Direct Coombs test-negative autoimmune hemolytic anemia and low-affinity IgG class antibodies

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Autoimmune hemolytic anemia, in which the direct antiglobulin test (DAT) is negative or weakly positive, may be due to low-affinity autoantibodies. We describe two such cases. An 8-year-old male presented with weight loss, jaundice, a hemoglobin of 33 g/L, reticulocytes of 306 x 10^9/L, and haptoglobin of < 0.1 g/L. The DAT was negative. After washing the red blood cells (RBCs) with saline at 4°C, the DAT was positive for IgG and an eluate contained an IgG3 autoantibody, thus confirming a diagnosis of autoimmune hemolytic anemia (AIHA). Red cell transfusions and corticosteroids were given with eventual complete recovery. A 73-year-old male had a hemoglobin of 89 g/L and haptoglobin of < 0.1 g/L. The DAT was initially negative but was positive for IgG using cold-washed (4°C) RBCs; it was also positive with unwashed cells in the DiaMed system and an eluate contained IgG1 autoantibody. AIHA was therefore confirmed and prednisolone started but continued hemolysis necessitated splenectomy before full recovery occurred. Although RBCs may be strongly sensitized with low-affinity autoantibodies in vivo, the IgG is easily removed when RBCs are washed at room temperature for a DAT. The DiaMed system that uses unwashed RBCs overcomes this problem, but cold washing the RBCs at 4°C must be used when preparing eluates.
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Autoimmune hemolytic anemia (AIHA), in which the standard agglutination direct antiglobulin test (DAT) is negative or weakly positive, is an uncommon but well-recognized phenomenon.[1-6] We describe two cases in which low-affinity autoantibodies were implicated and discuss other possible causes for a negative DAT in AIHA.

Case Reports

An 8-year-old male presented with a 7-day history of malaise, weight loss, abdominal pain, fever, and dark urine. Physical examination revealed a pale, slightly jaundiced child with mild splenomegaly and a generalized ejection systolic murmur. The hemoglobin level was 33 g/L, reticulocytes 306 x 10^9/L, haptoglobin < 0.1 g/L, and bilirubin 43 μmol/L (normal ranges: hemoglobin 130–180 g/L, reticulocytes ≤ 100 x 10^9/L, haptoglobin 0.4–2.0 g/L, and bilirubin ≤ 17 μmol/L). Because there was no evidence of other disease or infection, a diagnosis of AIHA was proposed. However, the DAT was negative, even though crossmatching showed incompatibilities with all donor red blood cells (RBCs) tested. Repeat testing after washing the red cells at 4°C showed that the DAT test was, in fact, positive for IgG and a RBC eluate contained an IgG3 autoantibody. These findings confirmed the diagnosis of AIHA. The patient was treated with blood transfusions, prednisolone, and folic acid, with good effect. He was discharged from the hospital after 12 days with a hemoglobin level of 94 g/L, still taking large doses of prednisolone and folic acid. He continued to make good progress and the dose of steroids was gradually reduced in accordance with his clinical condition and blood counts. Treatment was stopped after 7 months. He was well when last seen 16 months after presentation with a hemoglobin level of 134 g/L and a reticulocyte count of 55 x 10^9/L.

A 73-year-old male was referred with a tentative diagnosis of autoimmune hemolysis. Blood tests showed a hemoglobin level of 89 g/L, reticulocytes 300 x 10^9/L, and haptoglobin of < 0.1 g/L. The DAT was initially negative. However, using RBCs washed at 4°C, the test was positive for IgG and an eluate contained an IgG1 autoantibody. The DAT was also positive with unwashed RBCs used in the DiaMed-ID gel system. The diagnosis of AIHA was therefore confirmed. Tests for systemic lupus erythematosus (antinuclear factor) were negative; the serum contained an IgG paraprotein, which was thought to be benign and not related to the hemolysis. Treatment with prednisolone was started. The initial response was good in that the reticulocyte count remained increased and the hemoglobin level rose to 119 g/L. The dose of steroids was gradually reduced over the next few months, but the hemoglobin fell to 90 g/L and splenomegaly (14 cm by ultrasound; normal < 9 cm) was evident 10 months after presentation. Hemolysis continued in spite of azathio-

prine being added to his treatment and splenectomy was performed 2 months later; the spleen weighed 368 g (normal 120–200 g) and the histology was consistent with acquired hemolytic anaemia. Thereafter, recovery was rapid and prednisolone was stopped in 2 months. The patient was clinically well when last seen 17 months after diagnosis. A hemoglobin level of 12.1 g/L and haptoglobin of < 0.1 g/L suggested that the autoimmune hemolysis was still active but well compensated. There was no change in his IgG paraprotein at 13 g/L over this period.

Materials and Methods

The immunohematological investigations routinely employed at this Centre have been described previously.[7-10] They include DATs to detect RBC-bound IgG, IgM, IgA, C3c, and C3d by spin agglutination, and IgG, IgM, and IgA by enzyme-linked methods,[8] the cells being washed in phosphate-buffered saline (pH 7.0) at room temperature; in addition, serum and eluates are examined for RBC autoantibodies. For the present study, DATs and elutions were also performed using patients' RBCs washed at 4°C; the antiglobulin reagents were similarly kept at 4°C. In the second case, the DAT was also carried out using the DiaMed-ID gel method (DiaMed-AG, Cressier sur Morat, Switzerland) with unwashed RBCs and monospecific reagents.

The red cell eluates, prepared at 4°C, were also tested for IgG subclass using a spin agglutination indirect antiglobulin technique at 4°C with reagents specific for IgG1, IgG2, IgG3, and IgG4 (CLB, Amsterdam, Netherlands).

Results

The 8-year-old patient's RBCs were group O, D+C–E+c+e+. The usual agglutination DAT was negative using a polyspecific reagent and room temperature washing; repeat testing with RBCs washed at 4°C showed that they were, in fact, heavily coated with IgG and weakly sensitized with C3d. An eluate, prepared after cold washing the RBCs, contained an IgG3 autoantibody with no obvious blood group specificity. A strong autoantibody was detected in the serum by indirect antiglobulin test (IAT) employing low-ionic-strength saline (LISS).

The 73-year-old patient's RBCs were group A, D+C+E–c+e+. Using RBCs washed at room temperature, routine agglutination and enzyme-linked DATs were negative. However, the test was positive when the RBCs were washed at 4°C; in addition, the DAT was strongly positive for IgG with unwashed cells in the DiaMed system. Eluates made after cold washing contained an IgG1 autoantibody with no obvious blood group specificity. A weak IgG autoantibody was demonstrated in the serum using LISS. Repeat testing 17 months after presentation showed that the serum now contained a strong autoantibody, which was no longer of low affinity; the routine DAT was now positive with RBCs washed at room temperature.

Discussion

Up to 5 percent of patients with AIHA present with a negative DAT.[3] The possibility that low-affinity antibodies are the cause must be kept in mind and is well illustrated by the two patients whose RBCs were DAT negative until the tests were carried out on RBCs washed in cold saline. Using that technique, the DATs became positive and the diagnosis of AIHA was confirmed. It is thought that the RBCs were strongly sensitized in vivo with IgG, but the washing procedures in vitro effectively removed the immunoglobulin from the cells. Although such cases are rare, they are well documented and the Coombs negative presentation is typical; the autoantibodies involved are usually of the warm type,[3-6] although a case due to a low-affinity IgG cold autoantibody showing anti-Pr_a specificity has been described.[11] These reports stress the necessity to wash the red cells in cold (0–4°C) or LISS to slow down the loss of IgG from the cell surface, to use cold antiglobulin reagents, and to include controls (e.g., antiglobulin diluent) at 4°C to exclude a positive result being due to cold autoagglutinins.[3-6] In one series, for example, washing the RBCs at 4°C and at room temperature correlated with strong positive and negative/weak positive results, respectively, for IgG in the DAT; tests that were weakly positive at room temperature were negative when the cells were washed at 37°C.[6]

There could be several reasons for autoantibodies having low affinity: the fit with the corresponding antigen could be poor, relatively weak hydrogen bonding (rather than hydrophobic bonding) could be involved, the IgG could be attached by only one binding site (the equilibrium constant increases approximately 1000-fold when both sites bind),[12] or equilibrium constants of the autoantibodies might be particularly sensitive to small changes in pH, ionic strength, or temperature. Antibodies are heterogeneous in their affinity[5] and those with low affinity could represent a physiological extreme. Perhaps the affinity maturation process, whereby an ongoing immune response produces antibodies of continuously increasing affinity,[13] is abnormal and remains at an early stage. The late development of normal affinity shown by the

second patient's autoantibody would be in keeping with this theory.

Low affinity autoantibodies may be associated with severe hemolysis,[5,6] as in the first case in which the patient's hemoglobin level fell to 33 g/L. Such cases provide a good illustration of laboratory tests not correlating with the observed clinical situation and serve as a reminder that conditions in vitro may be very different from those in vivo.[6] The occurrence of severe hemolysis does not fit well with the view that RBCs sensitized with high-affinity antibodies are destroyed more efficiently,[6] a view that receives support from experiments in which RBCs were sensitized in vitro with similar amounts of serum and eluted autoantibodies, and labeled with ^{51}Cr. The cells coated with eluted autoantibody had the shorter survival, and it was postulated that this was because they had higher affinity.[14] Another suggestion for the occurrence of severe hemolysis is that the autoantibodies are heterogeneous in regard to affinity, and those antibody molecules having low affinity are not a major cause of the hemolysis but remain in the plasma after high-affinity molecules have been removed during RBC destruction.[6]

Column technology methods for DATs, which do not involve RBC washing, overcome the problem of Coombs negativity because of low-affinity autoantibodies, and we now employ the DiaMed system routinely whenever AIHA is suspected. However, the possibility of low-affinity autoantibodies being involved should not be forgotten, as the RBCs used to prepare eluates still need to be washed at 0–4°C. Otherwise, a positive column (DiaMed) test could be associated with a negative eluate and the results incorrectly interpreted as nonspecific adsorption, immune complexes, or drug-induced immune hemolysis, rather than AIHA due to low-affinity antibodies.

Other reasons for AIHA presenting with a negative DAT or one that appears too weakly positive for the degree of hemolysis can now be considered.

There may be increased amounts of autoantibody bound to the RBCs, but the number of molecules is insufficient to produce agglutination in the routine DAT.[1,2,6] In our experience, such cases are less frequent with the improvements in antiglobulin reagents. Nevertheless, cases still occur and in some instances sensitive enzyme-linked assays have shown small increases in immunoglobulins of more than one class acting synergistically to cause AIHA.[15-17]

Alternatively, hemolysis may be due to IgA or IgM class autoantibodies, whereas most antiglobulin reagents are standardized to detect IgG.[2,6,18-21] We previously have recommended that a comprehensive range of monospecific reagents be used to investigate patients with AIHA[10] so that cases due to IgA or IgM autoantibodies could be readily identified.

The autoantibodies and their corresponding antigens may have unusual characteristics or specificities. In some patients with AIHA, only complement was detected on the RBCs by routine testing but the presence of IgG was demonstrated at 4°C. Above 10°C, the autoantibodies apparently underwent configurational changes such that agglutination no longer occurred with the anti-IgG reagent.[22] Autoantibody activity may be dependent on RBC age; in one instance, a patient had AIHA with a predominantly young RBC population; the DAT was negative but the serum contained autoantibodies that reacted strongly with mature RBCs.[23] There may be variable antigen expression on the RBCs. A patient with chronic AIHA has been described in whom the DAT became negative during a severe hemolytic episode although the serum autoantibodies remained strongly reactive against a RBC panel. It was thought that the antigens against which the autoantibodies were directed had changed or had variable expression, resulting in a subpopulation of nonreactive cells that had not been removed from the circulation.[24] A case of warm type AIHA was reported in which marked depression of RBC Rh-antigen expression resulted in the patient presenting with severe anemia and a negative DAT, in spite of the serum containing strongly reactive IgG Rh autoantibodies.[25]

Another example of Coombs negative AIHA may be found rarely in patients with paroxysmal cold hemoglobinuria (PCH), in which severe intravascular hemolysis is associated with a negative DAT; usually C3d coating of the RBCs is evident in this condition.[26]

In summary, it is important not to rule out the possibility of an autoimmune etiology in a patient presenting with hemolytic anemia and a negative DAT on initial testing.

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