

Clinically and/or Serologically Misleading Findings Surrounding Immune Haemolytic Anaemias

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Keywords

AIHA · Positive DAT · Negative DAT · C₃d DAT · IgG DAT · Coombs-negative · Agglutination · Immune haemolysis · Autoimmune haemolytic anaemia

Summary

Autoimmune haemolytic anaemias (AIHAs) are well-characterized disorders. They can be differentiated from one another and from other non-immune haemolytic anaemias by clinical, laboratory and serological testing. However, several misleading clinical presentations and/or serological findings may result in misinterpretation, delay and/or misdiagnosis. Such failures are avoidable by adequate clinical and serological experience of the responsible physicians and serologists or, at least, by an optimised bidirectional communication. As long as this has not been achieved, unpleasant failures are to be expected. A true diagnosis of AIHA can neither be verified by clinical nor serological findings alone. Thus, a collective clinical and serological picture remains obligatory for fulfilling the criteria of optimal diagnosis and therapy. Ultimately, the majority of pioneer scientific and practical work in this field stems from scientists who were simultaneously involved in both the clinic and serology.

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Introduction

With the exception of untreated paroxysmal nocturnal haemoglobinuria (PNH), the unifying characteristic of all immune haemolytic anaemias is a positive direct antiglobulin test (DAT).

However, this finding is not always associated with autoimmune haemolytic anaemias (AIHAs), and immune haemolysis cannot always be excluded when the DAT remains negative. In general, results obtained by the DAT are of little or no value without relevant clinical information and detection of ‘true’ autoantibodies (aab) directed against autologous RBC antigens being capable of causing RBC destruction. In suspected cases, several aspects should be taken into consideration: medical history, signs, start, course, haemolytic character (extra- and/or intravascular haemolysis), current infections, co-morbidities and use of drugs. If haemolysis is recognisable, the DAT results are not only helpful in the verification of AIHA but also in its differentiation. In general, AIHAs are classified as warm, cold, paroxysmal cold (Donath-Landsteiner), mixed, and drug-induced. Each form is usually characterised by clinical and serological findings [1–5]. Confusion may occur for each type, but most commonly in AIHA of the warm type and in drug-induced forms.

In this article, the most relevant misleading findings will be addressed and critically discussed.

AIHA and Negative DAT

Several reports have repeatedly emphasised the occurrence of negative DAT in AIHA of the warm type, and the use of other methods for the detection of the causative autoantibodies (aab) in such cases [6–14]. The reasons behind this finding are attributed to either a low concentration or affinity of the aab, which may still play a role when the patients’ RBCs are washed prior to testing. In our experience, these aab can easily be detected when unwashed patients’ RBCs are tested, i.e. by using the gel or glass microcolumn technique. However, such aab are at times rather difficult to characterise. These aab may be considered as significant only if haemolysis is recognisable and the course of haemolysis is typical for AIHA.

In rare cases, the causative aab are transiently detectable in the serum and eluate or only in the eluate, but not by DAT [2, 3, 9, 12, 15, 16]. However, no eluate technique is currently available that invariably allows the detection of all types of aab [17–19]. In suspected cases, where the causative aab are not detectable by one method, at least one different technique should be used (fig. 1). An alternative method would be flow cytometry or other sensitive methods [10, 11, 13, 20]. By use of gel cards and different elution techniques, we were able to detect the causative aab in all cases tested in our laboratory during the last two decades.

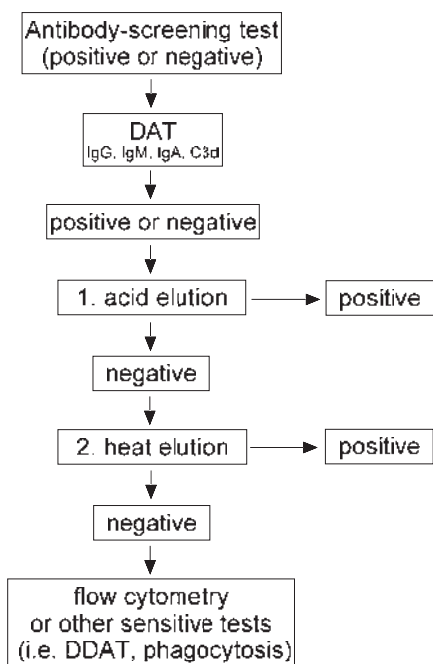
The reason why causative aab are not directly detectable by DAT in isolated cases but in the eluate is presumably the presence

of diminished antigens on autologous RBCs as a response to the haemolytic attack. In fact, such affected patients do survive haemolysis even without treatment, as long as the expression of the involved antigen has not been restored. This phenomenon is most frequently observed in patients with aab to Gerbich antigens [15, 21, 22].

Additional reasons for the occurrence of a negative DAT in AIHA of the warm type are non-complement-activating IgM or IgA aab. The former aab could be detected by sensitive methods rather than by agglutination tests [23]. In comparison, IgA aab may escape detection when anti-IgA is not included in the applied test system [9, 14, 15]. To confirm the nature of true IgA aab, the eluate from the patients' RBCs should also be tested by the indirect antiglobulin test using anti-IgA [15].

In conclusion, when sophisticated testing is employed, the causative aab in AIHAs, unlike those responsible for autoimmune thrombocytopenia, are detectable in the vast majority, if not all affected patients.

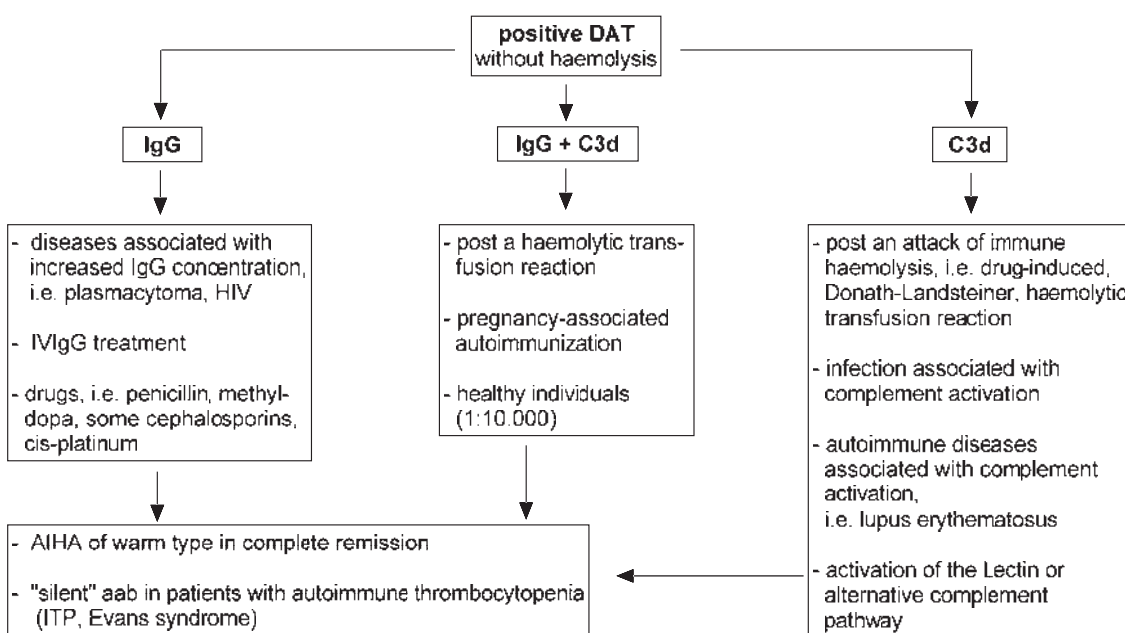
Fig. 1. Testing procedure in all cases suspected to have any kind of immune haemolytic anaemia. Antibody screening test and elution should invariably be included. In isolated cases, the causative autoantibody could be detected in serum and/or eluate but not on patient's RBCs (negative DAT). DDAT = Dual direct antiglobulin test.



Positive DAT in the Absence of Haemolysis

The observation of positive DAT in the absence of signs of immune haemolysis is relatively common. The significance of this phenomenon is evident in all laboratories dealing with RBC serology. Unfortunately, the vast majority of laboratories has only little or no information regarding the patients' underlying disease(s) and/or treatment. In such cases, the interpretation of a positive DAT remains largely speculative at the serological side. If the affected patient does not have signs of haemolytic anaemia, the positivity observed may be related to a number of in vivo occurrences that may result in an IgG- and/or C₃d-positive DAT (fig. 2).

Fig. 2. Most relevant instances observed to be associated with positive DAT without haemolysis at time of serological testing.



IgG-Positive DAT without Haemolysis

Given the fact that the RBC membrane is negatively charged and IgG molecules are positively charged, these cells are susceptible for unspecific IgG attachment onto their surfaces, leading to an IgG-positive DAT [25]. This may occur in cases where the IgG concentration is significantly increased due to the underlying disease, i.e. plasmocytoma and HIV, or due to treatment with immunoglobulins [3, 24]. In addition, these preparations may also contain antibodies to A and B RBC antigens. These antibodies may sometimes cause mild or, in rare cases, severe haemolysis [26]. Furthermore, some drugs may cause unspecific IgG adsorption onto RBCs, e.g. cephalosporins [3, 24, 27, 28]. In conclusion, less than 1% of patients with an IgG-positive DAT may have immune haemolysis [3, 24].

Drug-Induced IgG aab to RBCs

Some drugs may stimulate the production of IgG aab to RBCs, which do not always result in immune haemolysis. This phenomenon has most commonly been observed in patients who receive high doses of alpha-methyl dopa over a sustained period of time. A small number of these patients may develop haemolysis [3, 27, 28]. The list of drugs which have been implicated in causing a positive DAT is on the rise [27, 29]. Interestingly, in almost all cases, drug-induced aab do not appear to cause complement activation [2, 3, 27, 29]. The incidence of drug-induced aab is obscure, as these aab are either insignificant or result in immune haemolysis, which is usually indistinguishable from the 'so-called' idiopathic form [2, 3, 27, 29].

Drug-Induced IgG-Positive DAT

Penicillin is the prototype of an IgG-positive DAT. These antibodies are penicillin-dependent, do not cause complement activation and, in rare cases, may result in haemolysis [3, 27, 29]. Similarly, older generations of cephalosporins have infrequently been reported to cause unspecific IgG adsorption and drug-dependent IgG antibodies similar to penicillin-dependent antibodies [3, 27, 29]. The true incidence of such antibodies is unknown, as they are insignificant or remain uncharacterised.

Pregnancy-Induced IgG aab to RBCs

Pregnancy-related alloimmunisation is well-known, whereas little is known about autoimmunisation. The importance of this phenomenon is reflected by two aspects: Firstly, pregnancy-induced aab to RBCs are, unlike those from patients with true AIHA, usually harmless for both the mother and child; and secondly, their detection may result in confusion and inadequate responses at the serological and clinical side. We have previously described this phenomenon in 2001 [30] and again in 2015 [31]. The question why these aab do not appear to cause significant haemolysis is ob-

scure. Most importantly, alpha-methyl dopa is currently the standard drug for treatment of hypertension in pregnant women. Thus, in case of a positive DAT in pregnancy it has to be clarified whether or not this positivity might be related to the drug.

Transfusion-Induced aab to RBCs

RBC transfusion may stimulate not only the production of alloantibodies, but concomitantly aab or aab alone. Transfusion-induced aab may occur following serological transfusion reaction without significant haemolysis or following haemolytic transfusion reaction, and may persist for a long time in the circulation and/or on patients' RBCs (positive DAT and/or eluate). Thus, a sophisticated medical history regarding previous blood transfusions is indicated in all cases. We first recognised this phenomenon in 1984 [32], and since then this finding has been supported by various groups [3, 33, 34]. This type of aab appears to occur for short or long periods of time in patients who develop haemolytic transfusion reactions, leading to confusion with true AIHA. A typical example is described in this issue [22]. This phenomenon had previously led to the assumption that patients with AIHA frequently develop alloantibodies, which should be considered prior to RBC transfusion in patients with AIHA [35].

Agglutinating aab

Agglutinating aab are usually of the cold and only rarely of the warm type [1–3]. The vast majority of these belong to the IgM class. However, IgG and IgA aab may also infrequently cause direct RBC agglutination. In all cases of agglutinating aab, the results obtained by DAT cannot be correctly interpreted. In addition, the classification and characterisation of such aab are associated with difficulties that may be overcome either by treatment with dithiothreitol (DDT) or inhibition of eluates with specific antibodies [36].

Positive C₃d DAT in the Absence of aab to RBCs

One of the main prerequisites in understanding immunohaematology is an at least basic knowledge of the complement system, its functions and, most importantly, its interaction with RBCs. These cells express CD35 (CR1/C₃b receptor), CD55 (decay accelerating factor; DAF), and CD59 (membrane inhibitor of reactive lysis) complement regulatory proteins. The former protein plays a central role in understanding C₃d-positive DAT results. This component is the inactivated form of C₃b which is involved in phagocytosis, formation of C₃ convertase, and stimulation of complement activation [2, 3, 37, 38]. Thus, C₃d-coated RBCs represent cells which survive complement activation either by the classical pathway, i.e. due to drug-dependent antibodies or alloantibodies to RBCs, or due to activation of the alternative pathway in the presence of adjacent RBCs. While generation of C₃b via the classical

pathway is usually associated with haemolysis, the generation of C₃b via the alternative pathway does not cause significant haemolysis. The phenomenon that adjacent RBCs are susceptible to free C₃b in the circulation was demonstrated in 1984 [39] and later in 1992 [40]. This may give an explanation for the finding that DAT is positive with anti-C₃d in 8% of hospital patients. In comparison, all patients with AIHA of the cold or the Donath-Landsteiner type have a positive C₃d DAT. Patients with AIHA of the warm type show positive DAT with anti-IgG and C₃d in 50–60%, and with anti-C₃d in <13% [2, 3]. However, the true incidence remains speculative due to many diagnostic pitfalls.

Evidence Suggesting C₃-Independent C₅ Activation

The observation that complement activation, via the classical and the alternative pathway, invariably results in coating of adjacent RBCs with C₃d, but reactive haemolysis due to activation of the terminal complement components (C₅b-9) does not result in positive C₃d DAT can only be explained by direct activation of C₅. This hypothesis is supported by a previous report on one patient who developed intravascular haemolysis, similar to that observed in PNH. In the aforementioned case, we demonstrated C₅b-9 complexes on the RBC membrane, but did not observe any C₃d on survived RBCs [41]. Recent findings have demonstrated C₅ activation without C₃ activation [42]. Presumably, the finding in the former publication and the debate related to negative DAT in patients with PNH have led to the detection of DAF and, subsequently, CD55 and CD59 deficiencies on RBCs of affected patients [43–45].

Confusion of Cold Agglutinins with Warm Agglutinins and vice versa

Agglutinating cold aab with high thermal amplitude and a relative low titre may sometimes lead to confusion with warm IgM aab, and vice versa, i.e. agglutinating warm aab may be confused with cold agglutinins [36, 46]. In such cases, eluates from patients' RBCs (fig. 1), and a comparison of haemolysis tests at room temperature and 37 °C are usually helpful. While cold haemolysins are active at 20 °C but not or only weakly (dependent on temperature amplitude) at 37 °C, warm aab are predominantly active at 37 °C. In addition, in vivo agglutinations due to warm aab, remain stable at core temperature, for example, skin discolouration [46]. Finally, eluates from pre-warmed and washed (at 37 °C) RBCs of patients with AIHA of the cold type remains negative.

Donath-Landsteiner aab with AIHA of the Warm Type

The Donath-Landsteiner aab are usually quite weak and rarely strong enough to cause not only a positive C₃d DAT but also IgG DAT. In both cases, haemolysis is infrequently confused with AIHA of the warm type. However, Donath-Landsteiner haemolysis typically

occurs in children and rarely in adults. In addition, the disease is usually acute and spontaneously reversible within several days [47].

Additive-Dependent Agglutination

Several chemicals that are used as preservatives in commercial serological reagents, such as albumin, EDTA, citrate and low-ionic strength saline, have been implicated in causing anomalous blood grouping [48–55]. This phenomenon is characterised by the discrepancy between reactions of serum samples with RBCs in the presence and in the absence of the causative chemical. For example, the antibody screening test is positive and the cross-match is negative, or the autocontrol is positive but both the DAT and eluate are negative. The positive reactions are usually strong and may lead to confusion with alloantibodies directed against a high-frequency antigen or with agglutinating aab.

Negative Eluates

As discussed above, none of the available or used eluate techniques are capable of capturing the causative aab in all cases. The use of more than one technique is indicated in suspected cases (fig. 1). In our experience, this is the case in most patients with AIHA related to warm IgM aab, and in roughly 3% of all affected patients [2, 45].

Transplantation-Associated Immune Haemolysis

Many patients may develop mild or severe haemolysis following transplantation of haematopoietic stem cells, and less frequently, solid organs. In the former case, haemolysis is most often related to ABO blood group mismatches. Notably, these antigens are inherited independently of the HLA complex. In addition, some patients may develop aab and/or alloantibodies which may cause significant haemolysis [3]. The alloantibodies might be produced by the donor or recipient cells. Haemolysis related to the donors' antibodies is known as passenger lymphocyte syndrome [3, 57, 58], which usually persists for a short amount of time. In contrast, transplantation-associated aab may cause severely persistent AIHA.

Non-Immune Haemolytic Anaemia

Frequently, patients with hereditary or acquired non-immune haemolytic anaemia might initially be suspected to have AIHA. However, the latter form can usually be excluded in the absence of aab and/or C₃d on patients' RBCs [1–3].

Disclosure Statement

The author declares no conflict of interest.

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