Clinical severity in adult warm autoimmune hemolytic anemia and its relationship to antibody specificity

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Warm autoimmune hemolytic anemia (wAIHA) is a rare autoantibody (autoAb)-mediated immune disorder that affects both children and adults.[1-3] The pathology of the disease is caused by immunoglobulin G (IgG)-, IgM, or IgA-type autoAbs associated, or not, with molecules of the complement system, and directed against self-erythrocytes. Red blood cell (RBC) destruction is induced by activation of FcγR-bearing effector cells, mainly in the spleen after sequestration and trapping by splenic macrophages.[4-6] The most common RBC-antigen targets in wAIHA are major RBC membrane proteins, but few studies have investigated the clinical or biological relevance of such specificities[7,8] even though anti-Band 3 Abs have long been postulated to induce more severe hemolysis.[9]

In the present study, we determined the specificity of Ig autoAbs of wAHAI and explored whether there is a correlation between the specificity of the three-main autoAbs against membrane RBC proteins and the severity of the disease in a cohort of wAIHA patients. We also investigated the functional properties of eluted autoAbs using functional *in vitro* assays. We prospectively explored 52 samples sequentially obtained from 40 patients (24 women/16 men, mean age: 56 years [range: 21-98]) treated for AIHA in the French National Referral Center for adult immune cytopenias. Twenty-seven patients had primary wAIHA and 13 had secondary. Samples were obtained either recently after diagnosis (n=10), during (n=9), or after treatment (n=33). A full description of the cohort and of the methods are described in *Online Supplementary Material*. Investigations of patient RBCs included the direct antiglobulin test (DAT), antibody screening tests, and searches for antibodies in serum. IgM-type antibodies were never found alone on patient RBCs, but were mixed with other Abs in eight samples (15.4%, sequentially collected from four patients). Three patient samples (5.8%) were C3d positive alone, and the DAT was negative for four (7.7%). We found IgA autoAbs (always mixed with IgG) in seven patients (see also *Online Supplementary Table S1*). We tested the autoAbs obtained from the 29 patients with a positive DAT (*Online Supplementary Table S2*). Antibody specificities, deduced from negative reactions with specific antigen-null RBCs,[5,10] were Rhesus (RH) for 11 patients (monospecific for six), Band 3 for eight (monospecific for six), Glycophorin A (GPA) for seven (no monospecific), and unknown for three. Analysis of the patient samples sequentially collected during the follow up resulted in no significant changes in Ab specificity and only slight changes of the Ig-type classes and sub-classes (data not shown). We performed acidic elution of the autoAbs from patient RBCs. Thirty-five percent of the eluates contained several classes and sub-classes of antibodies. Forty-four samples (84.6%) contained IgG-type antibodies, mainly the IgG1 isotype (n=36; 69.2%), either alone (n=18; 34.6%) or mixed with other Abs, in contrast to IgG3 (n=8, 15.4%), which was always mixed with IgG1. The autoAbs demonstrated a large thermic range (as titers were similar when tested at +37°C or +20°C), and the titers were from 0 to 1024. Eluates were diluted to a final titer of between 8 and 128 for further studies; eluates with very low autoAbs titers (titer < 8) were excluded from subsequent investigations (11/52 eluates).

We first compared the relative clinical management of the patients as a function of autoAb specificity, assessed by immuno-hematological testing (*Online Supplementary Table S2*). There were no significant differences between the three autoAb specificity categories, nor for the three unknown eluate specificities, among the 29 patients, based on hemoglobin (Hb) levels at the time of diagnosis, the nadir of Hb counts during the course of the disease, or the number of lines of therapy, which is an indirect marker of the severity of wAIHA (Figure 1).

We conclude that specificity against RBC membrane antigens is not associated with the severity of the disease. Phagocytosis of autoAb coated RBCs in wAIHA may be the principal pathogenic mechanism underlying RBC destruction, except under certain conditions in which macrophages simply remove clusters of cell-bound IgG on RBCs coated with autoAbs; this phenomenon is called "antigen-shaving" or trogocytosis.[11,12] The testing of phagocytic reactivity of monocytes *in vitro* with Ab-coated RBCs is a useful parameter for evaluating *in vivo* immune RBC destruction in the diagnosis of AIHA or evaluation of the patient response to treatment.[13] For this test, normal adherent monocytes were overlaid onto RBCs, sensitized, or not, with eluted autoAbs and incubated for 2 hours at 37°C. Slides were then observed

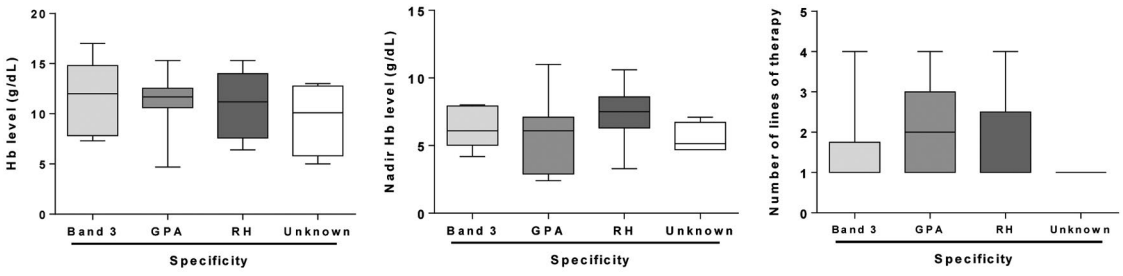

Figure 1. Parameters for clinical management of AIHA patients from the cohort as a function of autoAb specificity based on the results obtained from *in vitro* cellular investigations. Clinical management of the patients as a function of autoAb specificity. Hb levels of patients at the time of diagnosis (on the left), the nadir of Hb (in the center), and the number of lines of therapy (on the right) are indicated and represented as Boxes/Whiskers (min to max values); the horizontal bar in each box indicates the median. GPA: Glycophorin A; RH: Rhesus.

under a light microscope for the assessment of RBC phagocytosis and adhesion. Phagocytosis by monocytes was highly variable among the eluates tested (Figure 2A). The tested antibodies demonstrated a very large spectrum in their capacity to induce phagocytosis by CD14$^+$ monocytes: up to 50-60%, and even higher for seven autoAbs, almost higher than that of the positive controls of the test (Figure 2B). The phagocytic index of five individual eluates (one with specificity to GPA and two to RH) were very low, near or below (n=3) the baseline cut-off (Figure 2C). This variation may be due to varying IgG levels in the eluates as well as varying affinities of IgG for RBCs even though the eluates were assessed at comparable dilutions. We observed a similar range of variation for autoAbs against RH, Band 3, and GPA. The data compiled from 25 eluates showed a trend, although not significant (R^2=0.1143; *P*=0.0983), towards a correlation between phagocytic activity and hemoglobin levels (Figure 2D). As expected using this test, the functional activity of anti-RBC Abs correlated with Hb levels (although not significantly within our cohort).[13]

We evaluated erythro-trogocytosis (i.e., RBC trogocytosis) induced by autoAbs in eluates to explore the cellular contacts and directed membrane exchanges between RBCs and monocytes *in vitro*. Trogocytosis by monocytes was quantified using normal erythrocytes, pre-stained with the lipophilic dye PKH-67, followed by their sensitization with autoAbs, and then incubation with PBMCs. Synaptic membrane transfer was assessed as the acquisition of fluorescence from PKH-67-labeled RBCs by specific leukocyte subsets. This assay is thus more sensitive than the phagocytosis assay with monocytes, but less stringent.[4,11,12] Although the trogocytosis assay may have some limitations in assessing functional properties of Abs, it has been reported to predict the clinical consequences of immune blood cell destruction *in vivo*.[5]

We confirmed significant membrane transfer between non-sensitized donor RBCs and monocytes incubated together for 60 min (MFI of 2,311 for non-sensitized RBCs at T60 *vs.* MFI of 56 for T0, Figure 3A). Nevertheless, RBCs pre-coated with autoAbs induced a substantial level of membrane exchange with CD14$^+$ monocytes, which varied among the tested eluates, independently of the specificity of the autoAb (Figure 3B). The results of the CD14$^+$ trogocytosis experiments are represented as a MFI index ratio corresponding to the MFI for Ab-coated-RBCs/MFI for non-sensitized RBCs following incubation. There was no correlation between the results of monocyte-mediated RBC trogocytosis and either the Hb level of the patients (R^2=0.051; *P*=0.2679) or phagocytic index (R^2=0.0388; *P*=0.3454; *Online Supplementary Figure S1A, S1B*). We also found no significant correlation between anti-RBC Ab specificity and two mechanisms of RBC destruction measured by in vitro assays: phagocytosis and erythro-trogocytosis by CD14$^+$monocytes.

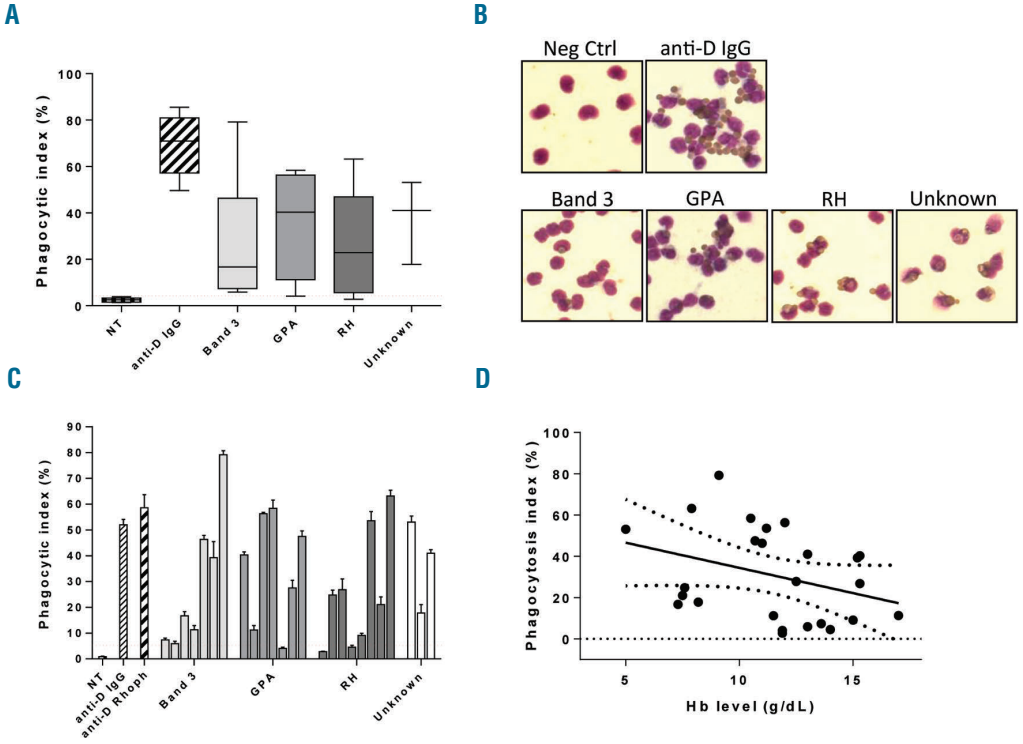

Figure 2. Determination of the phagocytosis index of monocytes against eluate-sensitized RBCs, as a function of autoAb specificity. (A) Reactivity of monocytes after incubation with normal RhD$^+$ human RBCs, either non-sensitized (NT), sensitized with an IgG directed against-RhD (anti-D IgG; positive control), or the eluates. The reactivity of monocytes (%) is indicated as a function of RBC treatment from at least three independent experiments; the horizontal bar in each box indicates the median. (B) Typical results for phagocytosis assays obtained after observation under a light microscope, reported as a function of autoAb specificity. (C) Phagocytic index for monocytes, showing results for each eluate used to sensitize RBCs. A positive control was also included using the polyclonal IgG anti-RhD rhophylac® (anti-D Rhoph) to sensitize RBCs. Results are shown as the means ± SD from at least three independent experiments. Values >5% were considered to be positive (dashed red line). (D) Correlation of the results between the Hb levels and the phagocytic index (R^2=0.1143; *P*=0.0983). GPA: Glycophorin A; RH: Rhesus.

In a previous study, we used a polyspecific Fc-Blocker to confirm the lack of involvement of Fc-receptors (FcR) in monocyte-mediated trogocytosis by Ig autoAbs.[4] We quantified the effect of the Fc-Blocker as well as that of anti-FcγRI/CD64 and anti-FcγRIII/CD16 mAbs on the autoAb-induced erythro-trogocytosis by monocytes (*Online Supplementaly Figure S2A, S2B*). AutoAb-activated CD14$^+$ trogocytosis was markedly reduced by pre-incubation of the monocytes with the Fc-Blocker (Figure 3C; see also *Online Supplementaly Figure S1C* for the representation of the inhibition index obtained for each specificity). Nevertheless, RBC trogocytosis was not totally abolished by a pan Fc-blocking mixture, with persistent intracellular PKH-67 staining detected in CD14$^+$ monocytes (Figure 3D); we also observed this result using higher concentrations of the pan Fc-Blocker (five-fold tested; *data not shown*).[4] This suggests that RBC membrane transfer to monocytes might contribute to RBC destruction by a mechanism other than classical Fc-mediated phagocytosis because intracellular RBC components were still observed after Fc-Blocker pretreatment. It has been shown that SIRPα also plays a role in the removal of aged RBCs through CD47 binding.[14] The exact mechanism explaining this observation remains to be investigated.

We also assessed antibody-dependent cell-mediated cytotoxicity (ADCC) of PBMC subsets towards sensitized-RBCs through degranulation using transient CD107a exposure at their cell surface.[15] Only very low levels of CD107a staining were detected on CD3$^-$CD16/56$^+$ natural killer (NK) cells for most of the autoAbs tested (n=25). We also assessed ADCC of CD3$^+$CD16/56$^+$ natural killer T (NKT) cells and CD3$^+$/CD8$^+$ T cells, which was negative (data not shown). Most values obtained for NK cells were just above or below the estimated baseline cutoff (*Online Supplementaly Figure S3A*). Two autoAbs induced weak CD107a MFI in NK cells and two others (one specific to GPA, the other to Band 3) triggered modest ADCC activity (above the baseline cutoff) for NK immune effector cells. Thus, the wAIHA autoAbs tested only weakly triggered the cytotoxic process of immune effector cells. Again, there was no correlation between Hb levels and ADCC (R^2=0.0236; *P*=0.4630; *Online Supplementaly Figure S3B*).

Taken together, our data demonstrate that the ability of anti-RBC AutoAbs to induce either phagocytosis, trogocytosis, and to a lesser extent, ADCC in wAIHA is not related to their antigen specificity. They also suggest that

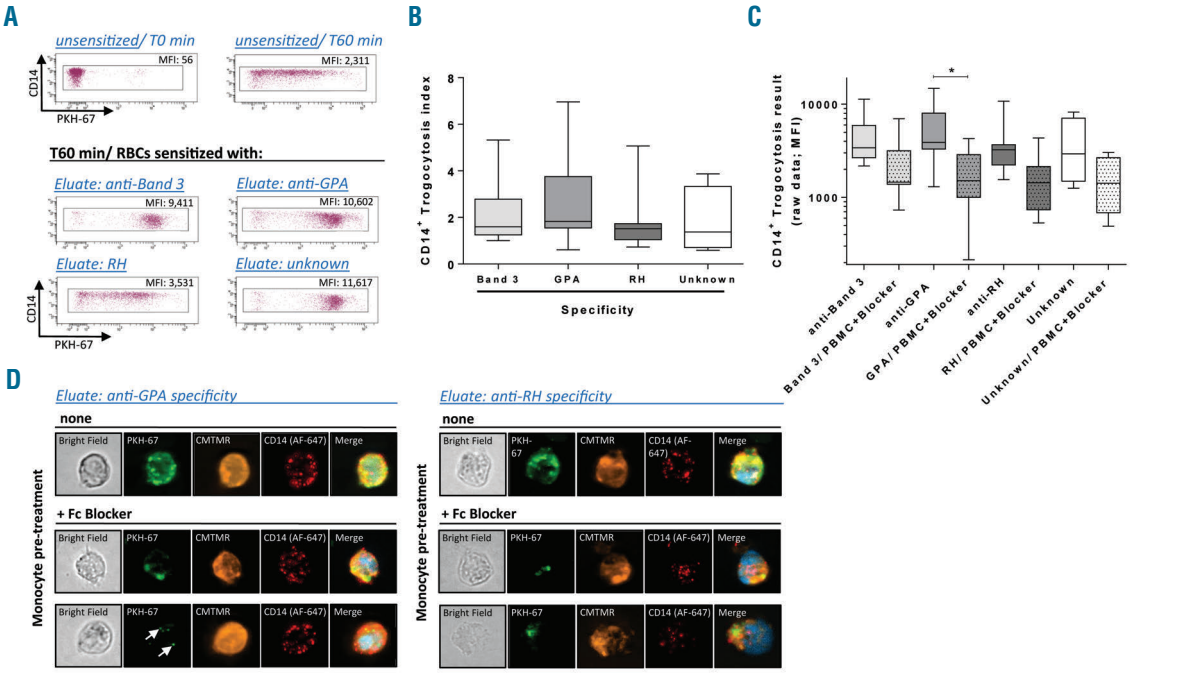

Figure 3. CD14$^+$-mediated trogocytosis and blocking with anti-FcγR blocking antibodies or a polyspecific Fc Blocker, with autoAb-coated RBCs. For erythro-trogocytosis experiments, PKH-67-labeled normal RBCs were incubated with PBMCs for 60 min. Spontaneous directed membrane transfer between RBCs and the gated CD14$^+$ monocyte population was quantified before (T0 min) and after coculture (T60 min), visualized as the acquisition of PKH-67 fluorescence by CD14$^+$ monocytes. (A) Results for trogocytosis obtained by flow cytometry with normal RBCs sensitized or not with the eluted autoAbs and incubated with monocytes for 60 min; monoclonal IgG anti-RhD served as positive control. Data were collected on a BD Canto 2 flow cytometer. Values of the MFI of gated populations is indicated. Similar results were obtained in at least two other experiments. (B) Results for the erythro-trogocytosis index visualized in monocytes as the ratio (PKH-67 CD14$^+$ MFI for eluate-sensitized RBCs)/ (PKH-67 CD14$^+$ MFI for non-coated RBCs). Results are shown as the means ± SD from at least three independent experiments; the horizontal bar in each box indicates the median. (C) Results for erythro-trogocytosis obtained by flow cytometry and expressed as raw data (PKH-67 CD14$^+$ MFI) with normal RBCs sensitized with the eluted autoAbs and then incubated for 60 min with monocytes treated or not with the pan Fc-Blocker. Results are from at least three independent experiments and represented as Boxes/Whiskers (min to max values); the horizontal bar in each box indicates the median. Results were only significant for specificity for GPA (*P*=0.0358; ANOVA with the Kruskal-Wallis multiple comparison test). (D) Fluorescence microscopy results for erythro-trogocytosis results by fluorescence microscopy with RBCs sensitized or not with the eluted autoAbs and then incubated with monocytes. Results for an anti-GPA eluate and an anti-RH eluate are shown from one representative experiment. Normal RBC membranes were stained with the lipophilic dye PKH-67 (green); PBMCs were loaded with CMTMR (orange) to visualize the cytoplasm of nucleated cells. RBCs and PMBS were then incubated together for 60 min at +37°C. The monocytes were then stained with Alexa-Fluor (AF)-647 coupled (red) mIgG anti-CD14 and mounted with a DAPI-containing mounting medium. Data were collected on a Zeiss Observer Z1 microscope and analyzed using Zen software. GPA: Glycophorin A; RH: Rhesus.

clinical severity may be dependent on the functional activity of the anti-RBC antibodies. Further studies are needed to investigate the activation state of the patients' immune cells, which control the level of RBC destruction by phagocytosis and erythro-trogocytosis, as a part of the complex pathogenesis of the disease.

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**PC and VL contributed equally to this work.*

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