on macrophages). Phagocytosis appeared to be a balance between positive signals (Fc/CR macrophage receptors) and negative signals (SIRPα ligated by CD47). In 2002, Oldenborg *et al.* described a lethal AIHA in CD47-deficient non-obese diabetic mice. In a later paper, Oldenborg (2004) suggested that CD47 was involved in the regulation of AIHA. He also suggested that because CD47 was reduced to 10–25% on Rh_{null} RBCs that the compensated HA, associated with this rare phenotype, was because of the low levels of CD47. Two groups studied the latter, in different ways, and came to a similar conclusion that the CD47 level was not the cause of the HA (Mouro-Chanteloup *et al.*, 2003; Arndt & Garratty, 2004).

Ahrens *et al.* (2006) reported that they could find no correlation of CD47 with the severity of HA in AIHA. These disappointing results were followed by another disappointing report on the CD47 story (Subramanian *et al.*, 2006). These authors used a *human* SIRPα as a probe and bone marrow-derived mesenchymal stem cells. These cells displayed CD47 but did not bind to SIRPα significantly. They concluded that Oldenborg's results in *mice* may not apply to *humans*! I would point out that there is an important statement in the original paper by Oldenborg *et al.* (2000), 'In contrast to splenic macrophages, bone marrow-derived macrophages do not phagocytose CD47-negative RBCs …', which may relate to some of the disappointing results above where splenic macrophages were not used.

Lack of correlation of clinical/haematological findings with in vitro serological results

Sometimes RBCs are found to be strongly coated with IgG and/or complement and yet are surviving normally. Sometimes, strong alloantibodies or autoantibodies are detected in patients' plasma and yet transfused incompatible RBCs appear to cause little or no morbidity. In contrast to these findings, some individuals have negative DATs and no detectable antibodies in their sera, yet appear to have AIHA. Other patients have no antibodies in their sera, detectable by routine procedures, yet have severe haemolytic transfusion reactions.

Such incidents have been well described in the literature for many decades, yet we still cannot explain many of them.

Presence of IgG and/or complement on RBCs is sometimes associated with relatively normal RBC survival or no morbidity

Sometimes we can explain this. For instance, IgG4 antibodies do not activate complement and do not react with macrophages, thus are clinically insignificant, but are detected by most anti-IgG. IgG2 antibodies activate complement poorly and only react with macrophages when the Fc receptor is of a particular allotype (possessed by 30% of Caucasians, but 85% of Asians). Thus, RBCs strongly sensitized with IgG2 antibody may survive normally in some patients, but in others have shortened survival. There are macrophage complement receptors for C3b and iC3b (the first breakdown product of C3b), but no efficient receptors for the next breakdown products of iC3b (C3dg or C3d). Thus, once RBCs are only coated with C3dg, they can yield

strongly positive DATs with anti-C3, but survive normally; the breakdown of iC3b to C3dg occurs within several hours *in vivo*.

So, the major question is why do RBCs coated with IgG1, IgG3 and/or C3b/iC3b sometimes appear to survive relatively normally? Good examples of this are (a) methyldopa-induced autoantibodies, where 15–20% of patients taking this drug make IgG (usually IgG1) autoantibodies, often with 3–4+ DATs, but most of these patients have no HA (Petz & Garratty, 2004) or (b) healthy blood donors who are strongly DAT+.

Quantity of RBC-bound IgG. Van der Meulen *et al.* (1980) used flow cytometry (FC) to compare the amount of IgG on strongly DAT+ RBCs from patients taking methyldopa with and without HA. They found a distinct difference and suggested a quantitative pathogenic threshold that was not obvious by the DAT but was obvious using FC. Garratty & Nance (1990) used FC to quantitate RBC-bound IgG on many more DAT+ patients than studied by Van der Meulen *et al.* (1980), with and without HA, and healthy blood donors with strongly positive DATs. Although they agreed that there was a relationship between the amount of RBC-bound IgG and the presence of HA (e.g. more chance that HA was found in the strongest DAT+ groups), they could not demonstrate any 'haemolytic threshold' that could be determined by FC. They concluded that although the amount of RBC-bound IgG was important, it was only one of several factors explaining the discrepancy.

Decreased activity of macrophages. Macrophages have been shown to have diminished reactivity in patients with diseases associated with immune complexes. Frank *et al.* (1979) showed that ^{51}Cr-labelled RBCs strongly sensitized with IgG anti-Rh(D) survived much better in patients with systemic lupus erythematosus than in controls. They postulated that this was because of the unavailability of Fc receptors because they were blocked by anti-DNA–DNA complexes. Rh+ foetuses have been shown to suffer with less severe haemolytic disease of the foetus and newborn (HDFN) in mothers with anti-D if the mothers also have antibodies to human leucocyte antigen (HLA)/DR that react with foetal macrophages (Dooren *et al.*, 1992). Kelton (1985) put forward a novel hypothesis in an attempt to explain why so few patients with methyldopa-induced strongly positive DATs (because of IgG1) had no HA. They postulated that α-methyldopa diminishes macrophage activity; he supported his hypothesis with convincing ^{51}Cr RBC

survival studies; no one has tried to confirm this, which is unfortunate, as many other drugs may have this effect and this may explain why we sometimes are unable to correlate serological with haematological findings.

Anti-idiotype. Masouredis *et al.* (1987a) found that protein eluted from DAT+ donor RBCs appeared to contain more IgG than could be accounted for by the RBC autoantibody. They suggested that perhaps 30% of the RBC-bound IgG was an anti-idiotype directed against the IgG RBC autoantibody. They confirmed and extended this finding later in 1987b and in 1989. It was suggested that perhaps anti-idiotype provided some protective mechanism in healthy DAT+ blood donors but, unfortunately, our own unpublished studies (Garratty & Postoway, 1990) showed that anti-IgG could be detected in eluates and plasma in both DAT+ donors and patients with AIHA; only a small number of donors and patients were studied; a larger study would be very worthwhile.

Transfusion of RBCs incompatible with alloantibodies in recipient plasma often cause no morbidity. In 1990, Ness *et al.* coined the term 'delayed serological transfusion reaction' (DSTR). This was a term applied to delayed transfusion reactions associated with the formation of alloantibodies, leading to a positive DAT (sensitization of transfused RBCs), but no evidence of HA (no clinical and laboratory signs). DSTRs were shown to be much more common than delayed haemolytic transfusion reactions (DHTRs). Table 3 shows the results of four reports comparing the two types of reactions. Data from 18 years of studying immune transfusion reactions at the Mayo Clinic found that 65% were DSTRs (Table 4). It should be noted that even antibodies generally considered to always be clinically significant, such as anti-E, -Jk[a], -Fy[a] and -K, more often caused DSTRs than DHTRs. No claim has been made that RBC survival is normal in DSTRs, only that no obvious laboratory or clinical signs of HA are noted.

It is interesting to note that patients who receive ABO incompatible blood also do not always show obvious signs of a HTR. Linden & Dressler (1992), studying patients who had received blood not meant for them, reported that 47% of 111 patients receiving ABO incompatible blood showed no obvious adverse effects. This report was followed by a similar result from the Quebec hemovigilance system, showing that 46% of 24 patients receiving ABO incompatible blood were reported as asymptomatic following transfusion (Robillard *et al.*, 2004).

Table 3. Delayed haemolytic transfusion reactions (DHTRs) vs. delayed serological transfusion reactions (DSTRs)

	Ness *et al.* (1990)	Pinkerton *et al.* (1992)	Heddle *et al.* (1995)	Vamvakas *et al.* (1995a, 1995b)	Pineda *et al.* (1999)	Mean
DHTR						
Per unit	1/9094	1/13681	1/11328	1/5405	1/6715	1/2424
Per patient	1/854	1/2537	1/2082	NA	NA	1/1824
DSTR						
Per unit	1/1605	1/3040	1/199*	1/2990	1/1612	1/1241†
Per patient	1/151	1/564	1/37*	NA	NA	1/358†

NA, not applicable.
*Heddle *et al.* were the only authors to include alloimmunization in their DSTR group (even if DAT was negative), hence the higher incidence.
†Heddle *et al.* data not included.

Immune haemolytic anaemia, but no antibodies detectable, by routine procedures

Sometimes patients with HA, apparently of an immune aetiology, have negative DATs and/or negative indirect antiglobulin tests (IATs). Negative test findings can be associated with AIHA and alloimmune HA (e.g. HTRs and HDFN). I have reviewed this subject in detail elsewhere (Garratty, 2005).

Table 4. DHTR and DSTR transfusion reactions at Mayo Clinic during a 19-year period (1980–1998) [Pooled data from Vamvakas *et al.* (1995a, 1995b) and Pineda *et al.* (1999)]

Specificity	Total number	DHTR	DSTR
E	184	47	137
Jk^a	95	45	50
Fy^a	62	26	36
c	54	18	36
K	62	16	46
C	22	8	14
Fy^b	12	4	8
S	7	4	3
Jk^b	17	3	14
e	12	3	9
C^w	5	3	2
Yt^a	2	1	1
A_1	2	1	1
Kp^a	1	1	0
Lu^b	1	1	0
D	1	1	0
M	2	0	2
Js^a	2	0	2
V	2	0	2
G	1	0	1
P_1	1	0	1
Co^b	1	0	1
Total	559	197 (35%)	362 (65%)

Autoimmune haemolytic anaemia. Patients with HA and haematological and clinical signs suggesting AIHA, but with negative DATs and IATs, have been described in the transfusion medicine literature for many years. Some of the early cases occurred before the role of complement was appreciated and appropriate antiglobulin sera (containing antibodies to relevant epitopes on RBC-bound complement) were available. Nevertheless, even after testing was improved, negative cases of unidentified cause continue to be encountered.

Worlledge & Blajchman (1972) reported that 3% of 333 patients with AIHA were DAT−; Chaplin (1973) reported a similar incidence. During a 10-year period in which Petz & Garratty (1980) studied 347 AIHAs (244 were warm type AIHA), they encountered 27 patients (11% of the warm type AIHAs), who were referred because of a negative DAT with routine reagents. Only 16 (59%) of these 27 were found to be DAT− when tested in our own referral laboratory (11 were found to react with our 'homemade' potent anti-C3). Thus, the true incidence in the series was 7%. Boccardi *et al.* (1978) reported that 11% of their AIHAs were DAT−, identical to the incidence in Petz and Garratty's referred patients.

There are at least three explanations for DAT− AIHA:

1 RBC-bound IgG below the threshold of the antiglobulin test, which requires 100–200 IgG molecules per RBC before agglutinates can be seen.
2 RBC-bound IgA or IgM. In the US, there are no Federal Food and Drug Administration (FDA)-licensed anti-IgA/IgM for use with RBCs. Immunological reagents can be standardized for the DAT (see Petz & Garratty, 2004, pp 210–11).
3 Low affinity autoantibodies. Such antibodies can be washed-off the RBCs when performing the DAT, particularly if the washes are with 37C saline.

Our approaches to investigate such patients can be found in Petz & Garratty (2004, pp 319–354) and Garratty (2005). Even using a battery of tests (including FC, enzyme-linked antiglobulin tests, concentrated eluates from RBCs, direct polybrene test and monocyte monolayer tests), we can only support the diagnosis of AIHA in 48% of the patients submitted to our laboratory (Garratty *et al.*, 2004).

Alloimmune haemolytic anaemia. HTRs with no detectable antibodies have been reported since the 1950s. In 1996, we reported 71 such patients encountered over a 10-year period (Garratty *et al.*, 1996). Seventy per cent of these patients had haemoglobinaemia and haemoglobinuria following transfusion of seemingly compatible RBCs. Eighteen per cent of the patients had antibodies detected by a simple unusual procedure, the indirect polybrene test (three anti-C, three anti-Jk[a], two anti-S, two anti-e, one anti-E and one anti-Jk[b]). These antibodies were not detected by other commonly used tests [including low-ionic-strength saline additives, enzymes, polyethylene glycol]. In eight patients, antibodies could not be detected, but phenotype-matched RBCs caused no HTRs. Three antibodies (anti-c, -C and -Vel) became detectable eventually, by routine testing.

NOVEL MECHANISMS THAT MAY EXPLAIN SOME OF THE DISCREPANCIES ABOVE

It is clear that we still cannot explain the lack of correlation of our serological tests and what we observe clinically in some patients, using the cartoons and principles that we have used to teach students for many years. We obviously need to start thinking 'out of the box' and produce more research in areas involving novel mechanisms, some of which are suggested below.

Can immune haemolytic anaemia be caused by only cellular immune mechanisms?

In 1981, I suggested that perhaps NK cells could be involved in DAT− AIHA and HTRs with no detectable antibodies (Garratty, 1981). Unfortunately, I was not able to prove it using NK cell assays and RBCs from DAT− AIHA, and RBCs incubated in sera from patients with HTRs and negative IATs. I only know of one publication supporting this hypothesis. Gilsanz *et al.* (1996) described a patient with an NK cell leukaemia who appeared to have a DAT− AIHA. The patient's NK cells reacted *in vitro* with autologous

RBCs but not allogeneic RBCs. The patient responded to treatment with cyclophosphamide; after 2 months of therapy, the NK cell counts were normal, the HA improved, and no NK lysis of autologous RBCs was observed *in vitro*. It is interesting to note that there is evidence that NK-cell-mediated thrombocytopaenia may occur. Garcia-Suarez *et al.* (1993) and Olsson *et al.* (2003) described NK cell cytotoxicity of platelets in autoimmune thrombocytopaenia (AITP).

Armed macrophages

Another area that may relate to negative serological findings in patients with HA is the role of so-called 'armed' macrophages. This term was first used by Sunada *et al.* (1985) who showed that monocytes from patients with AIHA were far more active, in phagocytosing IgG-coated RBCs, than monocytes from healthy individuals. They showed that the latter monocytes could be armed by incubating them in eluates from DAT+ RBCs, making them as active as monocytes from patients with AIHA. Hymes *et al.* (1990) demonstrated an identical phenomenon when studying platelets from patients with AITP (unfortunately, they did not reference the Sunada *et al.* publication from 1985). Hymes *et al.* (1990) showed that monocytes from patients with AITP bound to GPIIb–IIIa autoantigen 6·5-fold greater than normal monocytes. Normal monocytes became more active ('armed') following incubation with IgG from patients with AITP. These armed monocytes bound to GPIIb–IIIa autoantigens 5·8-fold greater than normal monocytes incubated with IgG-sensitized platelets. The binding by armed monocytes was shown to be via $F(ab')_2$.

Griffiths *et al.* (1994) showed that monocytes, passively sensitized with anti-D ('armed'), mediated adherent and phagocytic response to D+ RBCs. An important finding was that enhanced reactivity of armed monocytes with anti-D-coated RBCs occurred in the presence of 50% plasma, whereas interactions of normal monocytes were inhibited by 0·25% plasma. It was suggested that FcR1, which has a high affinity for monomeric IgG (e.g. IgG antibody not complexed to an antigen), is easily inhibited by IgG, and the Fc/IgG interaction might mediate arming of the macrophage. In contrast, FcRII and RIII, which have little affinity for monomeric IgG and are less susceptible to inhibition by free IgG, may mediate recognition of circulating IgG-sensitized RBCs, which is the classical route for RBC destruction. The IgG molecules in the plasma would always contain monomeric IgG that is not directed against RBC antigens and sometimes might also contain IgG antibodies directed to RBC

antigens. Both populations would attach to FcRI. If RBC antibodies are bound through this mechanism, they would be bound to the macrophage by their Fc domain, having their Fab domain projecting from the macrophage ('armed' macrophage); the Fab could react with RBCs having the putative antigen, thus the armed macrophage would now have RBCs on its membrane, leading to possible RBC death [note that these captured RBCs were circulating with no IgG antibody on them (DAT−)]. These interactions are illustrated in Fig. 2.

Griffiths *et al.* (1994) suggested that armed macrophages might explain HA without easily detectable antibodies. It may be that all early antibodies are adsorbed by the macrophages, creating an efficient system for removal of non-sensitized RBCs by armed macrophages. At this stage of increased cell destruction, no antibodies may be detectable in the plasma. This may well explain the RBC survival collapse curve noted when small volumes (<10 mL) of ^{51}Cr-labelled RBCs are injected into previously non-immunized individuals (Mollison *et al.*, 1997, pp 347–354), but it is more difficult to extend this as an explanation for DAT-negative AIHA and HTRs with no detectable antibody. In these conditions, one would think that antibody would eventually appear in the plasma, but perhaps antibody could be continually adsorbed by macrophages.
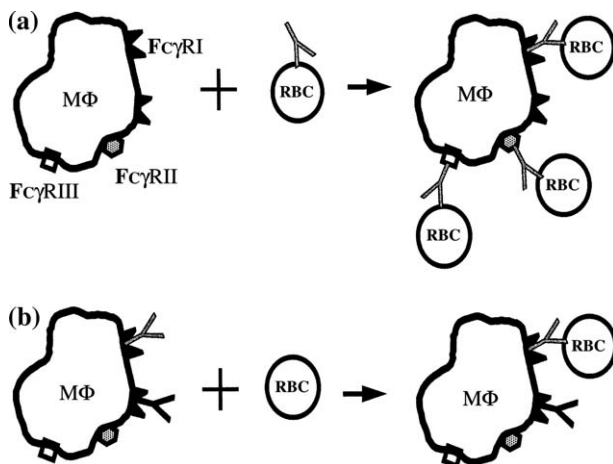

Fig. 2. a) Traditional view of extravascular RBC destruction. The Fc portion of the specific antibody attached to the RBCs reacts with macrophage [Fc (FcγRI, RII and/or RIII)]. (b) 'Armed' macrophage: Monomeric IgG, which can include some specific RBC antibodies, is adsorbed by macrophage FcRI receptors. The Fab portion of the 'specific' RBC antibody can react with non-sensitized RBCs, leading to the same end product as the traditional pathway.

Can HLA antibodies cause RBC destruction?

Remnants of HLA antigens are retained on mature RBCs, and sometimes HLA antibodies can react with RBCs. Such antibodies were originally called anti-Bg^a, -Bg^b and -Bg^c, which are anti-HLA-B7, -B17 and -A28, respectively. Such antibody reactivities are usually weak and clinically insignificant, but there are several reports suggesting they may be clinically relevant (Van der Hart *et al.*, 1974; Nordhagen & Aas, 1978; Panzer *et al.*, 1984, 1987; Weitkamp *et al.*, 1993; Benson *et al.*, 2003). One example of anti-Bg^a produced strongly positive results in a monocyte monolayer assay (Arndt & Garratty, 2004) suggesting clinical significance, and ^{51}Cr-labelled RBCs that were incompatible with HLA antibodies were shown to have reduced survival (Panzer *et al.*, 1984, 1987). Strong evidence that HLA may sometimes cause haemolytic transfusion reactions comes from observation of a woman with gastrointestinal bleeding who received 13 uncomplicated RBC transfusions in a 2-week period; with the 14th unit of RBCs, she developed shaking chills, nausea, vomiting and red urine (Weitkamp *et al.*, 1993). Lactate dehydrogenase was 2024 IU L^{-1}, haptoglobin <5 mg dL^{-1}, and there was a disproportionate decrease in haemoglobin. Similar reactions were seen with 3 of the next 6 units (2 saline washed). Post-reaction blood samples were grossly haemolysed but contained no RBC alloantibodies other than previously recognized anti-D and anti-Fy^b. Her HLA type was A1,26; B44,70; potent HLA antibodies specific for A2, A2-B17, A2-28 and A2-28-9 were present. The patient then received 10 units from HLA-compatible donors without a problem. Antibodies reacting 1+ by IAT against HLA-incompatible RBCs were detected 3·5 weeks after haemolysis began, reactive with each of the four donors implicated in the transfusion reactions but negative with RBCs of nine well-tolerated donors (13 units). HLA typing and lymphocyte cross-matching showed that all four donors implicated in haemolysis were incompatible with the HLA antibody, whereas the nine donors of tolerated units were HLA compatible. Thus, repeated, severe, asymptomatic haemolytic reactions occurred after transfusion of HLA-incompatible RBCs. The high titre (128) of the lymphocytotoxic antibody may explain these unusual reactions. In a similar case, a 37-year-old woman with no detectable antibodies had a DHTR following transfusion of two compatible units; transfusion of two more compatible units led to an acute haemolytic transfusion reaction during transfusion of the first 10 mL of the second unit (Benson *et al.*, 2003). The patient had fever, nausea, hypertension, haemoglobinaemia and haemoglobinuria. Investigation revealed

no obvious cause of the haemolytic transfusion reaction except for weak (microscopic) incompatibilities with the first unit of the second transfusion. The serologic reactions were found to be because of anti-Bg[a]; her serum contained anti-HLA-A2, -A28, -B7 and -B7 cross-reactive group (CREG). The patient was transfused with RBCs from an HLA compatible (negative for HLA-A2, -A28, -B7 and -B7 CREG) donor without incident. These results suggest that HLA-reactive alloantibodies should be investigated in patients with unexplained haemolytic transfusion reactions. Unfortunately, many patients have HLA antibodies, so the only proof to incriminate them is to show that RBCs from HLA-matched donors survive well.

Bystander/reactive lysis of RBCs

Dameshek (1965) described the phenomenon of lysis of 'innocent bystander' RBCs caused by complement activation initiated by antigen–antibody reactions remote from the putative RBCs. Thompson & Rowe (1968), Lachmann & Thompson (1970) and Thompson & Lachmann (1970) extended these findings, showing that the lysis could be associated with the membrane attack complex (C5b-9), without antibody or C3 being detected on the RBC membrane. Götze & Müeller-Eberhard (1970) showed that C5, 6 and 7, in the fluid phase, would cause the lysis of RBCs from patients with paroxysmal nocturnal haemoglobinuria (PNH). Sirchia et al. (1970) showed that if PNH RBCs were added to anti-HLA plus white cells, the RBCs were haemolysed. PNH RBCs are known to be hypersensitive to complement, and normal RBCs were not haemolysed in the two experiments above. So, can we apply this mechanism to explain any of our discrepant findings in patients without PNH? I think that we can in sickle cell disease (SCD).

Haemolytic transfusion reactions in SCD are very different to those seen in most other patients (Garratty, 1997; Petz et al., 1997). They are often associated with pain crises, reticulocytopenia (a decrease in the absolute reticulocyte count from the patient's usual level) and sometimes a lower haemoglobin/haematocrit, following transfusion, than pretransfusion, sometimes termed 'hyperhaemolysis' (Petz, 2006; Petz et al., 1997). This latter phenomenon has been described as being because of bystander lysis (King et al., 1997; Petz, 2006), suppressed haemopoesis (Petz et al., 1997) or both; autoantibodies may also play a role (Garratty, 1997 and 2004). How can bystander lysis be involved if it does not occur (in vitro) with normal RBCs? RBCs from SCD are, of course, not normal, and Test & Woolworth (1994) showed that sickle RBCs (especially irreversibly sickled RBCs) show increased susceptibility to reactive lysis. Unlike PNH, the quantity of CD55 and CD59 on sickle cells is normal; Test & Woolworth (1994) suggested that there may be a functional defect of CD59. It is of interest to note that some patients with thalassaemia suffer from 'hyperhaemolysis' (Sirchia et al., 1997) and their RBCs have also been shown to have similar findings concerning CD59 and cytolytic C5b-9 (Malasit et al., 1997; Salama et al., 2004). Hyperhaemolysis has also been observed in some patients with chronic diseases other than heamoglobinopathies (Treleaven & Win 2004; Darabi & Dzik, 2005) but CD55/59 were not studied in these reports. It is of interest to note that recently it was suspected that CD55 and CD59 may play a role in the pathogenesis of autoimmune haemocytopenias (Ruiz-Argüelles & Llorente, 2007).

Hyperhaemolysis in SCD can occur associated with a 'classic' DHTR but often occurs with no evidence of DHTR associated with RBC alloantibodies (e.g. it often occurs even when phenotypically typed RBCs are transfused or no alloantibodies are detectable). If RBC alloantibodies are not activating complement, leading to bystander lysis of autologous RBCs, then what could be the initiator of bystander lysis? Friedman et al. (1996) showed that 85% of SCD patients made platelet antibodies after 50 RBC transfusions; 48% made antibodies when the patients had <50 transfusions. About 50% of the platelet antibodies had HLA specificity. Heal et al. (1992) showed that 90% of unselected patients receiving ≥20 transfusions made antibodies to epitopes on foreign proteins (e.g. C2, C4, albumin and fibrinogen). They proved that such antibodies formed circulating immune complexes that could be cell-bound. I would suggest that HLA antibodies and antibodies to proteins, in SCD patients, could activate complement and cause reactive lysis, and hence, hyperhaemolysis. Win et al. (2008), in a recent review of hyperhaemolysis, suggested that interactions of activated macrophages with ICAM4 on RBCS may be involved.

Can antibodies damage RBCs independently to complement activation?

Many of us have taught our students that antibodies only damage RBCs by coating the RBC, and if IgG1, IgG2, IgG3 or IgA are present, they can react with specific macrophage Fc receptors; or if IgG1, IgG3 or IgM, they can activate complement, to produce a complex (C5, 6, 7, 8 and 9) that inserts itself into the RBC membrane, disturbing the osmotic balance of the RBC, leading to direct lysis of the RBC, or coating the RBCs with C3b or iC3b, that interact with

complement receptors on macrophages. It now appears that we may have not been correct and that antibodies perhaps may destroy RBCs by other mechanisms.

Destruction of RBCs by reactive oxygen species. In a series of papers, Wentworth's group showed that antigen–antibody reactions can generate hydrogen peroxide (H_2O_2) and ozone, in the presence of reactive oxygen species (ROS), from water, leading to the death of bacteria (Wentworth *et al.*, 2000, 2001, 2002). Nardi *et al.* (2001) reported complement-independent antibody-induced peroxide lysis of platelets in HIV-1-related immune thrombocytopaenia. They showed that anti-GPIIIa49-66 complexes from HIV-infected humans caused thrombocytopaenia in mice. Peroxide and other ROS caused platelet fragmentation/thrombocytopaenia. In a later paper, Nardi *et al.* (2004) suggested that this pathway might lead to effects on bystander cells other than platelets; they suggested megakaryocytes, or endothelial cells, but I would like to add the possibility of RBC lysis being caused by yet another bystander mechanism, but this time not involving complement (there are no data to support this suggestion). It is of interest that Coopamah *et al.* (2003) showed that when IgG anti-D-sensitized RBCs were incubated with white cells, hydrogen peroxide and other ROS were generated by monocytes and granulocytes. This is of interest to me as anti-D does not activate complement, but sometimes haemoglobinaemia is noted after transfusion of D+ units to a D− recipient with anti-D. This has been thought to be because of massive extravascular destruction (e.g. of a whole unit of blood or more) where haemoglobin is released from the RBC fragmentation and/or ADCC by the macrophages and escapes from the reticuloendothelial system into the circulation. More dramatic presentations of this have been reported with the therapeutic use of powerful anti-D in D+ individuals with autoimmune thrombocytopaenia (Gaines, 2000, 2005). I would suggest that maybe the above mechanisms might be involved to explain the clinical/laboratory findings with anti-D that mimic complement-mediated lysis but so far have not been clarified.

Another report also pertains to the above mechanisms. Lee *et al.* (2004) reported mice lacking leucine zipper transcription factor [nuclear factor (NF)-2-related factor (Nrf)], which mediates upregulation of antioxidant detoxification and antioxidant genes in apoptosis, led to an IHA. These mice had RBCs of abnormal shape, increased RBC-bound IgG (increased by 120%) and RBCs that were more sensitive to H_2O_2-induced lysis. It was suggested that a chronic increase in oxidative stress because of

decreased antioxidant capacity sensitizes RBCs with IgG and causes HA in Nr2-1-mice, suggesting a pivotal role for Nrf2-antioxidant responsive pathway in the cellular antioxidant defence system. There is no evidence, so far, that this could occur in humans.

Complement-independent destruction of RBCs by antibodies to glycophorins. Brain *et al.* (2002) were puzzled by the lack of correlation of laboratory and clinical findings in a patient who had an apparent severe AIHA caused by an autoanti-Pr. The severity of the HA (RBC survival showed a 3-day half-life) did not correspond with the minimal serological findings. The DAT was only weakly positive (only C3 on RBCs), the anti-Pr was an IgM kappa monoclonal agglutinin reacting to a titre of 32 at 4C, a titre of 1 at 30C and was non-reactive at 37C. Because Pr is found on glycophorin A (GPA), the authors wondered if antibodies to GPA affected the RBC membrane. They found that their patient's anti-Pr and several other anti-GPA (including an IgA anti-Pr, a murine monoclonal antibody, and lectins) led to an increase in RBC membrane permeability to sodium and potassium. Later work (M. Brain, University of Calgary, Canada, personal communication) has shown that calcium also enters the RBC. This could lead to the exposure of phosphatidylserine (PS) on the surface of the RBC membrane and subsequent interactions with the PS receptor on macrophages. This is an interesting hypothesis as we (Petz & Garratty, 2004, pp 180–182), and others, have seen several cases of HA associated with anti-Pr where the severity was not reflected by the serological findings. Head *et al.* (2005) found expression of PS on the RBC membranes of normal RBCs, following treatment with antibodies to glycophorin C (GPC). PS was not expressed when RBCs with variant GPC phenotypes (e.g. Gerbich variants resulting from deletions of exon 2 and 3, respectively) were substituted for normal RBCs.

The hypothesis by Brain *et al.* (2002) is a good example of thinking 'out of the box' and not trying to explain discrepant findings by older suggested mechanisms.

Can IgM antibodies cause shortened RBC survival independent of complement activation?

I have taught my students for years that IgM antibodies that do not activate complement (e.g. some anti-E) should not be classified as clinically significant as there are no IgM receptors on macrophages. There is little in the literature to suggest otherwise, but recently, Baudino *et al.* (2007) showed that murine IgM monoclonal agglutinating autoantibodies caused

HA in BALB/C mice. HA still occurred in C3-deficient mice. RBCs were not found in Kupffer cells in the livers of the mice (where complement-coated RBCs would be detected). In the spleen, they found an accumulation of agglutinated RBCs. Similar mechanisms have been described in mice before (Shibata *et al.*, 1990; Fossati-Jimack *et al.*, 1999). There are a few publications suggesting a similar phenomenon in humans with AIHA associated with IgM warm autoagglutinins (see reference list of Baudino *et al.*, 2007 and Petz & Garratty, 2004, pp 180–182), but most of these cases were associated with complement-activating IgM autoantibodies, so it is difficult to judge the relative importance of the agglutinin. Thus, the findings by Baudino *et al.* and others suggest that 37C-reactive IgM agglutinins may sometimes be clinically significant without complement activation being involved.

HOW DO NORMAL RBCS DIE?

Red cells survive for 100–120 days in the circulation. Every day about 2×10^{11} RBCs (about 40 mL) are removed from the circulation (Bocci, 1981). Why are they removed? As RBCs age, many changes occur (Table 5). The two most popular theories for their demise are that (a) a senescent cell antigen increases on band 3 of the RBC membrane as the RBC ages; (b) exposure of PS on the RBC membrane, which will react with the PS receptor on macrophages. Some have suggested that a form of apoptosis, termed erypoptosis, occurs (Bosman *et al.*, 2005, Lang *et al.*, 2005).

Many other mechanisms have been suggested and have been discussed extensively elsewhere (Bocci, 1981; Bratosin *et al.*, 1998; Arese *et al.*, 2005; Bosman *et al.*, 2005). Nevertheless, we really still do not know if one or more than one of these multiple suggestions is correct.

Table 5. Changes occurring as RBCs age

	Increased on old RBCs	Decreased on old RBCs
Senescent cell antigen	√	
Phosphatidylserine	√	
RBC-bound	√	
IgG/complement	√	
RBC sodium	√	
RBC volume		√
RBC lipid		√
RBC enzymes		√
RBC potassium		√
Sialic acid		√

WHAT IS A CLINICALLY SIGNIFICANT ANTIBODY?

If one asked an audience of blood bankers, haematologists, internists, pathologists and regulators (e.g. FDA) to define a clinically significant antibody or what they expect pretransfusion testing to achieve, I think you would get a very mixed response. Table 6 shows some of the responses you might get. It is not an easy question; it makes it easier for me if I focus on the word 'clinically'. To me, this should mean an antibody that is going to cause patient morbidity (e.g. following transfusion of blood that is incompatible *in vitro*). In the glossary of the Standards for Blood Banks and Transfusion Services (AABB, 2008), a clinically significant antibody is defined as an antibody that is capable of causing shortened cell survival. I do not like this definition for several reasons:

1 To satisfy this definition, the blood bank would have to perform ^{51}Cr-labelled RBC survival, or functional cellular assays, to determine if each antibody would cause decreased RBC survival in the recipient. Antibodies of any specificity (e.g. anti-D, -K, -Jk^a, -Fy^a, etc.) in a particular patient may not cause shortened RBC survival; as discussed previously, specificity is only one factor influencing pathogenicity. It is helpful, therefore, to always refer to antibodies as potentially clinically significant or having the potential to be clinically significant.
2 The definition does not refer to the clinical effect on the patient. For haematology patients, normal RBC survival is important, but for most patients, it would not matter if RBC survival was slightly diminished.

An example of why this AABB standard is too conservative for practical use concerns a standard that has been used by the FDA for decades, for evaluating any new product/process that might affect the unit of blood (e.g. new plastic containers, new anticoagulants and pathogen inactivation). The standard used is that 75% of the compatible RBCs should still be circulating after 24 h. This means that it is been acceptable for 25% of the RBCs to have shortened survival. It follows that if 4 units of blood (e.g. 42 days old) are transfused, then 1 whole unit could disappear within 24 h

Table 6. What is a clinically significant antibody?

Any detectable antibody?
Any antibody agglutinating or sensitizing RBCs at 37C?
Any antibody reacting by IAT?
Any antibody causing a readily discernable clinical reaction?
Any antibody causing laboratory signs of HA?
Any antibody causing decreased survival of transfused RBCs?

and still satisfy the FDA's standard. This would happen without any antibodies being involved. Yet, AABB standards want our pretransfusion testing to guarantee normal survival.

I much prefer the definition used by the British Committee for Standards in Haematology (Chapman *et al.*, 2004), 'Clinically significant antibodies are those that are capable of causing patient morbidity due to accelerated destruction of a significant proportion of transfused RBCs'. This is not perfect but is much more practical and of more value to blood transfusion services.

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