REVIEW

OPEN

Warm autoimmune hemolytic anemia: new insights and hypotheses

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Purpose of review
Warm autoimmune hemolytic anemia (wAIHA) is the most common of the immune hemolytic anemias. Although there are numerous case reports and reviews regarding this condition, some of the unusual and more recent findings have not been fully defined and may be contentious. This review will provide insight into the common specificity of the warm autoantibodies and hypothesize a novel mechanism of wAIHA, that is proposed to be linked to the controversial subject of red blood cell senescence.

Recent findings and hypotheses
It is now well established that band 3 on the red blood cell is the main target of autoantibodies in wAIHA. wAIHA targets the older red blood cells (RBCs) in about 80% of cases and, recently, it has been shown that the RBCs in these patients are aging faster than normal. It has been proposed that in these 80% of patients, that the autoantibody recognizes the senescent red blood cell antigen on band 3. It is further hypothesized that this autoantibody's production and potency has been exacerbated by hypersensitization to the RBC senescent antigen, which is processed through the adaptive immune system to create the pathogenic autoantibody. Recent publications have supported previous data that the senescent RBC antigen is exposed via a dynamic process, wherein oscillation of a band 3 internal loop flipping to the cell surface, creates a conformational neoantigen that is the RBC senescent antigen. It has also recently been shown that the cytokine profile in patients with wAIHA favors production of inflammatory cytokines/chemokines that includes interleukin-8 which can activate neutrophils to increase the oxidative stress on circulating RBCs to induce novel antigens, as has been postulated to favour exposure of the senescent RBC antigen.

Summary
This manuscript reviews new findings and hypotheses regarding wAIHA and proposes a novel mechanism active in most wAIHA patients that is due to an exacerbation of normal RBC senescence.

Keywords
aged RBCs, band 3, senescent red blood cell antigen, Type I wAIHA, warm autoimmune hemolytic anemia

INTRODUCTION

Warm autoimmune hemolytic anemia (wAIHA) is an autoimmune blood disorder whereby a patient produces an autoantibody to their own red blood cells (RBCs). It occurs at a rate of about 1 to 3 adults per 100,000 and can be idiopathic, without any known mechanism, or secondary to certain cancers, such as chronic lymphocytic leukemia, or drug administration, such as α-methyldopa or cefotetan [1,2▪▪,3▪,4]. More recently, wAIHA has been associated with use of checkpoint inhibitor therapies, such as anti-PD1 for treatment of certain cancers [5]. wAIHA is characterized by an acquired hemolytic anemia, sometimes life-threatening, a positive direct antiglobulin test (DAT) with gamma immunoglobulin (IgG), complement (C3d/g) or both [1]. The patient's eluate and serum contain antibodies to all RBCs without apparent subpopulation specificity. Historically, these autoantibodies have been associated with the Rh blood group system as they often do not react with rare Rh_{null} RBCs that lack Rh antigens [1]; although, there have been isolated reports of specific or mimicking antibodies to specific RBC antigens, such as D,

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KEY POINTS

- Warm autoantibodies primarily recognize band 3 and the older RBCs.
- Naturally present autoantibodies bind band 3 on older RBCs.
- wAIHA represents an exacerbation of the normal autoantibody-mediated red blood cell senescence.

Jk^{a,} E, Ge, Wr, that appear to resemble alloantibodies [1, see below].

SPECIFICITY OF AUTOANTIBODIES

Antibodies from patients with wAIHA react serologically with all RBCs that are tested and, historically, the autoantibody(ies) have been considered to have a wide variety of target specificities [1]. Early investigators determined that many of these autoantibodies failed to react with rare RBCs that lack Rh blood group antigens, such as Rh_{null} or, so-called ''dash-D-dash'' (-D-) RBCs [1]. Thus, warm autoantibodies were originally considered reacting with Rh antigens. Over the years, however, other reports of additional specificities for warm autoantibodies began to emerge [6–8], including Jk^a [1,9,10], Kell [1], Gerbich [11], Wright [12], glycophorin A [1,13–15], and the D and Lansteiner-Wiener (LW) antigens [1]. As more studies were undertaken to obtain a better understanding of the specificities of warm autoantibodies, it became apparent that band 3 on RBCs was involved and possibly a main target of these autoantibodies [13–15]. Subsequent publications began to support the idea that band 3 was a major target of warm autoantibodies [16–19]. It is now generally acknowledged that the main specificity of warm autoantibodies is to components of band 3 [19]. It is important to note that glycophorin A and Rh blood group antigens are linked to band 3, and that the Wright antigen lies on band 3 [20]. Loss of Rh antigens in Rh_{null} and/or -D- RBCs is known to have a conformational affect on the RBC membrane, resulting in an associated anemia. Thus, previous reports of warm autoantibody specificity to Rh antigens using Rh_{null} RBCs may have been due to an altered band 3 so that warm autoantibodies recognizing band 3 epitopes, particularly if conformational epitopes, failed to do so, giving the impression that the autoantibodies were directed to Rh rather than band 3.

BAND 3

Band 3 (AE1/SLC4A1) is a transmembrane anion exchange protein [21,22]. It works to exchange bicarbonate and chloride and is the most highly expressed RBC membrane protein. It functions in both structural and physiological capacities [22]. Band 3 provides cell mechanical support through its physical linkage to ankyrin and the cytoskeletal network [22–25]. Band 3's interaction with multiple proteins, including Rh, and in two specific complexes contributes to cellular morphology, conservation of cellular organization, and mechanical integrity [15,22–25]. Any changes in band 3's protein-protein interactions, such as Rh, could affect the band 3 ability to adopt some conformational states, including the autoantigen recognized by warm autoantibodies. Moreover, band 3 has been postulated to be a protein that can expose a neoantigen that becomes the senescent RBC antigen [26,27]; thus, band 3 is hypothesized to be responsible for RBC senescence [26,27,28▪▪]. However, this remains controversial (see below).

WARM AUTOANTIBODIES PREFERENCIALLY REACT WITH OLD RED BLOOD CELLS

In the 1980s, two different groups independently reported within one month of each other, on warm autoantibodies showing an association with the age of the RBCs [29,30]. Both groups reported that a significant proportion of the activity of warm autoantibodies from wAIHA patients reacted preferentially with older RBCs. Gray *et al.* [29] found that 4/5 (80%) of autoantibodies eluted from the RBCs of patients with wAIHA reacted weaker with reticulocyte-enriched compared to mature RBCs. They attributed this differential reactivity to the Rh system as they found that reticulocytes had less reactivity with anti-D than mature RBCs. Branch *et al.* [30] using a density gradient formulated to effectively separate reticulocytes from mature RBCs [30,31] found, in performing DATs on 24 patients with wAIHA, that 79% of patients had much less IgG on their autologous reticulocytes than on their mature, older RBCs. In 37% of patients, the DAT was negative in the reticulocyte-enriched fraction. They also showed that this differential reactivity was not due to Rh as they tested a number of different Rh antibodies on both the age-fractionated reticulocytes and older RBCs using normal healthy blood and found no differences in reactivity [31]. The autoantibodies that reacted better with reticulocyte-enriched RBCs were termed Type I and the patients as having Type I wAIHA. Those patients showing no difference or even slightly more reactivity with reticulocyte-enriched RBCs, were called Type II and patients as having Type II wAIHA. Other reports have supported these observations [1,19].

Recently, these two specificities of warm autoantibodies were confirmed in 22 patients having wAIHA using discontinuous Percoll gradients to separate reticulocyte-enriched vs. older RBCs [19]. These investigators also showed by Western blot and LC-MS that the warm autoantibodies were reacting with band 3 on the RBCs [19]. It has been suggested by these results that warm autoantibodies in the majority of patients having wAIHA may represent an exacerbation of normal RBC senescence [1,19,30].

RED BLOOD CELL SENESCENCE

Typically, RBCs live for approximately 115 to 120 days with a half-life of approximately 25 days [32,33▪▪]. These senescent RBCs are removed from circulation in the spleen or liver by macrophages; however, the exact mechanism of how these aged RBCs are removed by macrophages remains controversial [33▪▪]. Again, how RBCs die has been a subject of interest for many years and the mechanism remains a contentious topic [32,33▪▪,34▪▪,35▪▪]. In the 1980s, Marguerite Kay proposed that RBC senescence occurs due to band 3 changes in its external conformation during aging to create a neoantigen that is recognized by a naturally present autoantibody to this neoantigen [26,27,36,37]. This hypothesis has been supported by other investigators over the years [38,39]. Indeed, to date, this hypothesis as a mechanism of RBC senescence in humans has not been proven wrong; although, it has yet to be directly shown to be causative of RBC senescence, with a number of other hypotheses proposed [40]. In addition to the naturally present autoantibody to the RBC senescent antigen on band 3, other hypotheses include: (1) loss of cluster of differentiation-47 (CD47) don't eat me signal, (2) expression of phosphatidylserine (PS) on aged RBCs and (3) loss of sialic acid on aged RBCs, or a combination of these alone or in association with an autoantibody [40].

It is generally accepted that as RBCs age, they accumulate IgG on their membrane. What is not clear as yet is why? Proponents of the autoantibody to senescent RBC antigen hypothesis would argue that this increase in IgG as RBCs age is consistent with the hypothesis, while other proposed mechanisms, such as decreased CD47 or sialic acid, or increased PS exposure, have not been clearly shown to occur. Using animal models to study RBC aging *in vivo*, Singer *et al.* [41] showed that in mice, as RBCs age they accumulate IgG. Christian *et al.* [42] in dogs, using biotinylated autologous RBCs to follow the RBC lifespan, found that as the RBCs reach the limits of their lifespan (126 days) they accumulate greatly increased amounts of either IgG or complement [42]. In contrast, Hudson *et al.* [43] in mice that were deficient in either IgG or complement, showed that in these antibody/complement deficient animals that the mouse RBCs survived normally [43]; thus, did not support a mechanism of RBC senescent that involved autoantibody and/or complement [39]. The results of Hudson *et al.* [43] compared to Singer *et al.* [41] may simply indicate redundant pathways in mice for RBC senescence and/or differences in mouse strains. Mice have many differences in hematology and biochemistry compared to humans, indicating a high rate of age-dependent RBC clearance [44,45], and may not be a good model to study human RBC senescence [34▪▪,46]. Mouse RBC half-life is much shorter than in humans, mice have high retic counts compared to human, and have some nucleated RBCs in their peripheral blood, not found in humans [44–46].

Although animal models are often used to study human conditions, this question of the mechanism of RBC senescence is complicated by immediate removal of RBCs upon the end of their lifespan, making causative conclusions difficult. What is known in humans is consistent with accumulation of autologous IgG on aged RBCs [47]. Again, it isn't disputed that normal human RBCs accumulate IgG on their RBCs as they age, both *in vitro* and *in vivo* [1,36,47–49]. *In vivo,* support of a naturally present autoantibody to RBCs come from reports using normal human blood and specialized methods for the detection of IgG on the RBC membrane, such as Western immunoblotting [1,48]. Also, Franco *et al.* [49] using biotinylation of human RBCs found increased accumulation of autologous IgG at the end of the RBC lifespan [49]. *In vitro* aging of human RBCs in autologous plasma also results in accumulation of autologous IgG on the RBCs as they are stored, up to 60 days, allowing the IgG autoantibody to be eluted from the stored RBCs [35▪▪]. What is a problem with all of these studies is that there is no direct evidence that these autoantibodies are causative for the removal of the aged RBCs [34▪▪]. It is of note that the studies by Christian *et al.* [42] and Franco *et al.* [49], did not show increased PS exposure as the RBCs aged. Thus, PS exposure as a possible mechanism of RBC senescence is not supported by *in vivo* data and can be dismissed as a possible mechanism of RBC senescence. These investigators did not interrogate CD47 or sialic acid.

BAND 3 AND THE SENESCENT RED BLOOD CELL ANTIGEN

Studies mostly done by Marguerite Kay in the early 1980s investigating the mechanism of RBC senescence provided the first hypothesis that human RBC senescence was due to a naturally present

autoantibody directed to changes in membrane components of band 3 on aging RBCs [26,27, 36,37,50–55]. It was shown that the autoantibody binding was due to oxidative stress of the RBCs [56,57] Kay showed that the senescent RBC antigen had a molecular size between 55–65 kDa [37] and was comprised of two parts of the band 3 protein [53,54]. One peptide (peptide 1, aa 538–554) found on the 3rd external loop of band 3 and a second peptide (peptide 2, aa 812–830), which can access intracellular and extracellular environments [28▪▪,54–55] and, when expressed extracellularly, creates the neoantigen recognized by the autoantibody [28▪▪,55]. Furthermore, RBC aging and oxidation may favor formation of this neoantigen by influencing peptide 2's localization [28▪▪,58]. Support that both peptide 1 and peptide 2 are necessary for formation of the conformational RBC senescent antigen originally came from the work of Kay [53,54]. Recently, Badior and Casey [28▪▪] showed that peptide 2 was dynamic in its internal vs. external localization, and that it oscillates between internal and external location on the RBC membrane [28▪▪]. They hypothesized that the natural autoantibody to this conformational antigen would bind when both peptides were externally accessible and, as the peptide 2 was in a transient external state, autoantibody would bind and ''hold'' the conformational antigen until enough autoantibody accumulated to initiate the phagocytosis of the senescent RBC via activating Fc receptors on macrophages [28▪▪].

TYPE I WARM AUTOIMMUNE HEMOLYTIC ANEMIA ANTIBODY REACTS WITH BAND 3 RED BLOOD CELL SENESCENT ANTIGEN

It has been shown that wAIHA produces two types of warm autoantibodies, Type I that recognize older RBCs compared to reticulocyte-enriched RBCs, and Type II that recognize both younger and older RBCs equally [19,29,30]. Patients with Type I warm autoantibodies have autologous RBCs that appear to be aging faster than normal or Type II patients [19]. This hypothesis is based on the band 3 phosphorylation and discontinuous Percoll gradient patterns of Type I compared to Type II [19]. Type I patients show increased band 3 phosphorylation on lower density RBCs as well as enrichment of older (higher density) RBCs on Percoll gradients while Type II patients show little or no phosphorylation on band 3 and few to no low-density RBCs on Percoll gradients [19]. Band 3 phosphorylation is a hallmark of aged RBCs [59,60]. Investigators have suggested that these results may show that Type I wAIHA represents an exacerbation of normal RBC senescence [1,19,30,49]. Exacerbated aging of human RBCs has been previously reported *in vitro* due to human erythrocytes exposed to d-galactose [61▪▪]. Whether dysregulation of galactose metabolism [62▪▪] plays a role in the increased *in vivo* aging in Type I wAIHA requires further investigation.

We have unpublished data that shows that eluates from Type I wAIHA patients recognize band 3 in Western immunoblotting and specifically react with a band 3 fragment between 55–65 kDa (Fig. 1A), the molecular size consistent with the RBC senescent antigen proposed by Kay [37]. We have also used synthetic peptides spanning band 3 aa 538–554 and aa 812–830 that when used together can significantly inhibit the Western blot reactivity of an eluate from a patient having Type I wAIHA (Fig. 1B, C). Investigators have suggested that the RBC senescent antigen is comprised of a conformational antigen made up of these two amino acid sequences on band 3 [28▪▪,53,54]. Also, Petz and Garratty [1] reveal in their book, ''Immune Hemolytic Anemias'' that they were able to inhibit warm autoantibodies that reacted preferentially with aged RBCs (Type I wAIHA) with a synthetic peptide, derived from band 3, that was provided by Dr Marguerite Kay that represented the red blood cell senescent antigen [1]. These results led them to agree with previously published work [30] that Type I wAIHA is the pathogenic result of normal RBC senescence, which would be similar to pathogenic cold agglutinin autoantibodies [1]. Whether Type I wAIHA is a pathogenic version of normal RBC senescence remains to be proven; however, with RBCs perhaps aging faster in these patients, able to present increased amounts of senescent antigen resulting in increased autoantibody response to the increased stimulation, it is possible.

CYOTKINE PROFILE IN WARM AUTOIMMUNE HEMOLYTIC ANEMIA

A recent publication has examined the cytokine/chemokine profile in 54 patients having wAIHA [63▪▪]. These investigators confirmed previous reports of increases in production of TNFα and IL-10. Of note was their novel findings of two cytokine/chemokines increased in their study cohort. Both interleukin-8 (IL-8/CXCL8) and interferon gamma-inducible protein (IP10/CXCL10) were significantly increased. Both, along with TNFα, are biomarkers of inflammation. What is of interest in relationship to this review is that IL-8/CXCL8 can recruit and activate neutrophils. Activated neutrophils can produce reactive oxygen species (ROS), such as O_2^-, H_2O_2 or OH^-, which could result in RBC oxidative stress and increased RBC aging [64▪▪,65▪▪,66,67]. Excessive ROS production may affect membrane

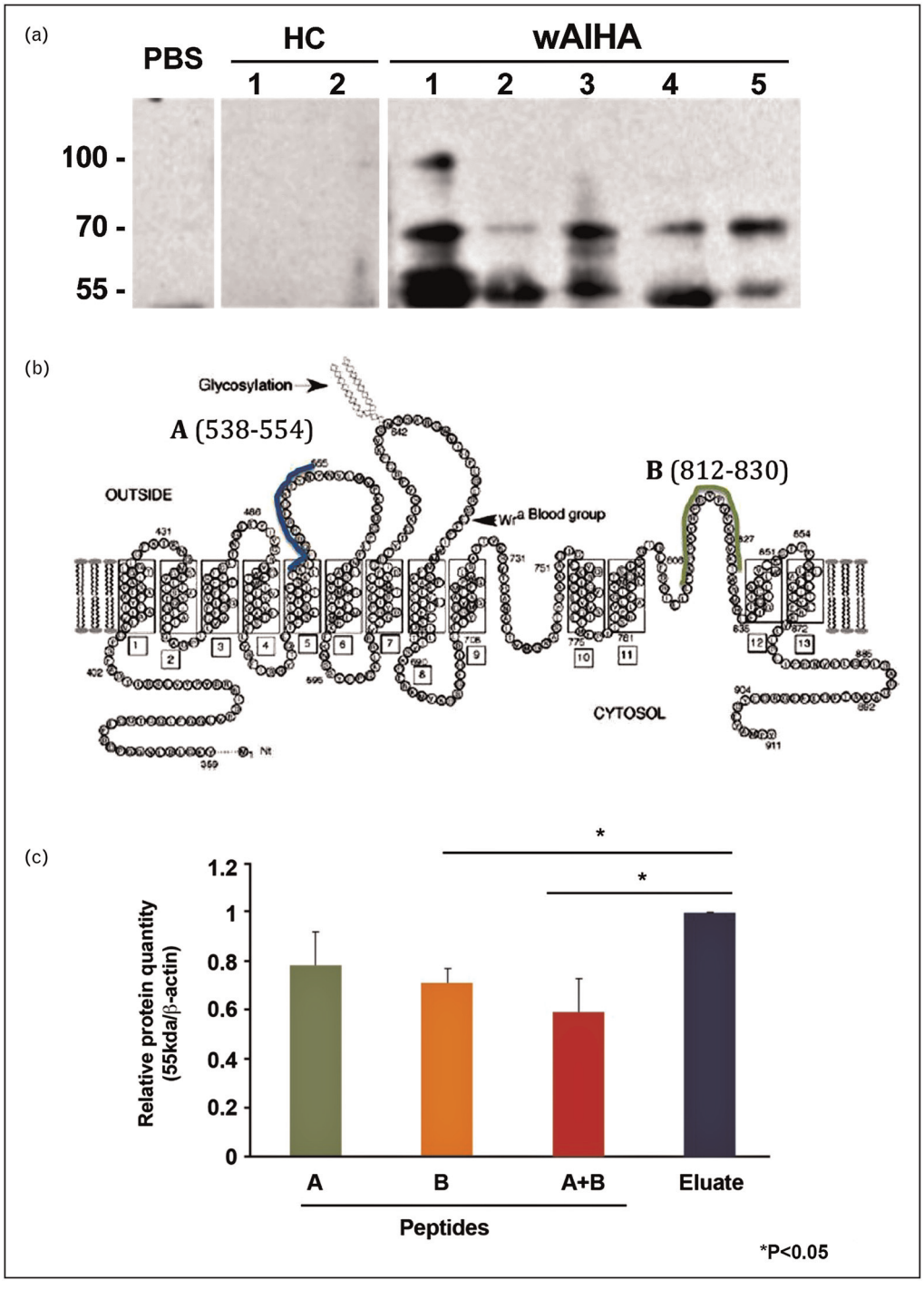

FIGURE 1. Warm autoantibodies from Type I wAIHA recognize 55–65 kDa band 3 fragment and can be inhibited by peptides proposed to form the senescent RBC antigen. (A) RBC ghosts were lysed and run on 10% sodium dodecyl sulfate polyacrylamide electrophoresis gel. Gel was transferred to nitrocellulose membrane and probed with PBS, eluates from 2 healthy controls (HC) and eluates from five patients with Type I wAIHA. (B) Schematic diagram of band 3 and the two peptides, A and B, proposed to be able to form the senescent RBC antigen (Modified from Zhu Q, Lee D W, Casey J R [69]. (C) Peptides A and B were used to inhibit the warm autoantibody used in the Western blot. Results represent the ratio of the 55 kDa band 3 result compared to beta-actin with either peptide A or B alone or in combination, compared to eluate without any inhibition (Eluate). $^{*}p < 0.05$. HC, healthy control; PBS, phosphate buffered saline; wAIHA, warm autoimmune hemolytic anemia.

lipids, integrins and cytoplasmic proteins in various circulating cells, including RBCs [66,67]. These effects are particularly critical for RBCs, which may become dysfunctional. First, excess ROS can cause oxidation of polyunsaturated fatty acids in the RBC membrane, bringing about a profound modification of the membrane lipids' lateral and transversal distribution and organization at the nanoscale level [63▪▪,64▪▪,65▪▪,66]. This may contribute to band 3 oxidative stress resulting in increased display of senescent RBC antigen and help to explain the increased aging of RBCs in wAIHA. Indeed, Iuchi *et al.* [67] have reported an increase in autoimmune hemolytic anemia in NZB mice under conditions of oxidative stress, and suggested that oxidation-mediated RBC autoantibody production is a direct result of ROS-mediated oxidative stress. This observation is consistent with the hypothesis that human RBCs under oxidative stress, which results in increased aging and presentation of conformational senescent RBC neoantigen, results in faster aging of the RBCs and development of Type I wAIHA.

CONCLUSION

The published findings implicating a naturally occurring autoantibody directed to a conformational antigen on band 3 that is responsible for red blood cell senescence is substantial and compelling. However, for the most part it is circumstantial and not as yet shown to be causative. It is a conundrum that is difficult to figure out how to overcome. If autoantibody is responsible for removal, one can conceive of circulating RBCs accumulating the autoantibody recognizing band 3-associated senescent antigen, reaching a threshold of opsonization, and being removed from the circulation [28▪▪]. There is no dispute that either *in vitro* or *in vivo* aged RBCs accumulate IgG autoantibody, while evidence so far shows that PS is not increased as RBCs age and no evidence for CD47 or sialic acid involvement. This leaves the hypothesis that RBC senescence is most likely due to autoantibody recognizing a neoantigen on the aged RBCs. However, *in vitro* density gradients would not provide aged RBCs that present the maximum senescent antigen as those RBCs would have already been removed after reaching a threshold of antibody sensitization as previously described [28▪▪]. *In vivo* models are required to settle this controversy. Biotinylation of human RBCs has proven that the oldest RBCs have the most IgG detectable on the cells but do not provide proof that removal is antibody-mediated via Fc receptors on macrophages. Perhaps using biotinylation of dog RBCs as previously reported [42], that have a similar lifespan, 116–125 days, as human RBCs could be isolated and used in *in vitro* phagocytosis assays to determine if monocyte-macrophages can recognize these opsonized cells and phagocytose them. This testing could also be done in humans with autologous enriched for the oldest IgG-opsonized RBCs. Type I wAIHA may represent a highly potent version of the normal autoantibody to senescent RBC antigen but this is currently speculation. It is known that Type I wAIHA autoantibody results in phagocytosis, both *in vitro* and *in vivo* [68]. Perhaps if Type I warm autoantibodies could be used to isolate and purify the antigen recognized and this be shown to be similar to peptides 1 and 2 reported by Kay [54] and Badior and Casey [28▪▪], this would be helpful. Until more studies can be done in humans, the controversy over how RBCs die will continue.

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Conflicts of interest

There are no conflicts of interest.

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- ▪ of special interest
- ▪▪ of outstanding interest

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