

Involvement of the IgE-basophil system and mild complement activation in haemophilia B with anti-factor IX neutralizing antibodies and anaphylaxis

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Introduction: Patients with haemophilia B who develop factor IX (FIX) neutralizing antibodies (inhibitors) after FIX infusion are at high risk of hypersensitivity reactions upon FIX re-exposure, but the underlying mechanisms are incompletely understood.

Aim: To investigate biomechanisms of FIX hypersensitivity.

Methods: A cellular antigen stimulation test (CAST) was employed to evaluate leukotriene C4 (LTC4) release from basophils stimulated by FIX in three treated children with haemophilia B, one of whom developed FIX inhibitor and experienced anaphylaxis following FIX re-exposure. Anti-FIX IgE and IgG antibodies and markers of complement activation (C5b9, C3d and iC3b) were measured in plasma, the last also after FIX infusion. Ten healthy children served as controls.

Results: The patient who developed anti-FIX inhibitors and anaphylaxis had a non-sense mutation in FIX gene (p.Arg298Stop) and, compared to controls, had higher plasma levels of specific anti-FIX IgE (2.285 vs 0.084 OD^{492 nm}), with marked LTC4 release from his FIX-stimulated basophils (519.8 vs 39.9 pg/mL). Further, he had higher plasma levels of anti-FIX IgG of all the four subclasses (total IgG 1.180 vs 0.120 OD^{492 nm}) with FIX neutralizing activity (1.5 BU); mild complement activation occurred during FIX-induced anaphylaxis (C5b9 increased from 258.5 to 351.1 ng/mL). The same parameters were normal in the two patients who tolerated FIX infusion.

Conclusion: In the patient with haemophilia B who experienced anaphylaxis after FIX, but not in the patients with haemophilia B who tolerated FIX, the CAST assay showed FIX-induced LTC4 release, which was associated with high plasma levels of specific anti-FIX IgE and IgG antibodies.

KEYWORDS

anaphylaxis, complement, factor IX, haemophilia B, immunoglobulin E, leukotrienes

1 | INTRODUCTION

Haemophilia B, a congenital bleeding disorder due to the plasma deficiency of coagulation factor IX (FIX), is rarer than haemophilia A, occurring in approximately 1 in 30 000 live birth males vs 1 in 5000 for haemophilia A.¹ Replacement therapy with clotting factor concentrates

is the mainstay of haemophilia treatment, and the development of neutralizing antibodies against the exogenous clotting factors (inhibitors) represents the main complication of this therapy. The prevalence of inhibitors in patients with severe haemophilia B is around 2%-4%, ie, 10-fold lower than in those with severe haemophilia A.² This difference is attributable to the higher prevalence in haemophilia B of

missense gene mutations rather than the more severe intron inversions frequent in haemophilia A.^{2,3} Other potential tolerogenic mechanisms may be the high degree of homology between FIX and other vitamin K-dependent clotting factors, which would confer some degree of tolerance.⁴ However, in contrast to patients with haemophilia A, those with haemophilia B complicated by inhibitors are at risk of developing hypersensitivity reactions up to anaphylaxis following FIX replacement therapy.⁵⁻⁸ Specific IgE antibodies,^{5,9} responsible for mast-cell activation and anti-FIX IgG1 antibodies,¹⁰ which activate the complement system with generation of anaphylatoxins, have been described in haemophilia B patients with inhibitor. However, the mechanisms underlying the increased risk of hypersensitivity reactions in these patients are still incompletely understood. Classical allergic reactions are due to IgE-mediated activation of mast cells and basophils with release of preformed mediators such as histamine or of newly formed mediators such as leukotrienes and platelet-activating factor (PAF). A functional cellular antigen stimulation test (CAST) based upon the determination of sulphido-leukotrienes (LTC₄, LTD₄, LTE₄) produced by interleukin-3 (IL-3)-primed basophils stimulated *in vitro* by allergens¹¹ has been developed and used to diagnose allergy to drugs, insect venoms, foods and inhaled or occupational allergens.¹¹ With this background and gaps of mechanistic knowledge, we chose to employ this *in vitro* cellular antigen stimulation test for the detection of leukotriene C₄ (LTC₄) release induced by FIX in three patients with haemophilia B, one of whom developed anti-FIX inhibitors and experienced anaphylaxis following FIX infusion. Further, specific anti-FIX IgE and IgG1,2,3,4 antibodies were measured in patients' plasma, and markers of complement activation such as C3d, iC3b and the terminal complement complex C5b9 were evaluated before and after FIX infusion.

2 | PATIENTS AND METHODS

2.1 | Index case description

He is a 2-year-old boy with no family history diagnosed with severe haemophilia B at the age of 16 months due to easy bruising. After diagnosis, he was treated on demand with intravenous infusions of recombinant FIX concentrate (BeneFIX[®], Pfizer Limited, Sandwich Kent, UK), mainly for soft tissues and muscle haematomas and a post-traumatic joint bleed. After a total of 7 days of exposure (EDs) to this replacement product over a period of 12 months, an anti-FIX inhibitor was detected in the occasion of a routine laboratory check. The first measured titre was 2.0 Bethesda Unit (BU), and this value was confirmed at a second test performed 2 weeks apart. In the attempt to induce immunological tolerance, he started regular home treatment with BeneFIX[®] 33 IU/kg 3 times weekly, at a time when his baseline inhibitor titre was 1 BU/mL. After the first 7 infusions, the inhibitor titre rose again to 2.0 BU. On the occasion of the fourth home infusion, the patient experienced fleeting flushing on his face. After 14 additional FIX infusions without complications and symptoms, he experienced face and body flushing, coughing fit and retching in the frame of the next two FIX exposures. The first time these reactions occurred immediately after the infusion end and had a rapid

spontaneous resolution, but at the time of the next infusion, the reaction started during the infusion and resolved only after antihistaminic drugs. In order to investigate the mechanism underlying these hypersensitivity reactions after FIX exposure, we chose to give the next BeneFIX[®] infusion in hospital under medical control in the presence of an anaesthesiologist.

On the occasion of this last infusion of FIX given at a dose of 500 IU (ie 33 IU/kg) administered over 5 minutes (ie 0.8 mL/min), the patient experienced face flushing, coughing fit and retching immediately after the end of the infusion. He had a high heart beat rate up to 120 bpm, dyspnoea with desaturation down to 88% from 99% and gasping, but no apparent bronchospasm. At this stage, oxygen and an intravenous antihistaminic drug (ie clorfenamin 2 mg) were used. After two additional minutes, he developed urticaria on his face with palpable weals on the cheeks and marked swelling of the upper lip. An intravenous corticosteroid (ie hydrocortisone 100 mg) was administered, with partial resolution of symptoms within 5 minutes.

2.2 | Controls with and without haemophilia B

To compare the results of the laboratory tests (see below) with those of the index case, we chose two age-matched children (both 2 years old) with severe haemophilia B who were regularly treated with twice weekly prophylactic infusions of BeneFIX[®] and who had not developed anti-FIX inhibitors. These volunteers were chosen among 44 patients with severe haemophilia B regularly followed at the Angelo Bianchi Bonomi Haemophilia and Thrombosis Center, of whom 10 were younger than 12 years old. In all the three patients, blood samples were obtained before and after FIX administration. In our case-list, no haemophilia B patient with FIX inhibitor and without anaphylactic reactions was available for the study. The genetic analysis of the three patients with haemophilia B involved in the study revealed p.Arg298Stop (nonsense) mutation in the index case, p.Cys95Tyr (missense) and 118-121 delGTTT (deletion in the donor splicing site of intron A) in the other two patients, respectively. Ten healthy children (age range 2-7 years, median 4 years) served as normal controls. The study was conducted following the ethical principles of the Declaration of Helsinki, regulatory requirements and the code of Good Clinical Practice. The parents of the patient and controls gave their written informed consent for blood sampling and laboratory testing at the time of FIX infusion.

2.3 | General laboratory methods

The IgE specific to FIX were measured, and the cellular antigen stimulation test (CAST) was performed as a functional assay of type I hypersensitivity for FIX. Moreover, IgG specific to FIX were measured, and their inhibitory activity on FIX was assessed. Both before and after the FIX infusion followed by anaphylaxis, complement activation markers were evaluated. Skin tests with FIX were not performed in the index case because his parents failed to give consent owing to their fear of a new anaphylactic reaction.

2.4 | Factor IX inhibitor

A Nijmegen-modified Bethesda assay was employed; dilutions of plasma are prepared in imidazole buffer and incubated with an equal volume of normal pooled plasma at 37°C. The control consists of normal pooled plasma incubated with an equal volume of buffer. At the end of the 10 minutes incubation period, residual factor IX was assayed using a standard 1-stage APTT-based assay, and the inhibitor titre was calculated from a plot of residual factor IX activity vs inhibitor units. The dilution of test plasma that gave a residual factor IX closer to 50% but within the range 30%-60% was chosen to calculate the inhibitor titre. A Bethesda Unit (BU) is defined as the amount of an inhibitor that neutralizes 50% of 1 unit of FIX in normal plasma after incubation.

2.5 | Anti-FIX IgE and IgG

Anti-FIX IgE and IgG were measured by sandwich ELISA. Recombinant FIX (BeneFIX[®], Pfizer Limited, Sandwich Kent, UK) was coated overnight onto microtitration plates at a concentration of 10 µg/mL, and after washing, the residual binding sites were blocked with bovine serum albumin 1%. After further washes, plasma dilutions (1:10, 1:100 and 1:1000) from patients and controls were added and incubated for 45 minutes at room temperature. After washing, we added goat anti-human IgE (ε chain specific) (Sigma, St. Louis, Mo, USA) for the determination of specific IgE and, after further washing, donkey polyclonal anti-goat IgG peroxidase conjugated (Santa Cruz Biotechnology Inc, Dallas, Texas, USA). For the determination of specific total IgG, we added a goat anti-human IgG (γ-chain specific) HRP conjugate (Sigma, St. Louis, Mo, USA); for the measurement of IgG subclasses 1, 2, 3, and 4, mouse monoclonal anti-human IgG1 or IgG2 or IgG3 or IgG4 were added, and after further washing, rabbit polyclonal anti-mouse IgG peroxidase conjugated. All the reactions were revealed by orthophenylenediamine (Sigma Chemical, St. Louis, Mo, USA).

2.6 | Cellular antigen stimulation test

This assay measures the amount of leukotrienes, mainly leukotriene C4 (LTC4), produced by blood basophils after in vitro stimulation by an allergen. Six millilitres of peripheral venous blood was collected in acid citrate dextrose (ACD) tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). After centrifugation for 10 minutes at 4°C at 550 g, the buffy coat layer was collected and centrifuged again at 4°C for 10 minutes at 550 g. The supernatant was then decanted and cells resuspended and mixed by gentle shaking with 500 µL of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) calcium buffer (HEPES 20 mM, NaCl 130 mM, KCl 5 mM, CaCl₂ 7 mM, MgCl₂ 3.5 mM, HSA [human serum albumin] 1 mg/mL, pH 7.4), containing interleukin-3 (IL-3) (2 ng/mL) and 10 µL of heparin (5000 UI/mL, Liquemin, Roche, Basel, Switzerland). We prepared four polystyrene tubes for patients and healthy subjects. In the first tube, we added 50 µL of the aforementioned human recombinant FIX at the

TABLE 1 Plasma levels of IgE and IgG to factor IX (FIX), cellular antigen stimulation test (CAST) and inhibitory activity on FIX in three (HB) patients with haemophilia B and ten normal controls. The first patient (HB1) experienced anaphylaxis after FIX infusion

	Anti-FIX IgEOD ^{492 nm}	CAST-ELISA ^a LTC4 release (pg/mL)		Anti-FIX IgGOD ^{492 nm}					FIX Inhibitor Bethesda U
		Buffer	FIX	Total	Subclass 1	Subclass 2	Subclass 3	Subclass 4	
HB1	2.285	92.8	519.8	1.180	0.740	1.259	1.193	0.973	1.5
HB2	0.086	55.3	58.8	0.040	0.156	0.300	0.184	0.262	none
HB3	0.071	50	30.3	0.056	0.170	0.325	0.350	0.302	none
Controls									
median	0.084	45.0	39.9	0.120	0.109	0.261	0.280	0.309	none
min-max	0.071-0.103	33.0-82.5	26.7-59.1	0.025-0.149	0.022-0.157	0.052-0.316	0.051-0.365	0.057-0.365	

OD, optical density; LTC4, leukotriene C4.

^aFinal basophil number/mL: HB1=21 740; HB2=27 320; HB3=24 030; Controls=median 24 670, min-max 20030-29970.

final concentrations of 50 UI/mL. As positive control, we used two tubes (second and third); in the second tube, 50 µL of monoclonal anti-FcεRI antibody (10 µL/mL –Bühlmann, Allschwil, Switzerland) was added. In the third tube, 50 µL of monoclonal anti-IgE antibody (Sigma, St. Louis, Mo, USA) was added at a final concentration of 10 µg/mL. In the fourth tube, 50 µL of stimulation buffer alone was added to evaluate basal values without stimulation. Then, 50 µL of the patient cell suspension and 900 µL of HEPES calcium buffer were added to each tube (final concentrations of basophils ranged between 20 000/mL and 30 000/mL); the tubes were gently shaken, incubated at 37°C for 40 minutes and then centrifuged for 5 minutes at 4°C at 1000 g. Finally, 100 µL of the supernatant was collected and frozen at -20°C for less than 1 month until analysis by ELISA (enzyme-linked immunosorbent assay), following the manufacturers' instructions (CAST-ELISA, Bühlmann Laboratories, Allschwil, Switzerland).

2.7 | Complement activation markers

The complement terminal complex C5b9 was measured in plasma by using the immunoenzymatic method from Quidel Corp. (San Diego, CA, USA); intra-assay and interassay coefficients of variation (CVs) were 6.8% and 13.1%, respectively. As markers of C3 activation, C3d and iC3b were measured by immunoenzymatic methods from MyBioSource (San Diego, CA, USA) and Quidel Corp. (San Diego, CA, USA), respectively. Intra-assay and interassay CVs were lower than 10% for C3d and lower than 14% for iC3b.

3 | RESULTS

3.1 | Anti-FIX IgE and the cellular antigen stimulation test (CAST)

After the FIX infusion, the patient with haemophilia B who developed anaphylaxis showed much higher plasma levels of specific anti-FIX than the two treated haemophilia B patients with no FIX inhibitor and the 10 untreated normal controls (Table 1). The patient with anaphylaxis showed a markedly higher LTC4 release from his FIX-stimulated basophils than the two patients with haemophilia B who

tolerated FIX and the controls. As a positive control, basophils from all subjects (patients and healthy controls) were stimulated with anti-IgE and anti-FcεRI antibodies, showing a marked LTC4 release in all cases (630.0-3200.0 pg/mL).

3.2 | Anti-FIX IgG and FIX inhibition

At the time of blood sampling before FIX infusion, the haemophilia B patient with FIX hypersensitivity reactions had plasma levels of inhibitory activity of 1.5 BU/mL (Table 1), not detected in the two haemophilia B patients without inhibitor nor in normal controls. The patient with haemophilia B who developed FIX anaphylaxis had much higher plasma levels of specific anti-FIX IgG than the two patients with haemophilia B who tolerated FIX and normal controls. The anti-FIX IgG antibodies detected in the haemophilia B patient with anaphylaxis were equally distributed among the four subclasses (IgG1,2,3,4), with much higher levels than the other two patients with haemophilia who tolerated FIX and normal controls (Table 1).

3.3 | Complement measurements

In the three patients with haemophilia B, complement parameters were measured before and after FIX infusion. At baseline, all had normal levels of markers of complement activation (Table 2). The patient who developed anaphylaxis showed a small increase in complement markers 20 minutes after the FIX infusion at the time of the occurrence of anaphylaxis (Table 2): C5b9 from 258.1 ng/mL to 351.1 ng/mL, C3d from 0.099 µg/mL to 0.153 µg/mL and iC3b from 75.0 µg/mL to 106.6 µg/mL. No postinfusion increase in the complement activation markers was observed in the two patients with haemophilia B tolerant to FIX (Table 2).

4 | DISCUSSION

The patient with FIX inhibitor who experienced anaphylaxis had a nonsense mutation in FIX gene, consistently with previous findings that deletions or nonsense mutations abolishing the production of

TABLE 2 Plasma levels of complement activation markers in three patients with haemophilia B (HB 1-3) and 10 normal controls. Patient HB1 experienced anaphylaxis after FIX infusion. In the patients with HB, plasma samples were collected at baseline in all subjects and at different time intervals after FIX infusion in patients with HB (20 min, 2 h and 24 h)

	C5b9 (ng/mL)				C3d (µg/mL)				iC3b (µg/mL)			
	Baseline	20 min	2 h	24 h	Baseline	20 min	2 h	24 h	Baseline	20 min	2 h	24 h
HB1	258.5	351.1	292.0	178.7	0.099	0.153	0.150	0.139	75.0	106.6	101.1	101.8
HB2	199.0	178.6			0.121	0.101			21.1	28.5		
HB3	182.3	191.5			0.104	0.107			24.8	26.7		
Controls												
median	269.4				0.132				24.1			
min-max	114.5-315.4				0.101-0.157				14.1-91.8			

the protein predispose to the development of FIX inhibitors.^{2,12-14} He had specific anti-FIX IgE and IgG antibodies after FIX infusions, but not the two patients who tolerated FIX administration. In the patient who experienced anaphylaxis, basophil-bound IgE induced cell activation after challenge with FIX, as demonstrated by the marked release of LTC₄ by FIX-stimulated peripheral blood basophils. Further, only mild activation of complement was observed during anaphylaxis. Taken as a whole, these findings indicate that in the patient with FIX-induced anaphylaxis, there was an involvement of the IgE-basophil system, with a possible supporting role of complement activation.

The demonstration of specific FIX IgE antibodies in the anaphylaxis patient is in agreement with previous studies carried out in haemophilia B patients with hypersensitivity reactions due to FIX infusion.^{5,9} In addition, we demonstrated that these IgE antibodies were functionally able to mediate the release of LTC₄ by FIX-stimulated peripheral blood basophils by means of a cellular antigen stimulation test (CAST) that measures sulphidoleukotrienes in the supernatant of antigen-stimulated leucocytes from peripheral blood. This test, that can be easily performed and has been largely used for the investigation of allergy to drugs, food and inhaled allergens, was employed for the first time in our haemophilia B patient with anti-FIX and FIX-induced anaphylaxis. Because the parents of our patient did not consent to skin tests, we were seeking for an ex vivo functional test and chose to perform CAST, which was able to reproduce in vitro an allergic reaction with no risk for the patient.

Even though the main IgG subclass of FIX inhibitors in haemophilia B has been reported to be IgG₄,⁴ IgG₁ antibodies have been detected in patients with allergic reactions to factor IX replacement therapy.¹⁰ These findings indicated that IgG₁ antibodies might be implicated in the allergic responses, probably through complement activation. In contrast, IgG₄ subclass antibodies, which are non-precipitating and fail to activate the complement system, exhibited a strong positive correlation with the functional inhibitor assay.⁴ All the four subclasses of anti-FIX IgG were detected in the patient with inhibitor and FIX-induced anaphylaxis. Because IgG₁ and IgG₃ activate the classical complement pathway, their presence might explain the slight complement activation observed after FIX infusion only in the haemophilia B patient with anaphylaxis but not in the two other patients. Complement activation was modest, so that it is unlikely to be the main mechanism involved in the anaphylactic reaction even though it may have played a supporting role.

In conclusion, the patient with haemophilia B carrying a nonsense mutation in the FIX gene, who developed a FIX inhibitor and then an anaphylactic reaction after FIX infusion, had high plasma levels of specific anti-FIX IgE (functionally active on basophils), anti-FIX IgG of all the four subclasses (exerting an inhibitory activity on FIX) and a mild activation of the complement system during the postinfusion anaphylactic reaction. Hence, the CAST assay can be proposed as a diagnostic test for the investigation of hypersensitivity in patients with FIX deficiency, particularly in those unwilling to undergo skin tests.

In our patient, the CAST assay was performed after the hypersensitivity reaction was well established, thus one could argue about its diagnostic utility; however, the marked abnormality of CAST assay in a patient with a demonstrated allergic reaction to FIX imposes the need to evaluate this test before the allergic reaction has developed. Future prospective studies in patients with haemophilia B, evaluated at baseline and in periods during treatment with FIX, may help assessing the predictive value of CAST assay for allergic reactions in these patients.

DISCLOSURES

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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