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Evidence Suggesting Complement Activation and Haemolysis at Core Temperature in Patients with Cold Autoimmune Haemolytic Anaemia

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Summary
Background: It is unclear why haemolysis may somewhat persist in patients with cold autoimmune haemolytic anaemia (cAIHA) at 37 °C (core temperature). **Methods:** Seven patients with cAIHA were included in this study. Serological testing was performed using standard techniques. Bound autoantibodies (aab) on patients' RBCs were analysed by the direct antiglobulin test (DAT), dual antiglobulin test (DDAT) and flow cytometry (FC) using pre-warmed RBCs (37 °C). Temperature-dependent complement binding was determined by incubation of patients' serum samples with group O RBCs and fresh serum complement. **Results:** The DAT was strongly positive with anti-C3d in all cases, independent of season and outside temperature. Haemolysis usually improved during warm periods of time, but decompensated following febrile infections, and persisted throughout the year, though exposure to the cold was strictly avoided. In addition, trace amounts of IgM aab were infrequently detectable on patients' RBCs even at 37 °C, and complement activation was demonstrated following incubation of RBCs with the causative aab at 37 °C. **Conclusions:** Binding of trace amounts of IgM aab at 37 °C may provide an explanation for the durable C3d-positive DAT and haemolysis in patients with cAIHA.

Introduction

Based on clinical and serological findings and historical aspects, autoimmune haemolytic anaemia (AIHA) can be classified into four types [1–5]: i) warm type (optimal reactivity of autoantibodies (aab) detected at core temperature (37 °C)); ii) cold type (optimal reactivity of aab at 0–4 °C, decreasing with increasing temperature, and abolished at 37 °C); iii) mixed type (both warm and cold aab are simultaneously involved); and iv) cold type in paroxysmal cold haemoglobinuria (Donath-Landsteiner AIHA) in which the aab are usually of IgG class with a low titre, but are able to strongly activate complement.

Haemolysis in patients with AIHA of the cold type (cAIHA) is extremely variable. In general, the significance of aab in these patients appears to be dependent on their thermal amplitude (the highest temperature at which the aab still exhibits in vitro reactivity with RBCs) rather than on their concentrations [6–10]. The vast majority of affected patients have aab with thermal amplitude below 30 °C. Cold aab with higher thermal amplitude have only been described in isolated patients with a fatal outcome [5, 8, 10]. Moreover, haemolysis may persist even when the patients are strictly kept warm [6–10]. Thus, other factors, i.e. febrile infections, may trigger haemolysis [10, 11]. Based on our experience, haemolysis in affected patients may improve but does not completely abolish at high temperatures, i.e. in summer. The question why haemolysis persists despite high temperatures and strict avoidance of cold weather remains obscure. This phenomenon cannot easily be explained by either a high titre or high thermal amplitude of aab [8, 12]. The question how cold agglutinins cause haemolysis at core temperature has not yet been addressed.

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Table 1. Most relevant data of studied patients

Patient no.	Age, years	Sex	Diagnosis cAIHA	Observation period, years	Haemoglobin*, g/dl	LDH*, U/l
1	80	f	primary	3	6.1–11.1	259–826
2	59	f	primary	3	8.7–11.2	283–759
3	77	m	primary	2.5	11.8–13.2	319–392
4	64	m	secondary[a]	1.5	7.6–15.9	192–1,396
5	72	f	primary	2	8.4–11.2	475–685
6	46	m	primary	6	10.0–13.9	229–442
7	62	f	secondary[b]	4	6.7–10.4	450–1,748

LDH = Lactate dehydrogenase.
*Minimal and maximal value during observation period.
[a]Underlying disease: multiple myeloma.
[b]Underlying disease: Waldenström's macroglobulinaemia.

Table 2. In vitro complement-binding assay using different incubation mixtures

Experiment	Test sample	Reagent from donor	Temperature, °C	EDTA
1	patient's serum	complement (serum)	37	after incubation
2	patient's serum	complement (serum)	28	after incubation
3	patient's serum	complement (serum)	20	after incubation
Ctr 1	patient's serum	complement (serum)	37	prior to incubation
Ctr 2	donor serum	complement (serum)	37	after incubation
Ctr 3	patient's plasma	plasma	37	after incubation

Ctr = Control.

Patients and Methods

Patients

The 7 patients included in this study were diagnosed with primary (n = 5) or secondary (n = 2) cAIHA (table 1). Active haemolysis commonly observed for cAIHA was present in all patients with detectable cold aab and abnormal laboratory parameters for haemolysis, including anaemia, undetectable haptoglobin, increased bilirubin and lactate dehydrogenase.

Serological Testing

Serological testing including antibody screening, direct antiglobulin test (DAT), titre and thermal amplitude of aab, the haemolysis test, and the dual antiglobulin test (DDAT) for detection of weak IgM aab were performed as described elsewhere [13–15]. Briefly, for DDAT, patients' RBCs were incubated with rabbit IgG anti-human IgM. After washing, cells were incubated with goat anti-rabbit IgG as a secondary antibody, and then examined for agglutination using Liss/Coombs gel cards (Biorad, Cressier sur Morat, Switzerland). in order to avoid any exposure to cold prior to serological testing, the blood samples from patients were collected in our laboratory under strictly controlled conditions. If indicated, samples were immediately incubated at 37 °C and washed with pre-warmed saline.

Flow Cytometry

The RBCs of 6 patients (patients no. 1–6) were tested for class IgM antibodies following pre-incubation and washing at 37 °C or 20 °C. For standardisation, a control was prepared by coating RBCs with a human monoclonal IgM anti-Jk(a) at different titres [16].

Briefly, 10^6 RBCs in 50 µl phosphate-buffered saline (PBS) were incubated with 5 µl rabbit anti-human anti-IgM Phycoerycin (PE; Southern Biotech, Birmingham, AL, USA) for 30 min at room temperature. After washing with PBS, cells were adjusted to 10^6/ml and analysed by flow cytometry (MACSQuant® Flow Cytometer, Miltenyi Biotech, Bergisch Gladbach, Germany). Approximately 10,000 events were collected for each sample, and the mean fluorescence intensity was determined.

Temperature-Dependent Complement Binding Assay

To assess the in vitro interaction between cold aab and donor RBCs, fresh serum and EDTA blood samples (blood group O) were collected from healthy blood donors. After washing O RBCs with PBS, 20 µl of the cells (haematocrit 50% in PBS) were incubated for 15 min at 37 °C, 28 °C or 20 °C. Serum or plasma of patients and donors were pre-warmed to the same temperatures. After careful temperature equilibration, 100 µl patients' serum (containing cold aab) and 100 µl donors' serum (containing fresh complement) were added to the pre-warmed RBCs (table 2). After incubation for 30 min at the defined temperatures, 10 µl EDTA (1.1%) was added to stop complement activation. The cells were centrifuged and the supernatant examined for haemolysis. Cells were washed and analysed with the antiglobulin test using gel cards with monospecific AHG serum (anti-C3d (1) and anti-IgM, DC screening I; Bio-Rad) and anti-C3d (2) (DAKO, Hamburg, Germany), diluted 1/40, or NaCl.

Another patient (no. 8) with harmless anti-I aab (thermal amplitude of 4 °C and normal clinical and laboratory findings) was included as control in this assay.

Results

Serological and Clinical Data

All patients presented here had chronic and active cAIHA. The titres and thermal amplitudes of the causative aab were strongly or moderately increased (table 3). All patients had strongly positive DAT with anti-C3d, but only 1 patient had positive DAT with anti-IgM. The DDAT was positive in all patients. However, when DAT or DDAT were performed after washing three times strictly at 37 °C, IgM was not detectable (data not shown). Heat and acid eluates were also negative. The C3d positivity did not appear to be dependent on the outside temperature. All patients were informed to avoid any exposure to the cold. Indeed, by doing so, haemolysis

Table 3. Most relevant serological findings

Patient no.	Aab titre		TA*, °C	DAT				DDAT	Haemolysis	
	4 °C	20 °C		IgG	IgM	IgA	C3d		20 °C (RBC/E-RBC)**	37 °C (RBC/E-RBC)**
1	512	4	28	–	+/–	–	++++	+	+/+	–/+
2	256	4	22	–	–	–	++++	+	+/+	–/+
3	128	4	22	(+)	–	–	++++	+	+/+	–/–
4	256	8	25	–	–	–	+++	+	+/+	–/–
5	4,096	64	26	–	–	–	++++	+	+/+	–/–
6	16	2	24	(+)	–	–	+++	+	+/+	–/–
7	64	,2	25	–	–	–	++++	+	+/+	–/–

Aab = Autoantibody; TA = thermal amplitude; DAT = direct antiglobulin test; DDAT = dual antiglobulin test; E-RBC = enzyme-treated red blood cells.

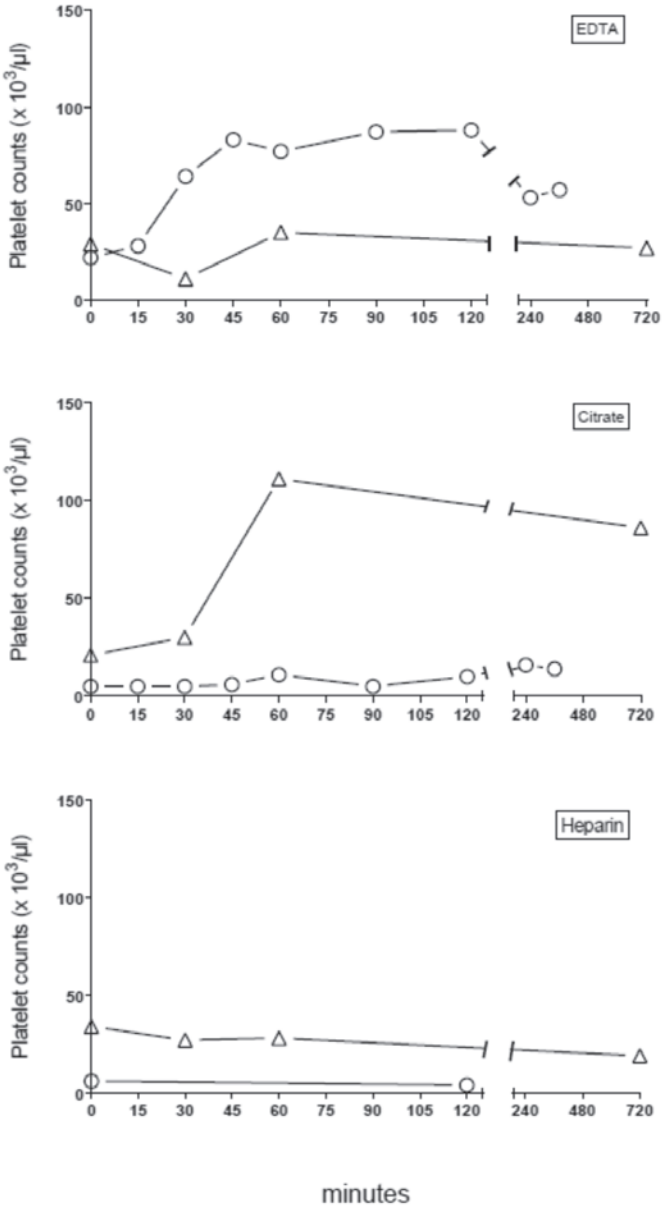

Fig. 1. Laboratory data of patient no. 1 during the observational period of 3 years. Upper panel: Filled diamonds: haemoglobin in g/dl; circles: lactate dehydrogenase in U/l; RBC: RBC unit transfusion. Lower panel (x): mean daily temperature in patients' living area in °C.

Table 4. Flow-cytometric analysis of IgM bound to RBCs

Patient no. / sample	Mean fluorescence intensity (arbitrary units)		IgM binding
	37 °C	20 °C	
1	1.0	5.6	–
2	1.2	1.8	(↑)
3	1.3	1.6	(↑)
4	1.0	4.3	–
5	1.0	4.6	–
6	0.7	0.6	–
Anti-Jk(a) 1:200		11.9	↑↑↑↑
Anti-Jk(a) 1:400		6.0	↑↑↑
Anti-Jk(a) 1:800		3.6	↑↑
Anti-Jk(a) 1:1,600		2.0	↑
Anti-Jk(a) 1:3,200		1.3	(↑)
O RBC (NC)		0.9	–

NC = Negative control; ↑↑↑↑ = very strong; ↑↑↑ = strong; ↑↑ = moderate; ↑ = weak; (↑) = very weak.

particularly improved during summer, but signs of haemolysis remained recognizable throughout the year (fig. 1). During observation, haemolytic attacks occurred mostly after exposition to cold and sometimes following febrile infections. Blood transfusions were required in four patients. Patients no. 1, 2 and 4 had 1–4 transfusion episodes with 1–2 transfused RBC units, and patient no. 7 required more than 100 RBC units.

FACS Analysis

Mean fluorescence was significantly increased in the standardisation control up to an IgM anti-Jk(a) titre of 3,200 (table 4). In comparison, two samples from patients' RBCs (no. 2, 3) that were pre-warmed and washed at 37 °C showed an increase of mean fluorescence of about a similar size than the weakest standardised control. In the other four cases (patients no. 1, 4, 5, 6), the increase of mean fluorescence was not significant as compared to the negative control. In contrast, at 20 °C, IgM was detectable in five of six cases. Patient no. 6 showed only weak reactivity at 4° C as can be seen by the low aab titre at 4 °C (table 3). This may explain the negative results in FACS analysis at 20 °C and 37 °C.

Table 5. In vitro complement binding on RBCs incubated with patients' serum samples and complement at different temperatures

Patient no.	Temperature, °C	Haemolysis	AGT	
			anti-C3d (1)	anti-C3d (2)
1	37	–	3+	4+
	28	+	4+	4+
	20	+	4+	4+
2	37	–	2+	4+
	28	+	3+	4+
	20	+	4+	4+
3	37	–	4+	4+
	28	+	4+	4+
	20	+	4+	4+
7	37	–	3+	4+
	28	+	3+	4+
	20	+	4+	4+
8*	37	–	–	–
	28	–	–	–
	20	–	–	–
Ctr**	37	–	–	–

AGT = Antiglobulin test; Ctr = control.
*Patient no. 8 with weak cold aab and without haemolysis.
**Controls 1–3 (table 2) were negative for all patients tested.

Temperature-Dependent Complement Binding Assay

In vitro haemolysis of O RBCs was observed with all serum samples of patients no. 1 to 3 and 7 that were incubated at 28 °C and 20 °C (table 5). IgM was not detectable in the monospecific anti-globulin test following incubation with patients' serum independent of the incubation temperature. Despite the fact that thermal amplitude of the causative aab ranged from 22 to 28 °C (table 3), complement binding was detectable in these patients at all temperatures up to 37 °C (table 5). All of the applied controls (table 2) were negative. In contrast to the other patients, control patient no. 8 did not show complement activation at temperatures from 20 °C to 37 °C. The results strongly indicate that IgM cold aab may bind to RBCs at much higher temperatures than suggested by the thermal amplitude obtained by agglutination.

Discussion

It is generally believed that the causative aab in patients with cAIHA bind on patients' RBCs, when a portion of their blood becomes 'cold' in the periphery, resulting in complement activation during rewarming in the circulation. Thus, every effort is attempted to avoid any exposure to low temperatures, which may allow the attachment of the antibodies to their binding sites. However, thermal protection is not invariably effective as has been introduced and demonstrated here. The haemolysis persisted throughout the year, even when patients strictly avoided any exposure to the cold. In addition, the haemolysis may decompensate during febrile infections while core temperature increases rather than decreases. The latter phenomenon might be explained by unspecific stimulation of macrophages or C3b-mediated phagocytosis. This is supported by the fact that haemolysis in patients with cAIHA is largely related to activation of the early (C1–C3) rather than the terminal complement components (C5b–9), resulting in extravascular rather than in intravascular haemolysis [17, 18]. A different explanation might be related to an unspecific antibody production due to the acute infection. Whatever the mechanism may be, the haemolysis observed in patients with cAIHA cannot be solely explained by exposure to the cold. In fact, there is evidence that trace amounts of cold aab do remain attached to RBCs at 37 °C, leading to complement activation. This is supported by the fact that one IgM molecule is required to fix one molecule of C1 [19]. Thus, the possibility that a few IgM aab may remain attached to RBCs at 37 °C is conceivable. Previously, it has been demonstrated that cold antibodies may react with isolated antigens from RBC membrane but not with intact RBCs at 37 °C [20]. In addition, the same authors have measured C3 fixation and lysis of PNH RBCs at 37 °C in the presence of cold aab and complement. Although they did not comment on aab and complement fixation on RBCs at this temperature, in one case 1.5 % of the aab remained attached to the cells, and in both cases described,the antibodies led to complement fixation of 4,500 or 1,000 molecules per cell [6]. Similarly, in our study, complement activation was demonstrated in different ways: i) persisting haemolysis and C3d positivity in vivo, independent of the outside temperature (fig. 1, tables 1, 3); ii) in vitro haemolysis of enzyme-treated RBCs in the presence of aab and complement at 37 °C (table 3); iii) activation of complement at 37 °C using allogeneic RBCs and cold aab from different patients with active haemolysis (table 5); and iv) infrequent detection of trace IgM aab on autologous RBCs by DAT, DDAT and FACS (tables 3, 4).

The question whether these bound antibodies may represent a subpopulation of cold aab with high affinity and high thermal amplitude or weak warm aab remains speculative. We were unable to characterise these antibodies by elution techniques. The second question, whether this 'subpopulation' of aab, if existing, might be responsible for complement activation at 37 °C and persisting haemolysis at core temperature also remains obscure. This hypothesis, however, is attractive in regard to two aspects: such a 'subpopulation' may play a key role in the variability of haemolysis between patients who have similar antibodies but different clinical pictures; and it may have potential impact on treatment. It is unclear why immunosuppressive drugs are infrequently effective in the treatment of patients with cAIHA, a condition resembling that of AIHA of the warm type. Ultimately, isolated patients may develop a mixed-type AIHA [13].

Finally, the question why IgM could not be detected on prewarmed RBCs from all patients studied here, might be explained by weak binding of aab and warm washing procedures prior to testing. Ultimately, each washing is associated with some aab elution from the cells. Thus, the true amount of aab which remain

bound onto the RBCs at 37 °C must be higher than on washed cells. The persistent haemolysis during the year might be explained by binding of IgM aab also at core temperatures. This is supported by positive results obtained by FACS, DAT and DDAT and most importantly by in vitro fixation of complement onto RBCs in the presence of cold IgM aab at 37 °C. Further studies focusing on this point may help understanding the new aspects described and discussed here.

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Disclosure Statement

The authors declare no conflict of interest with regard to this paper.

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