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Laboratory perspective of Cold Agglutinin syndrome: A case study

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Introduction

Cold Agglutinin Disease (CAD) represents a rare hemolytic disorder, constituting 15-30% of cases within the spectrum of Autoimmune Hemolytic Anemias (AIHAs) [1,2]. The pathogenesis of CAD revolves around autoantibodies known as Cold Agglutinins (CAs), named for their capacity to induce erythrocyte agglutination at temperatures below 37°C. Those autoantibodies typically consist of monoclonal Immunoglobulin (Ig) M type, less commonly IgA or IgG, and they target the carbohydrate antigens on the erythrocyte surface, predominantly antigen I [3-5].

Recently, CAD has gained recognition as a well-defined clinicopathologic entity [1]. In CAD (previously known as idiopathic CAD), a clonal lymphoproliferative disorder of the bone marrow leads to the production of CAs [6,7]. The Cold Agglutinin Syndrome (CAS), on the other hand, is defined as a similar but heterogeneous group of CA-mediated AIHAs that can occur secondary to other clinical conditions, such as specific infections (Mycoplasma pneumonia, Epstein-Barr virus, SARS-CoV-2 infection, and others) and malignancies (especially B-cell lymphomas other than lymphoplasmacytic lymphoma and marginal zone lymphoma) [6-9]. The binding of circulating CAs to cell surface antigens leads to erythrocyte agglutination and the antigen-antibody complex activates complement-mediated hemolysis. The clinical manifestations of CAD are hemolytic anemia and cold-induced circulatory symptoms (such as livedo reticularis, acrocyanosis, etc.) [10].

Analysis of samples containing cold agglutinins present a significant challenge for clinical laboratories using automated hematology analyzers. The initial suspicion of cold agglutinins typically arises from unsuccessful attempts to analyze the Complete Blood Count (CBC). The electrical impedance method counts the cells by detecting and measuring changes in electrical resistance in a conductive liquid as they pass through a small aperture. In agglutination phenomena doublet or triplet RBCs are considered as singular entities, while large aggregations of cells are disregarded during enumeration. This causes falsely decreased RBC and Hematocrit (Hct) values, while indices such as Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) may exhibit falsely elevated.

In this case study, we present the laboratory diagnosis of a patient presented with cold agglutination by use of current guidelines [8].

Materials and methods

During our routine laboratory workload, a patient result with many flags was given by the analyzer which alarmed laboratory

specialist. Unusual values of Hct and RBC count showed a mismatch in traditional Rule of Three [11]. Additionally, MCV, MCH, and MCHC were recorded as spuriously high (Table 1).

First, visual inspection of the sample tube was performed to observe the presence of micro-aggregates (Figure 1). Second, the sample was centrifuged to visualize the appearance of sera, for a possible hemolysis and lipemia interferences. We invited the patient to our laboratory and collected three sample tubes for CBC. To overcome cold agglutination, the following procedures were applied:

Pre-warming

One of the tubes was placed in a water bath set to 37°C for one hour before blood collection, and it was promptly analyzed without allowing it to cool.

Treatment with 2-Mercaptoethanol (2-ME) (12)

A 2-ME solution (Sigma-Aldrich, Missouri, USA) with a concentration of 14.3 M was utilized. 10 µL of this 2-ME solution was added to the 3 mL whole blood sample tube to achieve a final concentration of 0.05 M. Subsequently, the tube was gently inverted to ensure thorough mixing. This tube was kept at room temperature for 30 minutes together with another CBC tube as control for comparison.

Evaluation of 2-ME on normal patient's samples

To observe the effect of 2-ME on CBC results in normal patients, we obtained two samples from each of three healthy volunteers. One of the samples treated with 2-ME, second sample was used for the control.

Biochemistry parameters (LDH, AST, potassium, total and direct bilirubin, haptoglobin, immunoglobulin G, A, and M) were studied for a definitive diagnosis of the patient. Direct Coombs test, Serum Protein Electrophoresis (SPE) and Immune Fixation Electrophoresis (IFE) free kappa and lambda light chains analysis were also conducted (Table 1). The whole blood samples were collected into 3.0 mL K2-EDTA tubes (BD Vacutainer®, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) with a vacutainer system. CBC analysis was conducted using the Sysmex XN-9000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Informed consent was obtained from the patient prior to sample collection.

Results and discussion

This was a case of Cold Agglutinin Syndrome (CAS) who presented to our laboratory with abnormal CBC results. Many flags were given by the analyzer "RBC abnormal distribution, dimorphic population, anemia, RBC agglutination?, Turbidity/Hb interference?" In evaluation of parameters, we saw that RBC, Hct and Hb values did not obey the traditional Rule of Three. According to this rule, Hct result is obtained by multiplying the Hb value by three, and the Hb result can be obtained by multiplying the number of RBC by three. When test results don't match this rule, Wintrobe [13] formulas cannot be applied, and the underlying etiology should be investigated. Besides a peripheral blood smear is recommended [14]. Hence calculated parameters, MCHC 215.9 g/dL and MCV 112.8 fL were questionable. We first evaluated the sample tube visually and saw the agglutination on the walls of the tube. To confirm RBC or platelet agglutination in the sample, the best way is blood smear examination under the microscope. Analysis of peripheric blood smear revealed clusters of erythrocyte agglutination. We centrifuged the tube to see whether there was hemolysis/turbidity which could interfere with MCHC values. The plasma was clear with no hemolysis and/or turbidity appearance.

In Sysmex XN analyzer, Hb is directly measured by a colorimetric method, using sodium lauryl sulphate to lyse RBCs in the sample. Thus Hb, not affected by cold agglutinins, stands as the only reliable parameter. Physiologically there is a small amount of free Hb in plasma and this amount is measured together with Hb released after RBC lysis and its effect is negligible. In case of in vivo hemolysis as in hemolytic anemias, this effect may be important, and these patients have increased MCHC values. In vitro hemolysis does not affect the actual Hb concentration but may cause a false low RBC count and Hct concentration and false high MCH and MCHC [15]. In such a case MCV is not increased and plasma seems reddish pink after centrifugation [16]. An abnormally high MCHC corresponding to an erroneously high Hb can also be seen in turbid samples with high lipid content because of spectral interference [17].

Erroneous results because of cold agglutinins on CBC analyzers is a challenge for laboratories. In our analyzer Sysmex XN, RBC and Hct is measured in the same channel. In impedance method with hydrodynamic focusing, a very narrow path is created and only one small cell can pass at a time. Thus, RBCs can be counted separately. Passage of each erythrocyte creates an electrical pulse between two electrodes and Hct is measured by the cumulative height of the electrical pulses. MCV is calculated as Hct/RBC [18]. MCH and MCHC are calculated according to Wintrobe formulas. When erythrocytes aggregate, large clumps cannot pass through the pathway, only isolated RBCs, or small RBC clumps (two or three RBC), are analyzed causing erroneously low RBC and Hct counts leading to abnormally high MCV. In such a case as a rule, the MCHC is usually >36,5 g/dl. This association of low RBC count, high MCV and high MCHC is almost pathognomonic of the cold agglutination error on CBC analyzer results [19]. Cold agglutinin effects on CBC parameters were shown on different analyzers like ABX Pentra 80 [20], Advia 60 Bayer [21], Coulter [22,23] and Sysmex [24].

We called back the patient and performed the procedures described in the methods section. Prewarmed tubes and 2-ME added tubes did not show agglutination. One of the tubes was forced to agglutinate at room temperature for 30 minutes together with 2-ME tube and the CBC results were compared. Some agglutination occurred in the control tube but not in 2-ME added sample (Table 2). Sample collection at 37°C and/or pre-heating before counting was already proposed in 1945 by Finland et al. [25] as a method for eliminating CA interferences. Warming the sample leads to elution of IgM antibody from the cell surface allowing the agglutinated RBCs to separate. Nevertheless, only microscopic observation after the procedure ensures the resolution of RBC agglutination since behavior of different types of CAS is heterogenous [26]. We performed peripheric blood smear of the initial sample and after prewarming procedure and saw the resolution of RBC agglutination (Figure 2a-b).

2-Mercaptoethanol (2-ME) is a sulfhydryl reagent that can be used to disperse IgM type agglutinins and treated samples can be used for ABO typing, antigen typing, or the direct antiglobulin testing and prevent the agglutination caused test errors in CBC counting [27]. Using a weak concentration of 2-ME, the cold IgM agglutinin can be removed from the coated RBCs without disrupting the IgG or complement coating the RBCs. 2-ME with a final concentration of 0.05 M has been traditionally

Table 1: Patient characteristics at presentation.

Parameters	Results	Reference ranges
RBC (10^6/µL)	0,39	3,9-5,3
Hb (g/dL)	9,5	11-14,7
HCT (%)	4,4	37-49
MCV (fL)	112,8	83-98
MCH (pg)	243,6	27-32,3
MCHC (g/dL)	215,9	31,8-34,7
WBC (10^3/µL)	2,6	4,5-12,6
PLT (10^3/µL)	329	171-388
Flags: RBC abnormal distribution, dimorphic population, anemia, RBC agglutination?, Turbidity/Hb interference?		
Parameters	**Results**	**Reference ranges**
LDH (U/L)	236	135-214
AST (U/L)	24	0-32
Potassium (mmol/L)	3,95	3,5-5
Total bilirubin (mg/dL)	0,45	0-1,2
Direct bilirubin (mg/dL)	0,16	0-0,30
Haptoglobin (g/L)	0,88	0,3-2
Immunoglobulin (Ig) M (g/L)	0,72	0,4-2,3
Ig A (g/L)	2,07	0,7-4
Ig G (g/L)	11,87	7-16
Free kappa light chain	20,7	6,7-22,4
Free lambda light chain	20,6	8,3-27
Free kappa/free lambda	1,005	0,31-1,56
Direct Coombs C3b, C3d	Positive	
Direct Coombs IgG	Negative	
Serum protein electrophoresis	M component not detected	
Immunofixation electrophoresis	Band not detected	
RBC-Red Blood Cells; Hb-Hemoglobin; HCT-Hematocrit; MCV-Mean Corpuscular Volume; MCH-Mean Corpuscular Hemoglobin; MCHC-Mean Corpuscular Hemoglobin Concentration; WBC-White Blood Cells; PLT-Platelets; LDH-Lactate Dehydrogenase; AST-Aspartate Aminotransferase.		

Table 2: CBC results after different treatments.

	Prewarmed	2-ME added*	Control* for 2-ME added
RBC (10^6/µL)	3,08	2,97	1,03
Hb (g/dL)	8,7	8,5	9
HCT (%)	27	26	11
MCV (fL)	87,7	87,5	106,8
MCH (pg)	28,2	28,6	87,4
MCHC (g/dL)	32,2	32,7	81,8
WBC (10^3/µL)	4,62	4,91	4,37
PLT (10^3/µL)	242	270	253
*incubated at room temperature for 30 minutes.			
RBC-Red Blood Cells; Hb-Hemoglobin; HCT-Hematocrit; MCV-Mean Corpuscular Volume; MCH-Mean Corpuscular Hemoglobin; MCHC-Mean Corpuscular Hemoglobin Concentration; WBC-White Blood Cells; PLT-Platelets.			

Figure 1: Blood sample tube from the patient exhibiting agglutination.

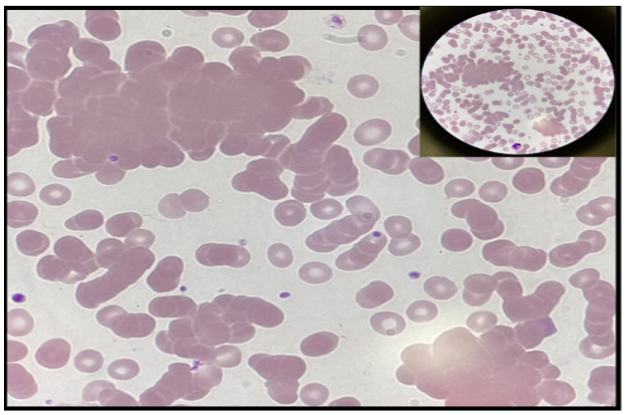

Figure 2a: Peripheral blood smear of the initial sample, 1000x.

used in Brucella agglutination test [28]. Effect of 2-ME on CBC results were also evaluated in 3 healthy volunteers, negligible differences were observed (Table 3).

In the case we present, the patient's WBC count was also affected by CAs. Platelet counts were stable in all the samples before and after treatments. CAs mainly attack erythrocyte surface antigen I, which are also shown on the surface of leukocytes and platelets [29,30] CA induced leucopoenia and thrombocytopenia were also shown in studies [31]. WBC increases were seldom as in the case of Ellidağ study [32]; it might be attributed to a change in the volume of blood cells i.e platelet and erythrocyte aggregates and immunoglobulins which might be falsely identified as leukocytes [16].

Erythrocyte agglutination in laboratory procedures and test results together with cold induced circulatory symptoms should lead the clinician suspect CAD. Chronic hemolysis, positive DAT, monospecific DAT positive for C3d, demonstration of CA at a significant titer, and absence of overt malignancy or specific infection that might cause secondary CAS must be demonstrated for a definitive diagnosis [1]. Detection of hemolysis is based on biochemical markers including indirect bilirubin, Lactate De-Hydrogenase (LDH), and haptoglobin [2]. Monoclonal IgMκ in serum or plasma (or, rarely, IgMλ or IgG) will also help diagnosis [8].

In the case we present, Direct antiglobulin test was positive for C3d and negative for IgG. Markers for erythrocyte hemolysis LDH, AST, haptoglobin, potassium, total and direct bilirubin

Table 3: CBC results of three volunteer's samples to observe 2-ME effect.

Parameters	Routine (1)	2-ME added (1)	Routine (2)	2-ME added (2)	Routine (3)	2-ME added (3)
RBC (10^6/µL)	5,00	4,86	4,52	4,44	4,70	4,64
HGB (g/dL)	14,30	14,00	13,60	13,70	13,10	13,10
HCT	44,00	42,30	42,40	41,50	40,50	40,00
MCV	88,00	87,10	93,70	93,50	86,20	86,10
MCH	28,70	28,90	30,20	30,80	27,80	28,20
MCHC (g/dL)	32,50	33,10	32,10	33,00	32,30	32,80
RDW-CV	13,30	13,20	13,70	13,80	13,60	13,70
RDW-SD	41,60	41,00	45,80	45,90	42,00	42,30
WBC (10^3/µL)	4,85	5,24	11,15	11,07	9,63	9,36
PLT (10^3/µL)	244	243	289	283	411	393

RBC-red blood cells; Hb-hemoglobin; HCT-hematocrit; MCV-mean corpuscular volume; MCH-mean corpuscular hemoglobin; MCHC-mean corpuscular hemoglobin concentration; RDW-red cell distribution width; WBC-white blood cells; PLT-platelets.

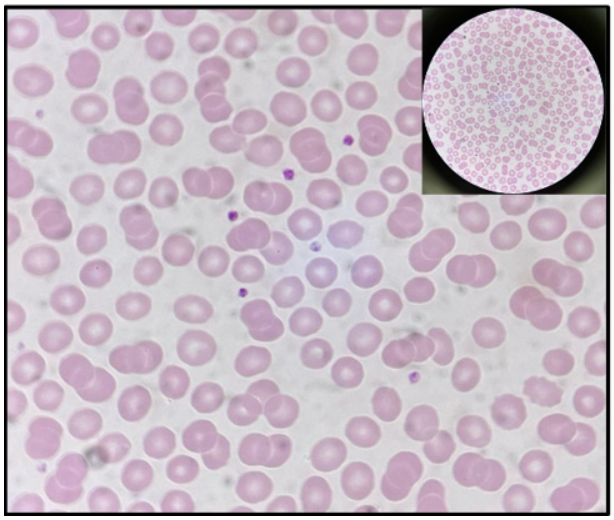

Figure 2b: Peripheral blood smear after prewarming procedure, 1000x.

were found in normal range. So, there were no evidence of intravascular hemolysis. Quantitative determination of individual immunoglobulins Ig M, Ig A, Ig G were all in normal range. Any kind of paraproteinemia was not determined by serum protein electrophoresis and there were no obvious pathologic chains in immunofixation electrophoresis. Though cold agglutinins are of IgM type in majority of cases, a weak IgG could be identified in 20% of cases. In 20% of all patients, a monoclonal protein had not been detected by standard laboratory methods [4].

In the retrospective evaluation of the patient's medical record, we saw that this patient was diagnosed with colon cancer in March 2023 and CA effects on CBC counts were observed afterwards. Thus, it was obvious that this was not a primary CAD, a CAS secondary to a malignant disease.

This is a case report of a Cold Agglutinin Syndrome presenting to the laboratory with interferences of cold agglutinins on CBC analysis. Besides resolving the cold agglutination, we emphasized on the potential support of laboratory on diagnosis of patient. Each laboratory should be aware of cold agglutination and have a procedure to overcome this challenge. Collaboration of clinician and laboratory in this specific issue is also recommended.

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