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# Direct Anti-Globulin Test and Clinical Diagnosis

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Takeshi Sugimoto

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## Abstract

This chapter focuses on anti-red blood cells antibody and anti-globulin test. The relationship between warm or cold antibody and hemolysis is explained. Direct anti-globulin test (DAT) is a useful clinical examination tool on the diagnosis of autoimmune hemolytic anemia (AIHA); however, false positive or negative results are sometimes detected. This chapter shows the disposition about the surroundings of the IgG antibody in DAT examination. In addition, this chapter contains pointing issue on the diagnosis of AIHA. Some pitfalls about diagnostic AIHA are presented from our experienced cases. To diagnose the background diseases such as lymphoproliferative disorders or autoimmune diseases under the analysis of secondary AIHA is important.

**Keywords:** direct anti-globulin test, autoimmune hemolytic anemia, secondary AIHA, complement activity, autoimmune diseases, lymphoproliferative disorders

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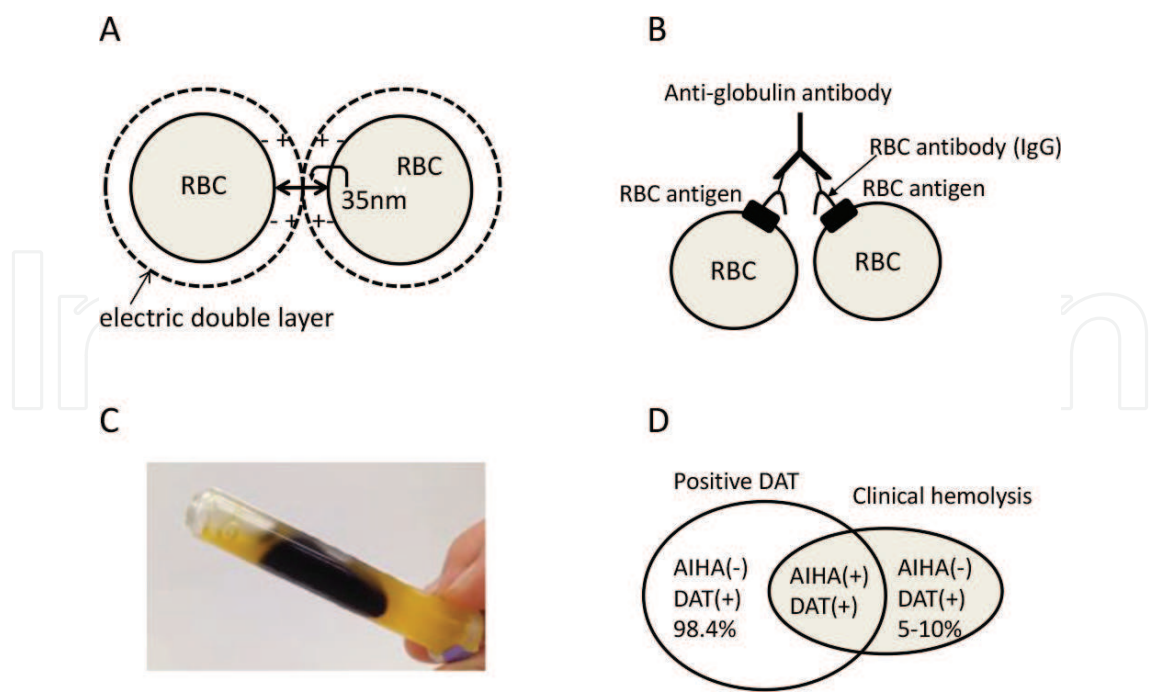
## 1. Introduction

Anti-globulin test is one of the standard examinations in clinical laboratory. This test is predominantly used in hematology area, transfusion medicine, or organ transplantation area. The positive status of anti-globulin test expresses the existence of antibody against the protein related to the blood type; however, the clinical application of this test is various. The potentiality of anti-globulin test is introduced in this chapter.

## 2. Anti-red blood cells antibody

Anti-red blood cells antibody is the antibody against the protein related to the blood type on the surface of red blood cells. Anti-red blood cells antibody is composed of autoantibody

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**Figure 1.** (A) The distance between RBCs is 35 nm. The surroundings of each RBCs obtain electric double layer. (B) The principle of coombs test, IgG-type antibodies bound to RBCs are united by anti-globulin antibody (Coombs antibody) with bridge-bindings. (C) Macroscopic aggregation in cold agglutinin disease (CAD). Aggregation part is floating on the plasma. (D) Schematic model about the relationship between direct anti-globulin test (DAT) and hemolysis. Only few percentage cases among positive DAT reach to hemolysis. In clinical AIHA, 5–10% of cases show negative of DAT. The figure is modified from original manuscript Ref. [1].

produced against self-red blood cells, and alloantibody produced against red blood cells of other persons. Antibodies classified according to thermal condition, warm antibody reacts to red blood cells (RBCs) mostly at around 37°C, and cold antibody reacts mostly at low temperature around 4°C. The main immunoglobulin antibody classes related to reaction to RBCs are IgG, IgA and IgM, in each of which light (L) chain has two types, that is, k and λ. Generally, IgM antibody is able to agglutinate by itself by reacting to RBCs (complete antibody). However, IgG antibody is not able to agglutinate alone (incomplete antibody) and requires activation of complement component to develop the agglutination. The capacity of binding of IgG antibody to complement is different among the IgG subclass, in which IgG3 followed by IgG1 is strong. In this context, high affinity of IgG antibody binding to complement brings about strong agglutination and hemolysis with activation of complement. **Figure 1B** shows a schematic model of the reaction with RBCs and anti-globulin.

### 3. Anti-globulin test

Anti-globulin test is a tool detecting RBCs which IgG antibody or complement bind to, and this test was developed by Coombs in 1945. There are two methods for anti-globulin test: direct anti-globulin test (DAT) and indirect anti-globulin test (IAT). DAT determines the existence of

IgG antibody or complement binding to RBCs antigen and IAT determines the existence of any IgG antibody reacting with RBCs antigen in the serum (or blood plasma). In the DAT method, anti-globulin antibody reagent (Coombs reagent) makes crosslink between IgG binding RBCs together, resulting in RBCs agglutination (**Figure 1A** and **B**). There are two kinds of anti-globulin antibody reagent: one is multispecific Coombs reagent reacting to any of IgG antibody, C3b or C3d component, and the other is mono-specific Coombs reagent reacting to only one component of them. When DAT shows positive result using multispecific Coombs reagent, the type of antibody will be determined by DAT using mono-specific Coombs reagent. Because the universal multispecific Coombs reagent is the cocktail reagent against IgG, C3b and C3d component, the antibody of IgA or IgM type is unable to be detected.

In clinical practice, DAT is useful for investigating AIHA, neonatal hemolytic disease, drug-induced immune hemolytic anemia (DIHA), and hemolytic transfusion reactions. DAT is also used for identifying the membranous protein sensitizing RBCs. IAT method is useful for irregular antibody examination, cross-match test for transfusion, mother's serum (blood plasma) examination in neonatal hemolytic disease, and identification of antibody eluted samples from antibody binding RBCs.

## **4. Autoimmune hemolytic anemia**

### **4.1. Warm antibody**

Warm antibody exerts to bind to RBCs mostly at around 37°C. Complement component will deposit on the surface of RBCs in activating warm antibody. The main detected protein by DAT is IgG antibody and complement component. The sensitized RBCs by warm antibody are recognized and received phagocytosis by hepatic and splenic macrophage, leading to cause extravascular hemolysis. Macrophage recognizes IgG antibody via IgG-Fc receptor (FcγR), in the subclass of IgG antibody IgG3 and IgG1 have strong affinity to macrophage. Therefore, IgG3 followed by IgG1 antibody are related stronger to causing hemolysis than IgG2 and IgG4 antibody. It is another mechanism of phagocytosis that macrophage recognizes and perform phagocytosis via C3b receptor protein deposited on the surface of RBCs. Warm antibody causes intravascular hemolysis, where IgM antibody reaction or antibody-dependent cell mediated cytotoxicity (ADCC) is involved. In clinical laboratory, warm antibody usually shows pan-hemagglutination character and tends to agglutinate all irregular antibody test panels. The main auto-antigens corresponding to warm antibody are Rh, band 3 and glycophorin A (GPA) protein [2, 3]. Blood-type specific warm auto-antibody (anti-E, -c, -e, -Kell, -Jk, -Ii, etc.) is detected in some of AIHA cases.

### **4.2. Cold antibody**

Cold antibody exerts to bind to RBCs at close to 0°C. The activation of complement components is possible to be proceeded on above 12°C. If the cold antibody binds to RBCs on the thermal range of complement activation, then intravascular hemolysis will occur. It is known

that there are two types of cold antibodies, namely cold agglutinin and Donath-Landsteiner antibody (DL antibody), each of which develops cold agglutinin disease (CAD) and paroxysmal cold hemoglobinuria (PCH), respectively. Each disease is divided into primary (idiopathic) and secondary type.

The immunoglobulin class in cold agglutinin (cold antibody) is mostly IgM type, and this single IgM antibody can activate C1 component at low temperature. If the cold agglutinin binds to erythrocyte in below body temperature, blood circulatory status is disturbed by intravascular hemolysis and hemagglutination. Circulatory disturbance emerges as pain and acrocyanosis in the extremities. As blood flows back to the central trunks from extremities, cold agglutinin leaves from RBCs. However, complement remains to be bound to RBCs, and complement cascade activates sequentially developing into intravascular hemolysis. On the other hand, RBCs escaped from hemolysis is going to receive hemophagocytosis in the reticuloendothelial system in the liver via remaining complement components C3b and C3d on RBCs, developing into extravascular hemolysis. Reflecting the abovementioned reason, DAT shows positive for complement component and negative for IgM antibody in CAD. Cold agglutinin usually has the specificity for Ii blood-type antigen. In clinical laboratory about CAD, RBCs have characteristics of self-aggregation on blood smear sample and macroscopic aggregation on sample tubes which disappear in warm condition (**Figure 1C**). Low titer CAD is known to be a subtype of the CAD, in which clinical manifestation accompanied by RBCs agglutination is not due to the quantity of cold agglutinin but to the activating thermal range of it. Mixed type AIHA is other subtype related to cold agglutinin.

PCH occupies in 1.7–10% of the hemolytic anemia. PCH comes from pediatric infectious diseases (measles, varicella, mumps, influenza, etc.) and adult idiopathic type in recent years. When patient exposes to cold circumstances, intravascular hemolysis appears attackingly several minutes to hours later. Patient raises hemoglobinuria, fever, abdominal and limb's pain. PCH is caused by DL antibody, which has the character of polyclonal IgG-type antibody and of strong hemolysin in low titer level. DL antibody reacts with C1 component in cold condition and called the biphasic hemolysin. DL antibody has the specificity for P blood-type antigen. During and immediately after the attacks of PCH, DAT with complement components shows positive. IAT test under cold condition shows positive with IgG antibody. The titer of serum complement becomes decreased due to consumption.

## 5. Relationship between DAT and hemolysis

DAT is a useful clinical examination tool at the diagnosis of AIHA during more than 70 years. Although RBCs autoantibody is observed at around 7% of all hospitalized patients [4], most of DAT positive cases indeed have no hemolysis [5]. The intensity of DAT reaction is generally related to the degree of hemolysis. Some AIHA cases show DAT negative status and other non-AIHA cases show DAT positive status (**Table 1**) (**Figure 1D**). Therefore, we need to consider whether the presence of RBCs autoantibody actually works for hemolysis or not and to discriminate the background disease in independent case. Also, in the diagnosis of



**False positive result**

- Hypergammaglobulinemia
- Intravenous immunoglobulin administration
- Anti-phospholipid antibody
- Infections
- Technical problem

**False negative result**

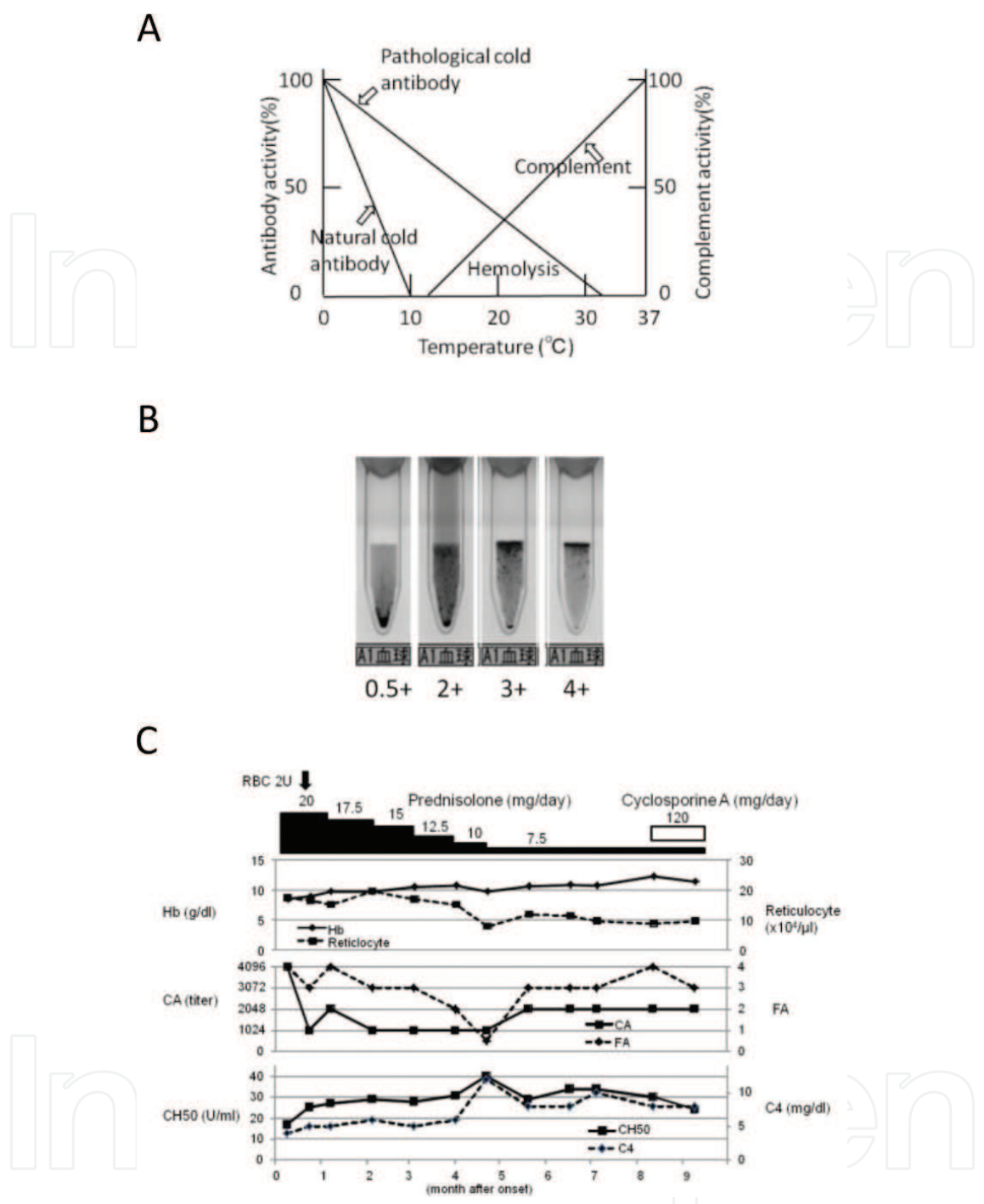
- Hemolysis caused by IgA or IgM antibody
- Below sensitivity level of amounting RBC-bound IgG antibody
- Low-affinity IgG antibody
- Technical problem

**Table 1.** Main causes of false positive and false negative results in direct anti-globulin test.

DAT negative AIHA, we should differentiate non-AIHA status like as mechanical hemolysis or thrombotic microangiopathy [6].

The following is the pointing condition about the occurrence of AIHA in clinical condition:

1. The amount of IgG antibody on RBCs. The situation of suspecting AIHA strongly by symptom irrespective of DAT negative status, Coombs negative AIHA should be considered. Autoimmune hemolysis can occurred in some condition even if the amount of IgG antibody on RBCs is below the detection limit of positive DAT, and 5–10% of all AIHA cases fall into this category [7]. The amount level of IgG antibody which reaches to DAT positive status is 200 or more molecules per each RBC in conventional irregular antibody test (tube test), and is 120–150 or more molecules per each RBC in column agglutination technology (CAT) [8, 9]. The newly high sensitive quantitative method for detecting RBCs-bound IgG antibody developed recently. Flow cytometric analysis (FCM), immunoradiometric assay (IRA) and enzyme-linked immunosorbent assay (ELISA) are useful methods for measuring low amount of RBCs-bound IgG antibody [10, 11].
2. The subclass of IgG antibody. As mentioned above, the cause of hemolysis depends on the affinity to IgG antibody and Fc gamma receptor (FcγR). IgG3 antibody having high affinity to FcγR mediates hemolysis with fewer amounts. Regarding this, RBCs bound with many complement components tend to become hemolysis regardless of the small amount of bounding IgG antibody [12].
3. Non-IgG antibody bound to RBCs. Conventional DAT becomes negative if the hemolysis occurred by IgA or IgM class antibody because universal Coombs reagent does not contain these classes of antibody [13]. DAT with the FCM method by using IgA or IgM antibody is able to detect autoantibody bound to RBCs.
4. Hyper-IgG status. A part of cases accompanied with hyper IgG status as a consequence of hyper-gammaglobulinemia or intravenous immunoglobulin administration show DAT or



**Figure 2.** (A) Schematic model about the relationship between antibody activity and complement activity in IgG antibody-mediated hemolysis. Each component has optimal activating temperature. Cold hemolysis requires adequate temperature in which both factor activate. (B) Evaluation of false positive result in the column agglutination technology (CAT) in cold agglutinin disease (CAD) (Ref. [16]). The agglutination in micro-bead matrix reaction shown as the sets of small dots is evaluated as false positive in CAT machinery, and the grade of false positive reaction was expressed by semi-quantitative scale (0.5+ ~ 4+). (C) Clinical course in the CAD case (modified from Ref. [16]). The degree of this false positive (FA) reaction in CAT is more correlated inversely than cold agglutinin (CA) to complement titer (CH50, C4).

IAT positive status [14, 15]. The reason of this is explained that plasma IgG antibody united to RBCs with nonspecific fashion. However, autoantibody for RBCs is produced by autoimmune mechanism in some of hyper-gammaglobulinemia cases. To analyze autoantibody for RBCs, examiner should pay attention to the existence of alloantibody hiding from autoantibody. If IAT is positive, free autoantibody for RBCs will be absorbed from serum (plasma) by using patient's own RBCs, and eluted autoantibody will be analyzed.

5. Thermal range of operating IgG antibody. Complement activity is essential to develop hemolytic reaction. RBCs autoantibody has optimal thermal range for bindings, and hemolysis requires adequate temperature in which complement activates and autoantibody binds to RBCs (**Figure 2A**). To take an example of CAD, not the quantity of cold agglutinin but the operating thermal range of cold agglutinin is important to emerge disease. We experienced a CAD case having blood type A (Rh+), in which the agglutination in micro-bead matrix area was interpreted to false positive. This agglutination in CAT is caused by cold agglutinin (IgM autoantibody) induced in CAD (**Figure 2B**). The degree of this false positive reaction in CAT seems to be correlated inversely to complement titer (**Figure 2C**). The range of temperature on activation of cold agglutinin is  $22 \pm 10^{\circ}\text{C}$  [17]. The temperature of operating CAT in the presenting case is between 18 and  $32^{\circ}\text{C}$ , where both cold agglutinin and complement component can activate, bring about the agglutination reaction in CAT test tube [16].

## 6. The pitfalls in the diagnosis of AIHA

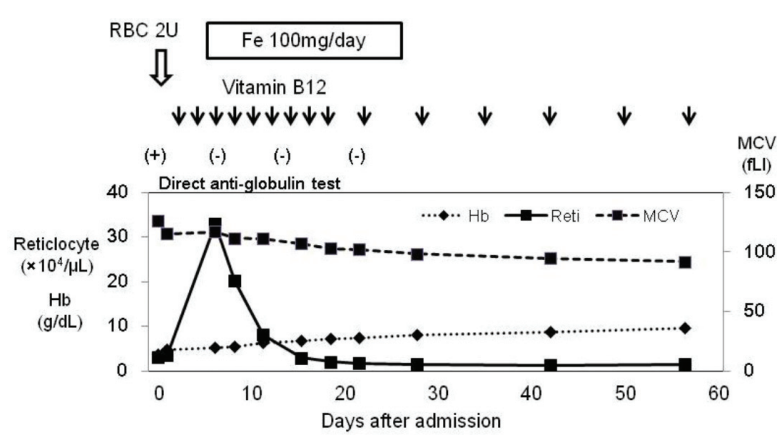
The pitfalls about AIHA diagnosis are described as follows:

1. In some cases, blood-type specific autoantibody is capable to be produced without receiving either previous transfusion or the history of pregnancy. As mentioned earlier, a part of AIHA cases have blood-type specific autoantibody, like as Rh type. Immunological disorders related to inappropriate gammaglobulin production seem to be a high risk group for occurrence. In this situation, discrimination between autoantibody and alloantibody for RBCs should be required. We experienced a 14-year-old female's Evans syndrome case with hereditary IgA deficiency. As she had no medical history of receiving transfusion or pregnancy, her hemolysis may be caused by JKa autoantibody [18].
2. Try to clarify AIHA or ineffective erythropoiesis. Hemolytic anemia is defined as the condition composed of anemia, increase of reticulocyte, rising serum indirect bilirubin and lactate dehydrogenase (LD) level and decrease of the serum haptoglobin level. AIHA is one of the hemolytic anemia occurred by autoimmune reaction. On the other hand, ineffective erythropoiesis such as megaloblastic anemia, sideroblastic anemia and myelodysplastic syndromes show similar laboratory data as hemolysis. However, the status of ineffective erythropoiesis is not the same status as that of AIHA, and most of erythroblasts are dying in the intramedullary



area by apoptosis in ineffective erythropoiesis. We experienced an 80-year-old female who has pernicious anemia accompanied with DAT positive status. Anemia was suspected of involving autoimmune mechanism by laboratory data expecting hemolysis; however, anemia was normalized with only vitamin B12 administration. As shown with this case, pernicious anemia cases with positive DAT status do not always be concerned with autoimmune mechanism (**Figure 3**) [19]. It is sometimes difficult to clarify AIHA and ineffective erythropoiesis at the onset of anemia.

- 3. Consider the background disease in secondary AIHA. Secondary AIHA is caused by infection, allogeneic blood transfusion, pregnancy, autoimmune disease and malignancy. We need to diagnose any AIHA cases carefully whether autoimmune disease or malignancy is exist (**Table 2**) [20]. In terms of autoimmune diseases, collagen diseases including systemic lupus erythematosus (SLE) or rheumatoid arthritis may sometimes complicate AIHA. Especially for SLE, the current SLE diagnostic criteria contain the existence of AIHA or DAT



**Figure 3.** Clinical course in the pernicious anemia case (modified from Ref. [19]). Autoimmune hemolytic anemia (AIHA) was suspected due to the hemolytic aspect and DAT positive status in initial presentation of this case. However, treatment with vitamin B12 administration improved anemia and the disappearance of hemolysis or DAT positive status turned out to be the diagnosis of pernicious anemia which was not involvement of true AIHA conclusively.

**Hematological malignancy**

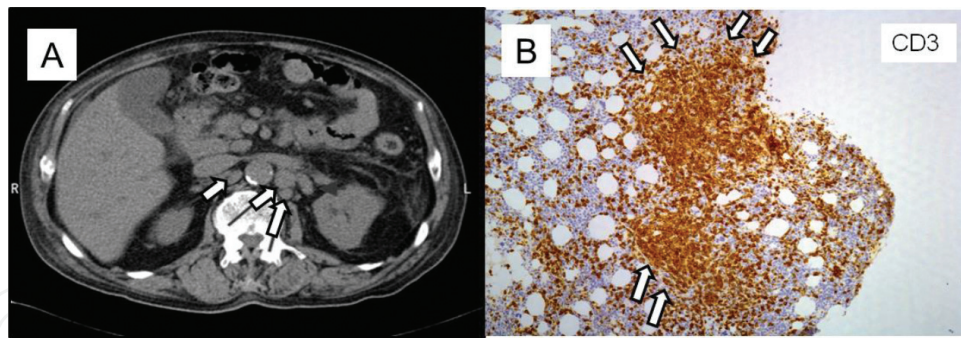
|   |           |
|---|-----------|
| Chronic lymphocytic leukemia (CLL)              | 4.3–9%    |
| Non-Hodgkin lymphoma excepting CLL/SLL category | 0.23–2.6% |
| Angioimmunoblastic T-cell lymphoma              | 17.8%     |
| Hodgkin lymphoma                                | 0.19–1.7% |

**Autoimmune disease**

|                              |          |
|------------------------------|----------|
| Systemic lupus erythematosus | 6.6–7.5% |
| Anti-phospholipid syndrome   | 9.7%     |
| Ulcerative colitis           | 1.4–1.7% |

Modified from Ref. [20] with refined WHO classification.

**Table 2.** Causes of secondary AIHA.



**Figure 4.** (A) Abdominal lymph nodes were swollen in CT scan (arrow) in an AITL case. (B) Bone marrow biopsy specimen (CD3 immunostaining (100×)). AITL cells infiltrate to bone marrow, which were shown as clear cells (arrow).

positive status [21], which is the reflection of high frequency of AIHA in SLE. AIHA is also complicated with inflammatory bowel diseases such as ulcerative colitis or Crohn's disease. Searching patient's medical history or following patient during clinical course is required for the diagnosis of background disease because these autoimmune diseases and AIHA are not always manifested at the same time. Anti-phospholipid antibody syndrome and idiopathic thrombocytopenic purpura are another responsible cause of DAT positive status; therefore, the possibility of existence of these diseases is also considered in DAT positive status.

4. About malignancy as a cause of secondary AIHA, lymphoproliferative disorders are predominantly observed than solid tumors. As shown in **Table 2**, angioimmunogenic T cell lymphoma (AITL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) seem to be the main cause. AITL is known to have symptoms of autoimmune diseases including AIHA. We show an AIHA case in which background disease is AITL (**Figure 4A**), and show another case involved in bone marrow (**Figure 4B**) [22]. In these presenting cases, computed tomography (CT) revealed abdominal lymphadenopathy. DAT positive status turned to be negative when AITL was in good control. In solid tumors, squamous cell carcinoma and adenocarcinoma are seen as a paraneoplastic syndrome [23]. To diagnose malignancy as the secondary disease of AIHA, especially for AITL and CLL/SLL, image diagnosing including CT scan or bone marrow examination is required.

## 7. Conclusions

The potentiality of anti-globulin test and pointing issue under the diagnosis of AIHA is explained. We need to make much of diagnosing the background disease such as lymphoproliferative disorders or autoimmune diseases when diagnosing AIHA.

## Conflict of interest

The authors have no conflict of interest.

## Author details

Takeshi Sugimoto

Address all correspondence to: takeshi\_sugimoto@kitahari-mc.jp

Department of Hematology and Oncology, Kita-Harima Medical Center, Ono, Hyogo, Japan

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