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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,

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reticulocytopenic patients will develop reticulocytosis compatible with an appropriate marrow response during the course of the disease [16,17]. In the face of a negative DAT, it is important to re-examine the blood film for evidence of spherocytosis and polychromatophilia in a case in which an alternative diagnosis is not evident. The progression to a more typical clinical picture (increased spherocytes and reticulocytes) may occur over time [16]. Over 90% (21 of 23 cases) of the patients reported in Table 1 in whom the blood smear was reported had spherocytes identified. Not unexpectedly, a similar very high prevalence of increased serum bilirubin and lactic dehydrogenase, and low haptoglobin was also evident.

About 30% of cases of WAT-AIHA with a positive DAT have a reticulocyte index equal to or less than 2× the basal index at the time of first analysis [16]. These patients are less likely to receive glucocorticoids and more likely to be transfused. Elevated bilirubin and lactic dehydrogenase were found in relatively reticulocytopenic patients as often as in those with an appropriate initial reticulocyte elevation, perhaps the result of ineffective erythropoiesis in relatively reticulocytopenic individuals, since the marrow in such cases often showed erythroid hyperplasia [16]. This relative reticulocytopenia may be the result of antibody sensitization of late erythroid precursors. Although the reticulocyte response in WAT-AIHA is variable, if the reticulocyte count is very high, for example, over 20%, the probability of an AIHA is high. No other acquired anemia presents with reticulocyte counts of such levels in adolescents and adults. We have seen the reticulocyte count over 90%, indicating that red cell survival is approaching that of the duration of the persistence of ribosomes in the circulating stress reticulocyte (3 to 5 days).

The clinical characteristics of DAT-negative, WAT-AIHA are not substantially different from cases with a positive reaction. The cases may be primary, without a predisposing or associated disease, or they may be associated, for example, with a lymphoproliferative disease or an autoimmune disease, such as systemic lupus erythematosus. The cases may be mild or severe. They may respond to glucocorticoids or splenectomy or they may not. Table 1 contains the earliest laboratory results reported on each patient. In some cases, progression led to more severe anemia and a more elevated reticulocyte count and hyperbilirubinemia as the course continued. These findings are in keeping with the natural history of the disease, regardless of the isotype of the antibody or the affinity or density of the antibody sensitization of the red cell. In a comparison of 154 patients with DAT-negative and 62 cases of DAT-positive WAT-AIHA in Japan, the former subjects had a statistically significant lower white cell count, reticulocyte count, mean red cell volume, serum total protein, and serum haptoglobin concentration than patients with DAT-positive, WAT-AIHA; but, the mean differences were quantitatively small and the standard deviations sufficiently great that the differences would be of no value in assessing an individual case. There was no difference in distribution by age, gender, primary versus secondary type, or frequency of accompanying thrombocytopenia and-or neutropenia (Evans syndrome). The mean red cell IgG density was approximately an order of magnitude less in DAT-negative patents (179 IgG molecules/red cell) than in DAT-positive patients (1397 IgG molecules/red cell) [46].

Alternative diagnostic considerations

In an adolescent or adult, severe anemia (<9 g/dL) with spherocytosis, reticulocytosis, and low or absent serum haptoglobin and a negative DAT is unlikely to represent hereditary spherocytosis. Although the latter disorder may be severe, such cases are usually detected in infancy or early childhood. Hereditary spherocytosis detected in adolescence or adulthood is usually mild and can be confirmed by other tests or family studies. The spherocytic hemolytic anemia that may be the prodromal phase of, or accompany, Wilson disease, or the intense spherocytic hemolytic anemia resulting from *Clostridium perfringens* α-exotoxin (lecithinase-C) are rare. For each, their affirmative diagnosis can be made by appropriate studies. The latter hemolytic anemia is profound and, usually, accompanies overt sepsis related to amniocentesis (amnionitis), abortion, liver abscess, biliary tract disease or surgery. It is not a subtle event. The blood film may contain lysed red cells (ghosts) and blood hemoglobin (relatively higher) out of proportion to the hematocrit (relatively lower), as a result of the profound intravascular lysis of red cells. The acute hemolytic anemia of Wilson disease, although often described as spherocytic, has contracted cells with small irregular projections that reflect Heinz body formation as a result of the oxidative effects of copper on hemoglobin. Zieve syndrome associated with severe, recent alcoholism, fatty liver, hyperlipidemia, and hemolytic anemia, also, is classified by some as a spherocytic hemolytic anemia but the red cells are usually not predominately classical spherocytes with smooth edges, decreased diameter on the blood film, and hyperchromatic symmetrical shapes. In addition, the clinical setting in which one finds Zieve syndrome, recent alcoholism, makes this consideration apparent. Paroxysmal nocturnal hemolytic anemia is another consideration. In one study, over 15% of patients with unexplained DAT-negative hemolytic anemia were found to have paroxysmal nocturnal hemoglobinuria by cell flow cytometry studies. This testing should look for the reduction or absence of CD59 on red cells and marked reduction or absence of fluorescently-labeled aerolysin, a protein produced by *Aeromonas hydrophila* that binds specifically and with high affinity to cell proteins that are anchored in the membrane by glycosylphosphatidylinositol, and, in this case, to these anchor proteins on normal neutrophils. An important consideration in the differential diagnosis of spherocytic hemolytic anemia is a delayed transfusion reaction. The laboratory findings may closely simulate an AIHA, including a positive DAT, as a result of alloimmunization.

The sensitivity of the direct antiglobulin test

Most commercial direct antiglobulin test reagents have a sensitivity that is above the threshold for red cell sensitization by IgG capable of inducing immune hemolysis. That threshold is not precise; it varies with the commercial antiglobulin reagent used. One reagent was not shown capable of detecting fewer than 500 IgG molecules/red cell [48], although another report found the reagent was not capable of detecting fewer than 150 IgG molecules/RBC [20]. A level of antibody sensitization below 150 molecules/red cell is capable of inducing hemolysis, as shown initially by the studies of Gilliland and colleagues of autoantibodies [14,15] and by Mollison and Hugh-Jones studying alloantibodies [49]. Kajii and colleagues, using an immunoradiometric assay, reported a mean IgG molecules/red cell of 133.1 in 64 cases of DAT-negative, WAT-AIHA as compared to 58.8 in non-immune types of anemia [44]. They had previously reported a mean of 33 IgG molecules/red cell in 100 healthy adults [50].They considered a value of 335 ± 72 (s.d.) IgG molecules/red cell as required to result in a positive DAT using a commercial reagent. They also concluded that a quantitative technique should be used to evaluate patients suspected of DAT-negative, WAT-AIHA and a statistical cut-off of 78.5 IgG molecules/red cell used to distinguish WAT-AIHA from other types of anemia with non-specific elevations in red cell IgG [44]. Since this approach does not have 100% sensitivity and specificity, the clinical features of the hemolytic anemia should be integrated into any diagnostic algorithm.

Although there is an imperfect correlation between the strength of the antiglobulin reaction and hemolytic rate, this relationship is also a function of the nature of the antibody. As early as 1955, Evans, using serial dilutions of the DAT reagent, showed that most patients had a decrease in strength of the reaction with either a partial or full remission of their immune hemolysis, but the test usually remained positive, often at a high titer, and sometimes was unchanged when hemolysis had abated. Reaction strength also varied either during the disease or a remission, unrelated to degree of hemolysis [11]. This finding has been confirmed repeatedly and speaks to other factors influencing hemolytic potency in addition to red cell antibody density or affinity, such as

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Table 1
Reports of direct antiglobulin test-negative, warm-antibody, autoimmune hemolytic anemia confirmed by an alternative test.

Year & citation	No of pts	Age (yrs)	Sex	Hb (g/dL) or Hct (ml/dL)	White cell count (10^9/L)	Platelet count (10^9/L)	Retic count (%)	Blood film	Total serum bilirubin (mg/dL)	Serum LDH (IU/L)	HPTG (mg/dL)	Spleen size	Confirming test
Case reports													
1971 [14]	7	22	F	32	NR	NR	5.6	NR	4.5	NR	NR	NR	CFAC
		22	M	38	2.0	14	3.0	NR	NR	NR	NR	NR	CFAC
		29	F	35	NR	NR	9.2	NR	NR	NR	NR	NR	CFAC
		29	M	18	NR	NR	38	NR	Jaund	NR	NR	EN	CFAC[a]
		37	M	27	Normal	Normal	52	Sphero	NR	NR	NR	500 g	CFAC[a]
		40	M	28	NR	NR	9.0	Sphero	NR	NR	NR	NR	CFAC
		55	F	18	NR	NR	27	NR	NR	NR	NR	NR	CFAC
1971 [21]	4	25	F	10	10.4	106	64	NR	1.5	1150	0	EN	Anti-IgA
		40	M	26	16.4	44	20	NR	1.5	455	<5.0	EN	Anti-IgA
		52	F	25	5.6	446	19	NR	NR	725	5.0	NP	Anti-IgA
		63	F	23	9.0	210	23	NR	1.1	680	0	NR	Anti-IgA
1972 [22]	1	45	F	5.6	6.0	185	34	Sphero	2.3	NR	20	EN	Anti-IgA
1974 [23]	2	12	F	5.8	13.3	129	44–60	Sphero	4.0	NR	0	NP	EP
		18	F	6.4	16.4	180	38–50	Sphero	2.7	NR	NR	EN	IAGT-pap
1975 [78]	1	49	F	10	20	150	18	NR	NR	NR	8	EN	IgM DAT
1976 [15]	9	3.5	F	29	NR	NR	8.4	NR	NR	NR	NR	NR	CFAC
		16	F	21	NR	NR	8.5	NR	NR	NR	NR	NR	CFAC
		28	M	25	NR	NR	15.6	NR	NR	NR	NR	NR	CFAC
		43	F	30	NR	NR	6.6	NR	NR	NR	NR	NR	CFAC
		54	F	28	NR	NR	26	NR	NR	NR	NR	NR	CFAC
		66	F	29	NR	NR	12	NR	NR	NR	NR	NR	CFAC
		66	M	27	NR	NR	8.1	NR	NR	NR	NR	NR	CFAC
		72	M	30	NR	NR	10.6	NR	NR	NR	NR	NR	CFAC
		76	F	17	NR	NR	20	NR	NR	NR	NR	NR	CFAC
1978 [24]	1	9	M	6.2	19.3	238	31	NR	2.2	NR	0	NP	Anti-IgA
1978 [25]	1	20	M	8.7	NR	NR	8.0	Sphero	4.3	389	0	EN	Anti-E in red cell eluate (concentrated) (IAGT)[b]
1981 [26]	1	26	F	12	NR	NR	18.6	NR	4.5	1220	15	NP	Anti-IgA
1982 [27]	1	71	F	8.6	14.4	467	13,5	Sphero	2.9	502	10	NP	Anti-IgA
1984 [28]	1	71	M	23	15.5	155	11.1	Sphero	5.5	1900	NR	ME	Anti-IgA
1984 [79]	1	75	F	7.3	11.0	NR	25.8	NR	3.7	860	0	NE	AAT
1986 [29]	1	5	M	5.9	19.4	508	34	Sphero	3.1	NR	0	NP	Anti-IgA
1987 [30]	1	51	F	10.6	9.1	733	28	Sphero	NR	575	20	NP	Anti-IgA
1990 [31]	1	28	F	5.2	11.1	NR	12	Sphero	5.5	NR	NR	EN	Anti-IgA

1992 [32]	1	54	F	5.0/8.5	NR	NR	9.8	NR	3.6	496	NR	NR	CSW
1996 [33]	5	21	F	4.2	23.0	308	230[c]	Sphero	Jaund	NR	<0.1	NP	Anti-IgA
		55	F	5.6	35.6	400	260[c]	Sphero	Jaund	NR	<0.1	NP	Anti-IgA
		59	F	9.1	4.2	360	217[c]	Roul	NR	NR	<0.1	NP	Anti-IgA
		65	F	5.8	13.1	513	210[c]	Sphero	NR	NR	<0.1	NR	Anti-IgA
		69	F	6.8	8.8	175	980[c]	Sphero	NR	NR	<0.1	NR	Anti-IgA
1997 [34]	2	8	M	3.3	NR	NR	30.6	NR	4.3	NR	<0.1	EN	CSW
		73	M	8.9	NR	NR	30	NR	NR	NR	<0.1	EN	CSW
1998 [35]	1	54	F	6.3	3.1	204	5.1	Sphero	14.9 (I)	588	<0.3	NP	IRMA
1999 [36]	1	0.4	NR	3.4	NR	NR	41	Sphero	5.1	NR	NR	EN	CSW
2001 [37]	1	49	M	8.5	NR	NR	6.0	NR	7.0	1000	NR	NR	FC
2003 [38]	1	48	F	7.4	N	N	29.5	Sphero	INC	INC	DEC	NP	Anti-IgA
2004 [20]	3	17	M	7.6	7.6	NR	10.8	Sphero	7.2	NR	NR	2 cm	CFAC
		20	M	8.7	NR	NR	8.0	NR	4.3	389	0	EN	IAGT
		49	M	5.7	DEC	DEC	20	Sphero	1.6	NR	0	4 cm	CFAC
2006 [39]	5	15	F	5.0	NR	NR	20	NR	4.8	1013	NR	NR	FC
		17	F	7.1	NR	NR	3.0	NR	3.8	937	NR	NR	FC
		31	F	6.5	NR	NR	2.0	NR	2.4	834	NR	NR	FC
		54	F	9.2	NR	NR	2.5	NR	3.3	695	NR	NR	FC
		62	F	8.3	NR	NR	9.0	NR	4.1	678	NR	NR	FC
2011 [40]	1	19	F	5.2	NR	NR	8.9	Sphero	1.5	1715	NR	NR	PEG
2013 [41]	1	36	M	4.3	13.1	194	36	Macro NRBC	7.5	1200	NR	NR	Anti-IgA
Serology laboratory reports													
1978 [19]	11	NR	6F 5M	Mean RBC count = 2.1×10^9/L	NR	NR	NR	NR	NR	NR	NR	Nr	IAGT-pap
1987 [42]	10	0–6	7F 3M	Mean = 6.8	Mean = 10.2	NR	Mean = 16.5	Abn (Sphero or Poik)	Mean = 11.6	Mean = 371	Median < 0.2	4 of 10	IgM RIAT
2004 [20]	27	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	CFAC[d]
2006 [43]	16	0–80	6F 10M	Mean = 6.7	NR	NR	NR	NR	NR	245	NR	NR	Acid eluate
2008 [44]	64	NR	NR	LOW	NR	NR	INC	NR	INC	INC	DEC	NR	IRMA
2012 [45]	50	18–75	30F 20M	Mean = 7.0	NR	NR	Mean = 3.7	NR	INC	Mean = 718	NR	NR	FC
2013 [46]	154	50. ± 25.8	84F 70M	8.0 ± 2.4	6.4 ± 3.6	175 ± 139	8.8 ± 11	NR	NR	577± 560	11 ± 2.8	NR	IRMA

Laboratory data at time of first admission or first data reported. AAT, automated anti-globulin test; CFAC, complement-fixation antibody-consumption test; CSW, cold saline wash; DAT, direct antiglobulin test; DEC, decreased; EN, enlarged; EP, erythrophagocytosis and adherence to macrophages in a skin window; F, female; FC, flow cytometry; Hb, hemoglobin; Hct, hematocrit; HPTG, haptoglobin; (I), indirect bilirubin; IAGT, indirect antiglobulin test; IAGT-pap, indirect antiglobulin test of eluate of patient's red cells coating papain-treated AB-negative red cells; INC, increased; IRMA, immunoradiometric assay for red cell IgG; Jaund, jaundice; LDH, lactic dehydrogenase; M, male; Macro, macrocytosis; No, number; Poik, poikilocytosis; Pt, patients, NR, not reported; NRBC, nucleated red cells; PEG, reticulocyte-enriched polyethylene glycol method; retic, reticulocytes; radioimmune antiglobulin test; Roul, rouleaux; Sphero, spherocytes.

[a] Intermittent positive direct antiglobulin test.

[b] Patient had autoimmune hemolytic anemia with negative direct antiglobulin reaction responsive to glucocorticoids with an anti-E eluted from his erythrocytes despite an E-negative red cell phenotype. Thought to be an auto-anti-Hr [25,47].

[c] Expressed as 10^9/L.

[d] These 27 cases were sent to a reference laboratory based on a negative direct antiglobulin test and a clinical disease compatible with warm-antibody type, immune hemolytic anemia. In the reference laboratory, none of the samples had a positive direct antiglobulin test for IgG, although 11 of the 27 had a weakly to strongly positive test for C3d. All 27 had IgG on their red cells as measured by the complement fixation antibody consumption test [20]. See text for further details.

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Table 2
Confirmatory tests for an immune basis of warm-antibody, direct antiglobulin (Coombs) test-negative hemolytic anemia.

I. IgG not detectable by direct antiglobulin test
A. Flow cytometry to detect red cell IgG [11,39,45,48,51,52]
B. Enzyme-linked anti-IgG assay [54-56]
C. Augmented sensitivity tests:
Direct polybrene (hexadimethrine bromide) (potentiating media) test [27,57,58]
Direct polyethylene glycol (potentiating media) test [40]
Povidone (polyvinylpyrrolidone)-augmented antiglobulin test [27,59,60]
D. Eluate with papainized red cells [19,23]
E. Concentrated eluate (increased ratio of antibody to antigen) assay [43,57]
F. Radiolabeled anti-IgG or staphylococcus protein A determination [44-50,61,62,85]
G. Column agglutination or gel test [39,45,53,63-65]
H. Complement fixation antiglobulin consumption test [14,40]
I. Monocyte monolayer assay [57,66-69]
II. Low affinity IgG antibodies
A. DAT after 4 °C or low ionic strength saline red cell wash [36,57]
III. Isolated IgA or monomeric IgM warm-reacting antibodies
A. Test with anti-IgA antisera [21,22,24,26–29,31,33,38,41,42,57] or anti-IgM or IgM radioimmune antiglobulin test [57,78-80]
IV. Natural-killer-cell-mediated hemolysis
A. ^{51}Cr-release assay (NK cell destruction of autologous but not ABH-matched allogeneic red cells) [69]

molecular subtype of Ig, steric orientation of Ig on the red cell surface, or the rate of removal of sensitized red cells in vivo (e.g. opsonin–macrophage interaction and efficiency) [20]. Thus, patients in remission from WAT-AIHA may have strongly positive DAT reactions. Garratty has discussed, comprehensively and with erudition, the matter of the insufficiency of red cell antibody-density as an explanation of hemolytic severity [51].

Efforts to standardize antiglobulin reagents, their application, and the interpretation of the agglutination reaction date back over five decades [18]. Chaplin described in detail the other technical problems that could result in a false positive or false negative DAT reaction because of inadequate performance of the test or inadequate controls in specific circumstances [18]. The variation among antiglobulin reagents, also, plays a role in the sensitivity of the test [20].

Mechanisms of hemolysis in cases with negative direct antiglobulin reactions

Three principal causes of WAT-AIHA with a negative DAT have been described: a) IgG sensitization below the threshold of detection of the commercial anti-IgG reagent, b) low-affinity IgG antibody sensitization, with red cell antibodies lost during the preparative washes before the direct antiglobulin reaction, and c) sensitization with an IgA antibody, sometimes shown to have specificity for an Rh blood group antigen, or rarely, warm-reacting, (monomeric) IgM without complement fixation, which many commercial DAT reagents cannot identify because they only contains anti-IgG and anti-C3.

Tests to determine antibody sensitization

Several tests have been used to confirm the presence of antibody sensitization of red cells in cases thought to be WAT-AIHA with a negative DAT and they are shown in Table 2 [14,19,21–24,26–29,31,33,36,38,40,41,45–47,52-70]. These tests are not usually performed in hospital clinical hematology laboratories, so that a confirmed diagnosis of WAT-AIHA in which the standard DAT is negative cannot be made in many instances.

The complement-fixing, antibody consumption test was the first to establish quantification of IgG molecules adsorbed to normal red cells (<35 IgG molecules/red cell) as well as on the red cells of patients with DAT-negative, WAT-AIHA (70–434 IgG molecules/red cell) [14,15]. It established that a low density of IgG can be hemolytic and that the DAT had a low, but measurable, rate of false negative reactions in this circumstance. In Table 1, we show the findings of 18 patients, aged 3 to 72 years who had immune hemolysis, low levels of red cell IgG antibodies, a negative DAT, but a positive complement-fixing, antibody-consumption test [14,15,20]. Table 1 also shows 27 cases that were sent to a reference laboratory for testing based on a negative DAT and a clinical disease compatible with WAT-AIHA [20]. In the reference laboratory, none of the patient samples had a positive DAT for IgG, although 11 of the 27 had a weakly to strongly positive test for C3d. All 27 had IgG on their red cells as measured by the complement-fixing, antibody-consumption test. The normal value in that laboratory was equal to or fewer than 25 IgG molecules/red cell. The IgG molecules/red cell on the patient samples ranged from 54 to 1400. The median value was 166 IgG molecules/red cell and 22 of 27 patient samples had fewer than 400 molecules/red cell [20].

Subsequent methods have been described to detect IgG-sensitized red cells below the threshold of the commercial direct antiglobulin test reagent (Table 2). Enzyme-linked anti-IgG assays and a variety of augmented sensitivity tests can be used. The “polybrene” or hexadimethrine bromide test is sensitive and effective for this purpose [57], and is nearly as sensitive as flow cytometry. However, it, like other methods listed in the table, is not generally available in clinical laboratories and cases should be referred to a reference laboratory if identification of the specific immunological sensitization is required. In a study of 800 specimens from patients with suspected DAT-negative, WAT-AIHA, a positive result for red cell sensitization was observed in 431 samples by one of several methods [70]. Fifty percent of 800 samples were positive using an antiglobulin reagent containing IgG, C3, or both. The results in the latter cases were often at or less than 1+ reactivity. This expert laboratory often found weak, but positive, DAT reactions in samples sent from less experienced laboratories that had interpreted the reaction as negative. The direct polybrene test was positive in 15 or 2.2% of 667 samples tested and the gel anti-IgG test was never the sole positive test. This laboratory stopped the use of the latter test in evaluation of suspected DAT-negative AIHA [70].

A second, less common, explanation for the occurrence of a negative DAT in patients with immune hemolysis is the presence of a low-affinity IgG antibody. Low affinity IgG has been associated with severe hemolysis [34,36]. These antibodies may be removed from the red cell surface during preparative washing of the cells at 37 °C or at room temperature. Cold washing with isotonic saline at 0 to 4 °C or with low ionic strength saline can prevent the loss of IgG from the red cell surface, thereby retaining a positive reaction to the commercial DAT reagent. In one laboratory low-affinity IgG was identified in 4.9% of 761 samples by using a low-ionic strength red cell wash technique maintained at 4 °C using refrigerated centrifuges [70]. Flow cytometric analysis of red cell IgG sensitization is another approach to detect a low-density, anti-red cell IgG antibody in clinical laboratories that have established this procedure [36,39,45,48,52]. Flow cytometry can be calibrated such that it can detect fluorescent-labeled anti-human IgG on red cells at a sensitivity greater than that of the commercial DAT reagent, decreasing the frequency of a false negative antiglobulin test. Appropriate distinctions between normal, abnormal but non-Ig-coated red cells, and Ig-sensitized red cells can be determined [57]. The percentage of fluorescent-labeled red cells and their mean fluorescent intensity then can be measured. Values for these variables have been determined for the red cells of healthy persons, patients with non-immune hemolytic anemias, and patients with DAT-positive, WAT-AIHA. This method has allowed identification of IgG-bound to red cells when the commercial DAT did not [37,39,45,48,52,53]. Although not reported to our knowledge, it should be possible to maintain the red cells at 4 °C until they are fixed for examination in the flow cytometer and, thereby, identify low-affinity as well as low-density IgG molecules sensitizing red cells. An attractive feature of flow cytometry is that many laboratories use this technique for other diagnostic purposes. Flow cytometry can also be used to detect IgA and IgM antibodies on red cells (see next paragraph), because specificity is a problem with these antibodies in an agglutination reaction as

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a result of the presence of heterophile antibodies and anti-light chain cross-reactivity; also, anti-IgA and anti-IgM reagents standardized for a DAT often are not available [20].

A third explanation for a negative DAT in a patient with WAT-AIHA is sensitization with an IgA antibody alone [21,23,24,26,30,31,33,38–41,71] or, rarely, a low molecular weight (monomeric) IgM that does not fix complement. Many commercial DAT reagents contain only anti-IgG and anti-C3. Thus, they cannot detect uncommon cases of IgA autoantibodies, shown occasionally to cause WAT-AIHA [52,72-74]. IgA or IgM may participate along with IgG, and C3 in red cell sensitization in cases of WAT-AIHA, but these red cells react with the DAT reagent containing anti-IgG and anti-C3 [75-77]. In the case of warm-antibody type, IgM antibody [42,57,78-80], the red cells may autoagglutinate spontaneously in the patient's plasma, may agglutinate spontaneously after washing several times and placed in a diluent, and may fix C3, resulting in a positive DAT with a commercial reagent containing anti-C3. Alternatively, cases in which red cells have been sensitized by IgM have occurred without C3 on the red cells and, thus, a negative DAT [57,70,78-80]. In a review of 49 patients who had IgM WAT-AIHA, only three had IgM only on their red cells [80]. In another large series of AIHA, no cases of IgM sensitization were found [70].

Table 1 contains the clinical features of 19 patients with anti-IgA as the genesis of their warm-antibody type, immune hemolysis [21,22,24,26–31,33]. They ranged in age from 5 to 71 years. Perhaps as many as 15–20% of cases of WAT-AIHA may have red cell sensitization with both IgG and IgA antibodies [4,33,34]; these cases are detected by their reaction with the anti-IgG component of the commercial anti-globulin reagent. In three reports, 0.03% (1 of 3625 cases), 0.65% (5 of 772 cases), and 2% (16 of 800 cases) of DAT-negative samples from patients with suspected WAT-AIHA were sensitized with IgA antibody alone [24,33,70]. Some cases of WAT-AIHA mediated by IgA have found the antibody to have specificity for Rh [24] or Rh antigen e [22,26,31], C [33], or D [71]. Some cases of IgA-mediated hemolysis include complement fixation to the red cell by the alternative pathway and, presumably, complement-coated, red-cell-mediated attachment to complement receptors on mononuclear phagocytes [26,33]. IgA has been shown to interact directly with the Fc receptors on mononuclear phagocytes and, perhaps, cytotoxic lymphocytes, thereby mediating hemolysis in the absence of detectable complement fixation, and resulting in a clinical picture indistinguishable from IgG-mediated WAT-AIHA [28]. A subset of mononuclear phagocytes contains receptors for IgA [81]. It is also possible that a very small array of co-sensitizing surface IgG molecules might play a role in macrophage removal [82]. Likewise, clearance of WAT IgM may be mediated by a very low density of IgG or C3 molecules, below detection of the standard DAT.

A fourth, rare, mechanism of “immune” hemolytic anemia, noted in Table 1, is not mediated by antibody sensitization but by natural killer cells. This mechanism was described in a patient with large granular lymphocytic (NK cell) leukemia [69]. The assay used chromium-51-labeled autologous and allogeneic, ABO identical red cells as targets in vitro. When exposed to the patient's purified NK cells, autologous red cells were lysed as determined by chromium-51 release, but the matched, allogeneic red cells were not.

The false positive direct antiglobulin test reaction

In studies of blood donors, otherwise healthy patients without anemia can have a positive DAT reaction. In one study, a positive test occurred at a rate of approximately one in 10,000 donors [83]. These positive reactions may persist for long periods without evidence of AIHA, they may disappear, or, very occasionally, an AIHA may develop later [84]. Other circumstances may, also, be associated with such a phenomenon, such as the coexistence of antiphospholipid antibodies [83]. Cytophilic or non-specific IgG adsorbed from plasma has been confirmed as the cause of a positive test in a proportion of cases by the inability to detect antibodies in the eluates of those samples of red cells. The frequency of a positive DAT to IgG is, approximately, 100 times more frequent in hospitalized patients than in healthy blood donors. This reaction may be a false positive resulting from the adsorption of cytophilic antibody, related to drug use, related to an alloreaction from prior transfusion, or be the result of Ig sensitization. In a small study comparing patients with DAT-positive AIHA, DAT-positivity in healthy individuals without anemia, and DAT-negative healthy individuals, the distribution of mean IgG molecules/red cell were 920, 307, and 54, respectively, with different distributions of IgG1 through IgG4 subclasses among the three groups [85]. The occasion of a DAT-positive reaction in a healthy individual or a patient should not cause diagnostic confusion if the clinical and other laboratory manifestations of WAT-AIHA are absent.

Management

In the absence of availability of (a) an alternative test for red cell sensitization by either low-density or low-affinity IgG, solitary IgA, or monomeric IgM in the absence of detectable C3 or (b) access to a reference laboratory to conduct such tests, but a patient with the clinical findings compatible with DAT-negative WAT-AIHA, the sequence of treatment is the same as for DAT-positive, WAT-AIHA [86-89].

Disclosure

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