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Direct antiglobulin ("Coombs") test-negative autoimmune hemolytic anemia: A review

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ABSTRACT

We have reviewed the literature to identify and characterize reports of warm-antibody type, autoimmune hemolytic anemia in which the standard direct antiglobulin reaction was negative but a confirmatory test indicated that the red cells were opsonized with antibody. Three principal reasons account for the absence of a positive direct antiglobulin test in these cases: a) IgG sensitization below the threshold of detection by the commercial antiglobulin reagent, b) low affinity IgG, removed by preparatory washes not conducted at 4 °C or at low ionic strength, and c) red cell sensitization by IgA alone, or rarely (monomeric) IgM alone, but not accompanied by complement fixation, and thus not detectable by a commercial antiglobulin reagent that contains anti-IgG and anti-C3. In cases in which the phenotype is compatible with warm-antibody type, autoimmune hemolytic anemia and the direct antiglobulin test is negative, an alternative method to detect low levels of IgG sensitization, use of 4 °C, low ionic strength washes to prepare the cells for the direct antiglobulin test reaction to permit retention and identification of low affinity IgG antibodies, and, if the latter are uninformative, testing for sensitization with an anti-IgA, and, if necessary, an anti-IgM reagent identifies cases of warm-antibody type, immune hemolysis not verified by a commercial reagent.

Introduction

In 1945, Coombs, Mourant, and Race showed the utility of an antiglobulin test to determine the presence of red cell antibodies in the serum of mothers whose newborns had hemolytic disease [1]. This test for maternal serum antibodies became known as the indirect antiglobulin test. Prior to their studies, the identification of red blood cell group antigens depended on a simple direct agglutination reaction between human red cells and human serum, introduced by Landsteiner in 1901. The finding of the presence of intraspecies, human serum isoagglutinins led to the identification of human blood groups. In 1946, Coombs and coworkers reported that the antiglobulin reaction also could detect sensitization of the red cells of infants with hemolytic disease of the newborn; this approach referred to as the direct antiglobulin reaction [2]. The tests allowed these investigators to identify incomplete (non-agglutinating) antibodies in cases of Rh-hemolytic disease of the newborn and in transfusion reactions in which the sensitized red cells only agglutinated after exposure to a serum antiglobulin reagent prepared from the serum of a goat or rabbit injected with human serum immunoglobulin. A later study by Coombs and Mourant indicated that the globulin sensitizing the red cell resided in the gamma globulin fraction of plasma proteins [3]. Over the next two decades, it became evident, first, that red cells in cases of cold-antibody type, immune hemolytic anemia were sensitized by a protein that was not in the gamma fraction of serum globulins, subsequently shown to be a component of the complement system fixed to the red cell as a result of the antigen-antibody reaction [4].

In 1943, Dacie and Mollison established that normal red cells had a normal survival in patients with familial (hereditary) spherocytosis [5]. Shortly thereafter, in 1946, Loutit and Mollison showed that normal red cells had a markedly shortened survival in patients with acquired spherocytic hemolytic anemia, indicating that, unlike familial spherocytic hemolytic anemia, the destructive factor was not intrinsic to the red cell but an extrinsic factor, presumably an antibody [6]. Virtually simultaneously, Boorman and coworkers used the direct antiglobulin test (DAT) to divide cases of spherocytic hemolytic anemia into congenital (familial) cases (i.e. hereditary spherocytosis) with a negative antiglobulin test and acquired cases with a positive test, and proved that the latter cases were the result of an immune reaction against the patients' red cells [7]. The DAT, known informally as the "Coombs test", was one of the most important advances in diagnostic hematology and in blood bank procedures. A more detailed history of the development of understanding of the nature of autoimmune hemolytic anemia can be found elsewhere [4,8].

In the 1950s, suspicion arose that despite this new and sensitive method of detecting red cells IgG antibodies, there were occasional cases in which the clinical findings were identical to warm-antibody

Abbreviations: DAT, direct antiglobulin test; Ig, immunoglobulin; WAT-AIHA, warm-antibody type, autoimmune hemolytic anemia.

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type, autoimmune hemolytic anemia (WAT-AIHA), but the DAT did not confirm that diagnosis. Some cases were reported in which the DAT became positive only after several weeks of illness [9]. Other cases were found in which the red cells did not react with the antiglobulin reagent but antibodies were present in the serum [10]. Rare cases were shown to be positive for red cell antibody sensitization, only after preparing an antiserum against the patient's own plasma proteins [11]. Descriptions of patients with acquired hemolytic anemia with a negative antiglobulin reaction suggested that in some cases antibody sensitization may be undetectable with an antiglobulin reagent. Examples of these observations include: one case in which a positive antiglobulin reaction was shown only after trypsin-treated cells were studied, a second in which pancytopenia was present and red cell agglutination was absent but leukocyte and platelet agglutinins were demonstrated, and a third in which leukoagglutinins were evident with a hemolytic anemia that was sometimes weakly positive and sometimes negative by the direct antiglobulin reaction. These cases were discussed in a comprehensive paper on the serology of immune hemolytic disease by Evans and Weiser in 1957 [12].

Red cell sensitization in autoimmune hemolytic anemia (AIHA) was sometimes positive with an antiglobulin reagent but that reaction could not be inhibited by high concentrations of serum gamma globulin. Initially, this type of reaction was referred to as the "non-gamma [globulin]", positive antiglobulin (Coombs) test. Subsequent studies confirmed that the sensitizing plasma protein was a component of the complement (C□) system, specifically C3d [4]. Thus, autoimmune hemolytic anemia could occur in which there was a positive DAT for IgG, for IgG and C3, or for C3 alone. Although it was suspected that IgG on the red cell surface was the complement-fixing agent in the case of IgG-negative, C3-positive, WAT-AIHA, the proof of this supposition was established by Gilliland, Leddy, and Vaughan in 1970, using a sensitive complement-fixing, antibody-consumption test [13]. In 1971, Gilliland and co-workers next used the test to show, conclusively, that the commercial reagent used to test for red cell antibody sensitization, in some cases of apparent WAT-AIHA, could not identify sensitization that was below, approximately, 500 molecules of IgG/red cell. They found that the red cell of healthy individuals had less than 35 molecules of IgG adsorbed to its surface. They described the first cases of hemolytic anemia in which the red cells were negative using the standard DAT but in which they could show an increase above background of red cell surface IgG molecules with a complement-fixing, antiglobulin consumption test, accompanying a clinical state indicative of WAT-AIHA [14,15]. At the University of Rochester Medical Center, where the test was developed, it was ordered by requesting the "micro-Coombs" test.

Here, we (a) summarize the features of cases of DAT-negative, WAT-AIHA reported in the medical literature since 1971, each documented by an alternative test capable of identifying red cell antibody sensitization, and (b) review the tests used to detect low levels of anti-red cell IgG, low affinity IgG, or sensitization with other classes of antibody, notably IgA, and, rarely, a warm-reacting, monomolecular IgM. Several cases of suspected WAT-AIHA have been reported, based on the presence of anemia, reticulocytosis, indirect hyperbilirubinemia, elevated serum lactic dehydrogenase, spherocytosis, sometimes mild splenomegaly, or some combination of these findings, with no other apparent explanation, but without verification of red cell antibody sensitization by either the DAT or an alternative method of detecting antibody sensitization of the red cell. We do not include these cases. We focus on those cases of DAT-negative, WAT-AIHA that have been verified to have antibody sensitization of red cells by an alternative test.

Methods

The identification of the reported cases of DAT-negative, WAT-AIHA was performed using three major search engines: PUBMED, OVID, and Google Scholar. The search strategy used a query of "Coombs negative autoimmune hemolytic anemia" as well as variations of this query including "immune hemolytic anemia" and "negative Coombs" or "negative direct antiglobulin test".

The inquiry to PUBMED yielded 222 citations from which those patients with a negative DAT with confirmation of WAT-AIHA by an alternative method were included in Table 1. A similar strategy was employed using OVID, but was less informative since the terms "negative Coombs test" and "negative direct antiglobulin test" were not recognized as categories in this database, and were searched as word phrases. A more salutary result was obtained when Google Scholar was employed for the search from which more than 10,000 citations were retrieved. Sorting these citations by relevance allowed selection of reports of DAT-negative, WAT-AIHA. We, also, examined the reference list of papers reporting WAT-AIHA with a negative DAT reaction and, thereby, obtained papers not identified in the prior searches. In some cases, the paper cited could not be found despite an exhaustive search by author, title, and journal. Some other cases may have been omitted, inadvertently, but we consider the cases detailed in Table 1 representative and comprehensive and covering over 45 years of observation. We, also, were directed to additional relevant papers by experts in the topic who read a draft of this paper.

Results and discussion

Incidence

The precise incidence of patients presenting with an anemia compatible with WAT-AIHA with a negative DAT is not known but has been estimated at 3 to 11% of all cases, and is dependent, in part, on the potency of the direct antiglobulin reagent used for testing [4,12,18–20].

Descriptive features

Table 1 lists, chronologically, the cases reported of WAT-AIHA with a negative DAT that were shown to have antibody (usually IgG, occasionally IgA, rarely monomeric IgM) sensitization of the red cells by an alternative method [14,15,20–46]. The specific test used to confirm the antibody sensitization is shown in the last column of Table 1. Table 1 is divided into two sections, the upper section describes case reports and the lower section describes cases based on serological reactions accumulated in testing laboratories. Other cases of presumed WAT-AIHA with a negative DAT have been published but these reports did not include a confirmatory test; they were based on typical clinical features without an alternative cause being identified. These cases have not been included in Table 1.

The classical clinical manifestations of WAT-AIHA include: a decreased hemoglobin concentration, reticulocytosis, increased serum indirect bilirubin, elevated serum lactic dehydrogenase, low to absent haptoglobin, spherocytes and polychromatic macrocytes on the blood film, and, sometimes, splenomegaly, each shown in Table 1 for the cases in which they were reported. Erythroblasts and erythrophagocytic monocytes may be seen in the blood smear. Hemoglobinemia may accompany severe hemolysis. The manifestations of AIHA are related to the intensity of the hemolysis. Haptoglobin levels may be higher than anticipated if hemolysis is mild and an accompanying inflammatory condition induces exaggerated haptoglobin synthesis (acute phase reaction). When all or the most compelling of these are present, i.e. anemia, reticulocytosis, spherocytes, low or absent haptoglobin, in the absence of a positive DAT, it can be decisive to use an alternative test to establish if there is antibody sensitization of the red cells or request such a test from a reference laboratory.

Although reticulocytosis is an important feature of AIHA, the reticulocyte count may not be elevated early in the course of the disease. In one comprehensive study of this question, the reticulocyte count was less than 4% in 20% of cases of AIHA, initially [16]. Sometimes,