A comprehensive diagnostic algorithm for direct antiglobulin test-negative autoimmune hemolytic anemia reveals the relative ratio of three mechanisms in a single laboratory

Toyomi Kamesaki*, Eiji Kajii

Center for Community Medicine, Jichi Medical University, Tochigi, Japan

Running title: New classification and diagnostic algorithm for DAT-negative AIHA

*Corresponding author: Toyomi Kamesaki, Center for Community Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
TEL: +81-28-544-2111; FAX: +81-28-544-4902; E-mail: kmskt@jsicht.ac.jp

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Abstract

Background: Direct antiglobulin test (DAT)-negative warm autoimmune hemolytic anemia (AIHA) is mainly caused by three mechanisms: red blood cell (RBC)-bound immunoglobulin (Ig)G below the detection limit of routine DAT; RBC-bound IgA or IgM; or low-affinity autoantibodies. Although most cases of DAT-negative AIHA are thought to be caused by RBC-bound IgG, and combinatory serological analyses are recommended, relative ratios of each mechanism have not been clarified. Methods: Two groups of patients with undiagnosed hemolytic anemia and negative conventional tube method DAT (TM-DAT) were investigated using anti-IgA and anti-IgM sera, or column agglutination method DAT (CM-DAT), respectively, in addition to radioimmunological quantitation of RBC-bound IgG. Results: Three of 73 patients with DAT-negative AIHA showed positive RBC-bound IgA and normal amounts of RBC-bound IgG. Another 3 patients were RBC-bound IgM-positive, but only one of these showed normal amounts of RBC-bound IgG. In another group of patients with DAT-negative AIHA, 4 of the 20 showed positive CM-DAT and negative CM-DAT after washing RBCs. Three of these patients had normal amounts of RBC-bound IgG. Five patients with positive CM-DAT both before and after washing RBCs had high amounts of RBC-bound IgG. Conclusion: Relative ratios of patients with DAT-negative AIHA resulting from RBC-bound IgG, RBC-bound IgA, RBC-bound IgM and low-affinity IgG were estimated as about 80%, 4%, 1% and 15%, respectively. A new classification and diagnostic algorithm for DAT-negative AIHA were proposed.
Introduction

The direct antiglobulin test (DAT), which can detect red blood cell (RBC)-bound immunoglobulin (Ig)G and complement, remains the most important serological assay for diagnosing autoimmune hemolytic anemia (AIHA) [1, 2]. However, 5-10% of patients with AIHA display negative DAT findings, representing ‘DAT-negative AIHA’ [3-5]. Most of these patients carry a certain amount of IgG molecules per RBC, displaying negative routine tube-method DAT (TM-DAT) findings but in vivo hemolysis [6]. We have previously proposed a cutoff value for the diagnosis of DAT-negative AIHA of 78.5 IgG molecules per RBC if RBC-bound IgG is measured before treatment [7]. As most antiglobulin sera used routinely for DAT do not contain anti-IgA or anti-IgM [1], some patients with DAT-negative AIHA have RBC-bound IgA or IgM autoantibodies and the clinical and hematological features of AIHA. When routine DAT is performed, RBCs are washed 3-4 times in large volumes of saline at room temperature, and low-affinity autoantibodies can be lost during this wash phase of TM-DAT [1]. However, such low-affinity autoantibodies are maintained after washing using cold saline or low ionic strength solution (LISS) [1]. A column agglutination method DAT (CM-DAT) that requires no washing procedure can detect low-affinity autoantibodies [8]. Although most cases of DAT-negative AIHA are thought to be caused by RBC-bound IgG, and combinatory serological analyses are recommended for diagnosis [1], the relative ratios of these mechanisms in patients with DAT-negative AIHA have not been exactly determined, partly because cases involving these mechanisms are relatively uncommon and serological data and clinical diagnoses are thus difficult to accumulate in a single laboratory. The aim of this study was thus to estimate relative ratios of patients with DAT-negative AIHA resulting from RBC-bound IgG, RBC-bound IgA or IgM, or low-affinity IgG autoantibodies in a single laboratory and to construct a comprehensive diagnostic algorithm using radioimmunological quantitation of RBC-bound IgG, CM-DAT and TM-DAT.
Patients and methods

This study consisted of two investigations undertaken during periods from 2009 to 2012 and from 2014 to 2015 at the Laboratory of the Center for Community Medicine of Jichi Medical University following approval by the Institutional Ethics Panel Committee. Informed consent was obtained from all individual participants included in the study. Anti-IgA or IgM sera have been commercially available in Japan since 2009, and a compact CM-DAT system has been used in our laboratory since 2014.

Testing for IgA or IgM autoantibodies

During the 3-year period from 2009 to 2012, a total of 628 patients from all over Japan were referred to our laboratory for quantification of RBC-bound IgG. Of these, 268 patients had undiagnosed hemolytic anemia (HA) and negative results from conventional TM-DAT performed at the referring hospital. We assessed these patients by TM-DAT using anti-IgG sera and anti-C3b+3d antibodies (Ortho Diagnostics, Rochester, NY), and anti-IgA and anti-IgM sera (Medion Grifols Diagnostics AG, Düdingen, Switzerland) according to the instructions from the manufacturers, and RBC-bound IgG levels were also measured using the radioimmunological procedures described below. To avoid detecting cold autoantibodies in TM-DAT, RBCs were washed 8 times using room-temperature phosphate-buffered saline (PBS; pH 7.0, 0.15 M).

Testing for low-affinity IgG autoantibodies

During the 7-month period from September 2014 to March 2015, 140 patients consulted our laboratory. Of these, 96 patients had undiagnosed HA and negative TM-DAT in our laboratory. We assessed these cases using CM-DAT containing dextran gels and anti-IgG antisera (Kainos Laboratories, Tokyo, Japan and...
Medion Grifols Diagnostics AG, Dübening, Switzerland), before and after washing RBCs with PBS. RBC-bound IgG levels were measured using the radioimmunological procedures mentioned below. Low-affinity IgG was verified by positive CM-DAT, negative CM-DAT after washing with PBS and positive CM-DAT after washing with LISS. To avoid detecting cold autoantibodies in CM-DAT, blood samples were incubated at 37 °C for 30 min.

Radioimmunological quantitation of RBC-bound IgG

Radioimmunological quantitation of RBC-bound IgG was performed in accordance with a previous report [7]. Briefly, whole-blood (5-ml) samples were taken with ethylene-diamine-tetra-acetic acid anticoagulation. Whole blood was centrifuged at 130×g for 10 min to prepare the RBCs. Supernatant and buffy coat were thrown away. Next, 200-µl samples of RBCs were washed twice with 14 ml of PBS and diluted in 14 ml of PBS. RBCs were passed through a cotton-wool column to eliminate neutrophils and monocytes [9]. RBCs were washed 4 times with 14 ml of PBS, then were suspended in 0.2 ml of PBS containing 2% bovine serum albumin (BSA). Anti-human IgG antibodies affinity-isolated from goat (Sigma-Aldrich Japan, Tokyo, Japan) were labeled with 125I (PerkinElmer Japan, Yokohama, Japan) using IODO-GEN (Pierce, Rockford, IL) as per the instructions from the manufacturer. Next, 125I-labeled goat anti-human IgG antibodies were diluted in PBS containing 2% BSA (Wako, Osaka, Japan) with a specific activity of 1,000-5,000 cpm/200 µl. A volume of 200 µl of washed RBCs was incubated for 1 h at 37 °C with 100 µl of diluted anti-human IgG. Toyo affinity beads (TOYOPEARL AF-Tresyl-650M; Tosoh Bioscience, Griesheim, Germany) were conjugated with human IgG (Sigma-Aldrich Japan) as per the instructions from the manufacturer. Next, 100 µl of a suspension of IgG beads was added to the mixture of 125I-labeled anti-human IgG and RBCs, and incubated at 37 °C for 30 min. RBCs were lysed by the addition of 120 µl of 20% Triton X-100 (Sigma-Aldrich Japan). These beads were washed 3 times with 6% Triton X-100, and radioactivity was measured using a gamma counter (Hitachi Aloka Medical, Tokyo, Japan). A standard curve was made using human IgG standards (5-40 ng IgG/ml; Takara Bio, Shiga, Japan). Percentage inhibition of binding was marked against each concentration of IgG and a fitting curve was made using the Fit Spline command of JMP for Macintosh version 12.2.0 statistical software (SAS Institute, Cary, NC) to extrapolate the concentration of IgG from the percentage inhibition of binding for each sample. After the number of RBCs was quantified, RBC-bound IgG levels were
calculated using the following formula: RBC-bound IgG level (IgG molecules/RBC) = 370 × \[\text{IgG} \times (\text{ng/ml}) \div (\text{number of RBCs/0.02μl})\]. Each attending physician was provided with the RBC-bound IgG level and specific DAT results within 5-10 days of placing the order.

Clinical diagnosis and clinical course survey

One year after consulting our laboratory, follow-up surveys with the attending physician were performed to evaluate the clinical diagnosis and course. The clinical diagnosis of HA was made by each attending physician using in vivo hemolysis (low hemoglobin concentration, high lactate dehydrogenase level, high indirect serum bilirubin level, low haptoglobin level, high percentage of reticulocytes, and/or high erythropoiesis level in bone marrow) and exclusion of other icteric anemic diseases without hemolysis, such as myelodysplastic syndrome, megaloblastic anemia, congenital dyserythropoietic anemia, erythroid leukemia, hepatobiliary diseases, and constitutional jaundice. AIHA was clinically diagnosed by each attending physician using DAT, steroid-reactivity, and exclusion of alloimmune HA and drug-induced HA on the basis of clinical history and course, with reference to the RBC-bound IgG value [2, 10].

Statistical analysis

We compared distributions of categorical variables among groups using the \(\chi^2\) test or Fisher’s exact test. We also analyzed the normality of laboratory continuous variables using the Kolmogorov-Smirnov test with Lilliefors significance correction. Most variables did not display normal distributions, so the Mann-Whitney U-test was used to determine differences between groups. Statistical significance was defined as a two-sided P value < 0.05. All statistical computations were calculated using JMP for Macintosh version 12.2.0 statistical software (SAS Institute, Cary, NC).
Results

DAT-negative AIHA resulting from IgA or IgM autoantibodies in the 2009-2012 study (Fig. 1)

A total of 199 surveys were returned for analysis (response rate, 74.3%). Mean age of participants was 51.1±26.6 years (range, 0-91 years), and 54.1% of participants were female. No significant differences in age or sex were evident between patients who did and did not reply (50.1±26.9 years vs. 53.5±25.9 years; 55.7% female vs. 50.0% female, respectively).

Among the 199 patients, 99 were clinically diagnosed with AIHA by the attending physician, comprising 12 patients with AIHA and positive DAT and 87 patients with AIHA and negative DAT results. Of the 87 patients with AIHA and negative DAT, 3 patients showed paroxysmal cold hemoglobinuria (PCH) with positive DAT for C3, 11 patients had cold agglutinin disease (CAD) (7 positive and 4 negative DAT for C3) and 73 patients had warm AIHA (DAT-negative AIHA, 14 positive and 59 negative DAT for C3). Three patients (4%) with DAT-negative AIHA were RBC-bound IgA-positive (one positive and 2 negative DAT for C3) and all had amounts of RBC-bound IgG under the cutoff value (78.5 IgG molecules/RBC) (Table 1). Another 3 patients (4%) with DAT-negative AIHA were RBC-bound IgM-positive. Two of 3 patients showed positive DAT for C3 and amounts of RBC-bound IgG over the cutoff value, but only 1 patient (1%) showed negative DAT for C3 and amounts of RBC-bound IgG under the cutoff value, suggesting the possibility of AIHA resulting from IgM, although low-affinity IgG could not be denied (Table 1). At the 1-year follow-up, all 3 patients with IgA autoantibodies were alive, but 2 of the 3 patients with IgM autoantibodies had died.

DAT-negative AIHA resulting from low-affinity IgG autoantibodies in the 2014-2015 study (Fig. 2)

Sixty-five surveys were returned for analysis (response rate, 67.7%). The mean age of participants
was 57.3±23.9 years (range, 1-88 years), and 50.0% of participants were female. No significant differences in age or sex were evident between patients who did and did not reply (56.1±23.1 years vs. 59.8±25.6 years; 53.9% female vs. 46.2% female). Twenty-six patients were clinically diagnosed by the attending physician as showing AIHA with negative DAT results. One of these patients had PCH with negative DAT for C3, 5 patients had CAD (2 positive and 3 negative DAT for C3) and 20 patients had warm AIHA (DAT-negative AIHA, 6 positive and 14 negative DAT for C3), including 7 with Evans syndrome. Of the patients with DAT-negative AIHA, 9 had positive CM-DAT results and 11 had negative CM-DAT results (i.e., ‘both TM- and CM-DAT (DATs)-negative AIHA’) before washing RBCs. After washing RBCs with PBS, negative CM-DAT results were obtained from 4 (20% of patients with DAT-negative AIHA) of the 9 initially positive patients (Table 2). Three (15% of patients with DAT-negative AIHA) of these 4 patients with negative CM-DAT results post-washing had RBC-bound IgG levels under the threshold value (78.5 IgG molecules/RBC), indicating a loss of RBC-bound IgG during the washing of RBCs and the initial presence of low-affinity IgG autoantibodies. RBCs in these 4 patients were confirmed to maintain positive result to both TM- and CM-DAT after washing using LISS. The remaining one (5%) of the 4 patients with negative CM-DAT results post-washing demonstrated amounts of RBC-bound IgG over the cutoff value, indicating loss of RBC-bound IgG during RBC washing, but also that RBCs were holding high-affinity IgG autoantibodies after washing (i.e., ‘both DATs-negative AIHA with low-affinity IgG autoantibodies’) (Table 2). All four patients showed negative DAT for C3 and were alive 1 year later. Five (25%) of 9 patients with positive CM-DAT remained positive after washing RBCs with PBS, and all five patients (i.e., ‘tube DAT-negative AIHA’) had amounts of RBC-bound IgG over the cutoff value, meaning that CM-DAT was more sensitive than TM-DAT, and patients with DAT-negative AIHA might decrease in the era of CM-DAT, but will not disappear. Two patients who maintained
positive CM-DAT results after washing with PBS and demonstrated normal levels of RBC-bound IgG (33±13 IgG molecules/RBC [7]) were clinically diagnosed with non-AIHA (myelodysplastic syndrome and HA of unknown origin). The ratio of primary and secondary AIHA in ‘tube DAT-negative AIHA’ or ‘both DATs-negative AIHA’ was 3/2 (systematic lupus erythematosus (SLE)), or 5/6 (3 idiopathic thrombocytopenic purpura, rheumatoid arthritis, SLE and acute lymphoblastic leukemia-post- peripheral blood stem cell transplantation), respectively, representing almost the same ratio (about 50%) seen in DAT-positive AIHA [1].

The discrepancy between the diagnoses and the DAT results provided for patients with cold AIHA

In the Results for the 2009-2012 study there were 11 patients diagnosed with CAD but 4 of these had no C3 detected by the DAT. And of 5 patients diagnosed with CAD in the 2014-2015 study, 3 did not have C3 detected by the DAT. Similarly, in the 2014-2015 study, a patient with PCH had no RBC-bound C3. Both CAD and PCH are characterized by RBC-bound C3 plus a high thermal amplitude, high titer cold agglutinin or positive Donath-Landsteiner Test, respectively. The diagnoses provided for these patients are inconsistent with the DAT results provided. This discrepancy could result from a retrospective review one year later.

A comprehensive classification and diagnostic algorithm of DAT-negative AIHA

On the basis of the above-mentioned data for DAT-negative AIHA, we propose a diagnostic algorithm and comprehensive classification for DAT-negative AIHA (Table 3). This classification is based on RBC-bound IgG levels and findings from CM-DAT both before and after washing RBCs.
Discussion

Our results estimated relative ratios of patients with DAT-negative AIHA resulting from RBC-bound IgA, IgM, low-affinity IgG autoantibodies or RBC-bound IgG autoantibodies under the threshold level of positive DAT as approximately 4%, 1%, 15% and 80%, respectively. Approximately 20% of patients with DAT-negative AIHA could not be diagnosed using RBC-bound IgG levels alone and 45% of TM-DAT-negative AIHA patients showed positive CM-DAT. CM-DAT is therefore recommended when TM-DAT-negative AIHA is suspected. Indeed, a previous report [11] by our research collaborators, which investigated 50 patients with suspected AIHA, revealed that CM-DAT was more sensitive than TM-DAT and that the threshold levels of RBC-bound IgG were about 100 and 200 IgG molecules/RBC, respectively.

In addition, our results suggested that a combination of radioimmunological quantitation of RBC-bound IgG and CM-DAT can classify DAT-negative AIHA resulting from RBC-bound IgG into four groups: ‘tube DAT-positive AIHA’; ‘both DATs-negative AIHA’ (so-called ‘DAT-negative AIHA’); DAT-negative AIHA resulting from low-affinity IgG autoantibodies; and ‘both DATs-negative AIHA with low-affinity IgG autoantibodies’ (Table 3). Such classification would decrease patients with undiagnosed HA and allow estimation of prognoses for each group.

Estimating the prevalence of each mechanism of DAT-negative AIHA is difficult, because patients with DAT-negative AIHA are scarce and sufficient serological data for clinical diagnosis cannot be accumulated in a single laboratory. In Japan, our laboratory is a unique reference laboratory for serological testing of AIHA, including the quantitation of RBC-bound IgG since 2006, and patients with AIHA (specifically, DAT-negative AIHA) were thus recruited in our laboratory, allowing follow-up with these patients to generate a database of clinically diagnosed immune HA and to reveal characteristics [12] and diagnostic cutoff values for DAT-negative AIHA [7].

Approximately 0.03-2% of patients with suspected warm-type AIHA reportedly have RBC-bound IgA only [13, 14], representing a much smaller proportion compared to our data in the present study, potentially due to the low rates of clinically diagnosed AIHA patients in those studies. The prevalence of patients with DAT-positive warm AIHA and RBC-bound IgM has been reported as 8%, but none of those patients had
IgM alone; 2% had RBC-bound IgM plus RBC-bound C3 (no IgG or IgA was detected) [1]. This 2% is slightly more than suggested in DAT-negative warm AIHA by our results. This variation is most likely because most RBC-bound IgM can directly agglutinate RBC or activate complement, yielding a “positive DAT”, and because monomeric IgM or incomplete activation of complement may cause a “negative DAT” [1]. Regarding severity and prognosis, patients with AIHA resulting from RBC-bound IgA reportedly display comparable severity and prognosis to patients with AIHA resulting from RBC-bound IgG, but the rare warm AIHA patients with RBC-bound IgM reportedly show more severe symptoms and worse prognosis than AIHA patients with RBC-bound IgG [1]. In our study, one patient showed detection of RBC-bound IgM but no IgG, IgA and C3, and showed good prognosis at 1 year after testing. This could be explained by the presence of low-affinity IgG autoantibodies not checked in our first investigation, suggesting the extreme rarity of pure IgM-type DAT-negative AIHA.

Positive results from TM-DAT were reportedly obtained from 5% of patients with DAT-negative AIHA if tested after washing RBCs using cold saline [14], and these patients show positive DAT results using the column agglutination method [15], again consistent with our results and supporting our proposal to identify low-affinity autoantibodies using pre- and post-washing column DAT. Lai et al. [15] reported negative DAT by routine tube test, but positive CM-DAT in a patient with clinically significant AIHA. However, Leger et al. [14] obtained a positive DAT for 37 (8.6%) of 431 patients with suspected DAT-negative AIHA when tested after washing RBCs using cold LISS, and two-thirds of their patients with positive TM-DAT after washing using cold LISS showed negative findings from gel testing. Although only a small number of patients were included in our study, we detected 4 patients (20%) with low-affinity IgG autoantibodies among 20 patients with DAT-negative AIHA and 3 (15%) of these 20 patients displayed low-affinity IgG alone, suggesting that the CM-DAT method does have a use in our setting. We also encountered another
patient with low-affinity IgG autoantibodies who showed positive CM-DAT after washing RBCs using LISS, but negative TM-DAT (data not shown).

However, some limitations must be considered for the present study. First, the clinical diagnosis and course of patients were retrospectively evaluated through the attending physicians 1 year after receiving the blood samples, which might have resulted in some unreliable data and selection bias for subjects included in our database. Second, samples sent to our laboratory were stored at 4°C for several days, which might have contributed to discrepancies in DAT results between our laboratory and those of the attending physicians.

In conclusion, our data revealed relative ratios of patients with DAT-negative AIHA resulting from RBC-bound IgG, RBC-bound IgA or IgM, or low-affinity autoantibodies, and we proposed a new classification and diagnostic algorithm for DAT-negative AIHA to assess the characteristics and prognosis of each group in future studies.

Acknowledgments

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Ethical Prerequisites

All procedures performed were in accordance with the ethical standards of the institutional and with the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996. Informed consent was obtained from all individual participants included in the study.

Disclosure Statement
The authors have no conflicts of interest to disclose.
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**Figure legends**

**Figure 1.** Testing for immunoglobulin (Ig) A or IgM autoantibodies and direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (AIHA) resulting from IgA or IgM autoantibodies.

PCH: paroxysmal cold hemoglobinuria, CAD: cold agglutinin disease, RBC: red blood cell

**Figure 2.** Testing for low-affinity immunoglobulin (Ig) G autoantibodies and direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (AIHA) resulting from low-affinity IgG autoantibodies.

PCH: paroxysmal cold hemoglobinuria, CAD: cold agglutinin disease, RBC: red blood cell
DAT: direct antiglobulin test, AIHA: autoimmune hemolytic anemia, Hb: hemoglobin, Reti: reticulocytes, WBC: white blood cells, Plt: platelets, RBC-Ig: red blood cell-associated immunoglobulin, RA: rheumatoid arthritis, PSL: adrenal cortical steroid (prednisolone), N.T.: not tested, *: over the cutoff value (78.5 IgG molecules per red blood cell) [7]

### Table 1. Six cases of DAT-negative AIHA with IgA or IgM autoantibodies

<table>
<thead>
<tr>
<th>Age years</th>
<th>Gender</th>
<th>Hb g/dl</th>
<th>Reti $\times 10^4$ µl</th>
<th>WBC /µl</th>
<th>Plt $\times 10^4$ /µl</th>
<th>Tube method DAT</th>
<th>RBC-bound IgG molecules/RBC</th>
<th>Cold Agglutinin titer</th>
<th>Comorbidity</th>
<th>Treatment/Response</th>
<th>1 year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>male</td>
<td>4.6</td>
<td>25.6</td>
<td>16220</td>
<td>44.6</td>
<td>-</td>
<td>-</td>
<td>3+</td>
<td>2+</td>
<td>225*</td>
<td>64</td>
</tr>
<tr>
<td>89</td>
<td>male</td>
<td>8.0</td>
<td>27.3</td>
<td>9200</td>
<td>29.7</td>
<td>-</td>
<td>-</td>
<td>3+</td>
<td>+</td>
<td>81*</td>
<td>32</td>
</tr>
<tr>
<td>62</td>
<td>female</td>
<td>10.2</td>
<td>32.8</td>
<td>11130</td>
<td>31.6</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>53</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>female</td>
<td>6.5</td>
<td>22.0</td>
<td>6270</td>
<td>26.2</td>
<td>-</td>
<td>-</td>
<td>3+</td>
<td>-</td>
<td>70</td>
<td>64</td>
</tr>
<tr>
<td>80</td>
<td>male</td>
<td>11.6</td>
<td>29.2</td>
<td>11920</td>
<td>17.3</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>83</td>
<td>male</td>
<td>4.4</td>
<td>19.5</td>
<td>7060</td>
<td>10.5</td>
<td>-</td>
<td>-</td>
<td>2+</td>
<td>-</td>
<td>43</td>
<td>32</td>
</tr>
</tbody>
</table>

DAT: direct antiglobulin test, AIHA: autoimmune hemolytic anemia, Hb: hemoglobin, Reti: reticulocytes, WBC: white blood cells, Plt: platelets, RBC-Ig: red blood cell-associated immunoglobulin, RA: rheumatoid arthritis, PSL: adrenal cortical steroid (prednisolone), N.T.: not tested, *: over the cutoff value (78.5 IgG molecules per red blood cell) [7]
Table 2. Four cases of DAT-negative AIHA with low-affinity IgG autoantibodies

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Hb (g/dl)</th>
<th>Retic ($\times 10^4/\mu l$)</th>
<th>PL (×10^4/μl)</th>
<th>Tube method DAT-IgG/IgA/IgM/C3</th>
<th>Column agglutination method DAT-IgG</th>
<th>Post-washing Column agglutination method DAT-IgG</th>
<th>RBC-bound IgG molecules per RBC</th>
<th>Diagnosis</th>
<th>Comorbidity</th>
<th>Treatment</th>
<th>1-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>male</td>
<td>7.7</td>
<td>4.4</td>
<td>20/9</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>109*</td>
<td>both DATS-negative AIHA with low-affinity IgG</td>
<td>Chronic kidney disease</td>
<td>No treatment</td>
<td>alive</td>
</tr>
<tr>
<td>69</td>
<td>female</td>
<td>6.8</td>
<td>4.1</td>
<td>17/0</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>71</td>
<td>Drug-induced liver dysfunction</td>
<td>No treatment</td>
<td>alive</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>female</td>
<td>7.8</td>
<td>13.5</td>
<td>7.2</td>
<td>-</td>
<td>1+</td>
<td>-</td>
<td>41</td>
<td>AIHA resulting from low-affinity IgG</td>
<td>AML-post-BMT</td>
<td>No treatment</td>
<td>unknown transfer to other hospital</td>
</tr>
<tr>
<td>47</td>
<td>female</td>
<td>7.9</td>
<td>13.4</td>
<td>9.3</td>
<td>-(&lt;\text{N.T./N.T.}&gt;=)</td>
<td>2+</td>
<td>-</td>
<td>14</td>
<td>Antiphospholipid antibody syndrome</td>
<td>PSL/effective</td>
<td>alive</td>
<td></td>
</tr>
</tbody>
</table>

DAT: direct antiglobulin test, AIHA: autoimmune hemolytic anemia, Hb: hemoglobin, Retic: reticulocytes, PL: platelets, Ig: immunoglobulin, AML: acute myelocytic leukemia, BMT: bone marrow transplantation, PSL: adrenal cortical steroid (prednisolone), both DATS: both tube method- and column agglutination method-DATS, N.T.: not tested, *: over the cutoff value (78.5 IgG molecules per red blood cell) [7]
Table 3. New classification and diagnostic algorithm for direct antiglobulin test (DAT)-negative autoimmune hemolytic anemia (AIHA) using a combination of radioimmunological quantitation of red blood cell (RBC)-bound immunoglobulin (Ig)G, tube method-DAT (TM-DAT) and column agglutination method DAT (CM-DAT).

<table>
<thead>
<tr>
<th>Classification for DAT-negative AIHA</th>
<th>TM-DAT</th>
<th>CM-DAT</th>
<th>CM-DAT after washing RBCs using PBS</th>
<th>RBC-bound IgG</th>
<th>Relative ratio in TM-DAT-negative AIHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>tube DAT-negative AIHA</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>over cut-off value</td>
<td>25% (n=5)</td>
</tr>
<tr>
<td>both DATs-negative AIHA</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>over cut-off value</td>
<td>55% (n=11)</td>
</tr>
<tr>
<td>so-called ‘DAT-negative AIHA’</td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
<td>under cut-off value</td>
<td>15% (n=3)</td>
</tr>
<tr>
<td>resulting from low-affinity IgG autoantibodies</td>
<td>negative</td>
<td>positive</td>
<td>negative</td>
<td>over cut-off value</td>
<td>5% (n=1)</td>
</tr>
<tr>
<td>both DATs-negative AIHA</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>over cut-off value</td>
<td>*</td>
</tr>
<tr>
<td>with low-affinity IgG autoantibodies</td>
<td>negative</td>
<td>positive</td>
<td>positive</td>
<td>over cut-off value</td>
<td>*</td>
</tr>
</tbody>
</table>

tube DAT-negative AIHA: TM-DAT-negative and CM-DAT-positive AIHA, both DATs: both TM- and CM-negative DAT, PBS: phosphate buffered saline, LISS: low ionic strength solution, *: Positive TM-DAT using anti-IgA or anti-IgM sera was detected in 4% or 4% of patients with TM-DAT-negative AIHA, respectively.
Fig. 1

Total patients with negative tube method DAT at the referring hospitals 2009-2012 (n=268)

- Unreturned for analysis (n=69)
- Returned for analysis (n=199)

Clinically diagnosed as non-AIHA (n=100)

Tube method DAT positive using anti-IgG (n=12)

- Clinically diagnosed as PCH (n=3)
- Clinically diagnosed as CAD (n=11)

Clinically diagnosed as AIHA (n=99)

Tube methods DAT negative using anti-IgG (n=87)

- Clinically diagnosed as warm AIHA (n=73) ‘DAT-negative AIHA’

DAT positive using anti-IgA (n=3(4.1%))

- RBC-bound IgG<threshold Value (n=3(4.1%))

DAT positive using anti-IgM (n=3(4.1%))

- RBC-bound IgG<threshold Value (n=1(1.4%))
Total patients with negative tube method DAT using anti-IgG in our laboratory 2014-2015 (n=96)

- Unreturned for analysis (n=31)
  - Clinically diagnosed as non-AIHA (n=39)
    - Clinically diagnosed as PCH (n=1)
    - Clinically diagnosed as CAD (n=5)
      - Column agglutination method DAT negative using anti-IgG (n=11(55.0%)) ‘both DATs-negative AIHA’
      - Clinically diagnosed as warm AIHA (n=20) ‘DAT-negative AIHA’
    - Clinically diagnosed as warm AIHA (n=20) ‘DAT-negative AIHA’
  - Returned for analysis (n=65)
    - Clinically diagnosed as AIHA (n=26)
      - Column agglutination method DAT positive using anti-IgG (n=9(45.0%))
      - Post-washing column agglutination method DAT positive using anti-IgG (n=5(25.0%)) ‘tube DAT-negative AIHA’
    - Post-washing column agglutination method DAT negative using anti-IgG (n=4(20.0%))
      - RBC-bound IgG<threshold Value (n=3(15.0%)) ‘AIHA resulting from low-affinity IgG autoantibodies’

Fig. 2