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Characterization of the anti-factor VIII immunoglobulin profile in patients with hemophilia A by use of a fluorescence-based immunoassay

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Summary

Background—The development of neutralizing antibodies, referred to as inhibitors, against factor VIII (FVIII) is a major complication associated with FVIII infusion therapy for the treatment of hemophilia A (HA). Previous studies have shown that a subset of HA patients and a low percentage of healthy individuals harbor non-neutralizing anti-FVIII antibodies that do not elicit the clinical manifestations associated with inhibitor development.

Objective—Assess HA patients' anti-FVIII antibody profiles as potential predictors of clinical outcomes.

Methods—A fluorescence immunoassay (FLI) was used to detect anti-FVIII antibodies in 491 samples from 371 HA patients.

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Authorship

Contribution: B.B. designed and performed the research, analyzed results, and wrote the paper; A.S.R. performed the research; A. L.D., M. D. T., D.B.B., and J.C.B. provided patient samples and contributed to the manuscript; C.H.M. analyzed results and wrote the paper.

Results—Assessments of antibody profiles showed that the presence of anti-FVIII IgG₁, IgG₂, or IgG₄ correlated qualitatively and quantitatively with the presence of a FVIII inhibitor as reported by the Nijmegen-Bethesda assay (NBA). Forty-eight patients with a negative inhibitor history contributed serial samples to the study, including seven patients who had negative NBA titers initially and later converted to NBA-positive. The FLI detected anti-FVIII IgG₁ in five of those seven patients prior to their conversion to NBA-positive. Five of 15 serial-sample patients who had a negative inhibitor history and a positive anti-FVIII IgG₁ later developed an inhibitor, compared to 2 of 33 patients with a negative inhibitor history without anti-FVIII IgG₁.

Conclusions—These data provide a rationale for future studies designed both to monitor the dynamics of anti-FVIII antibody profiles in HA patients as a potential predictor of future inhibitor development and to assess the value of the anti-FVIII FLI as a supplement to traditional inhibitor testing.

Keywords

Factor VIII; Factor VIII Deficiency; Hemophilia A; immunoassay; Inherited Blood Coagulation Disorders

Introduction

Hemophilia A (HA) is an X-linked inherited bleeding disorder in which coagulation factor VIII (FVIII) is absent or dysfunctional and is most commonly treated by infusion of plasma-derived or recombinant FVIII. A major complication associated with FVIII infusion therapy is that up to 30% of patients develop antibodies that inhibit the function of and/or induce immune dependent clearance of the infused product(1;2). Anti-FVIII antibodies, referred to as inhibitors, diminish the effectiveness of infusion therapy, and, in the case of high titer inhibitors, necessitate the use of FVIII bypassing agents(3) or immune tolerance induction therapy (ITI)(4;5). Patients who develop FVIII inhibitors face an increased risk of bleeding complications(6) and present substantial financial and patient management challenges to the healthcare system(7).

The Bethesda assay(8) for measurement of FVIII inhibitors was developed in 1975 and modified in 1995 to the Nijmegen-Bethesda assay (NBA)(9), which is the gold standard method in use today. The NBA utilizes the degree to which HA patient plasma inhibits the *in vitro* clotting reaction of healthy donor plasma as a means to assign FVIII inhibitor titers. More recently, assays utilizing chromogenic substrates(10), enzyme linked immunosorbent assays (ELISA)(11;12), surface plasmon resonance (SPR) (13;14), and fluorescent immunoassays (FLI)(15-19) have been developed to detect anti-FVIII antibodies in HA patients. Many previous studies have observed that there is some discrepancy between the results obtained from functional assays, such as the NBA, and those obtained using other testing methods(11;12;18). Although the assortment of FVIII inhibitor assays all share the common goal of identifying the presence of anti-FVIII antibodies, they have key fundamental differences that contribute to the generation of discrepant results. The NBA and chromogenic inhibitor (CBA) assays attempt to simulate *in vivo* conditions in order to detect FVIII-specific functional inhibition of the clotting process. For the purpose of these assays, functional inhibition of FVIII-dependent clotting is reflected in decreased extent or kinetics

of an *in vitro* clotting reaction(8;9) or the cleavage of a chromogenic substrate as a surrogate for clotting activity(10), but there is no direct measurement of FVIII-specific immunoreactivity. Alternatively, SPR, ELISA, and anti-FVIII FLI (α FVIII-FLI) inhibitor assays directly detect anti-FVIII antibodies, but do so without any means to assess the detected antibody's ability to inflict functional inhibition on FVIII. These differences, as well as the lack of uniformity among laboratories used to determine what constitutes a positive reaction, make it difficult to integrate the various test results in order to reach a definitive diagnosis of a clinically significant inhibitor.

Previous studies utilizing direct antibody detection methods (11-13;20;21) have shown that the Ig subtype and subclass composition of the anti-FVIII antibody response may be critical in assessing the clinical implications of the immune response. These studies implicated IgG₁ and IgG₄ as the most common anti-FVIII antibody subclasses present in NBA-positive patient samples. The current study investigates the composition of the antibody response in 371 HA patients, the largest group of patients studied to date, using an α VIII-FLI. The study examines the prevalence of anti-FVIII antibodies in HA patient plasma, evaluates the make-up of the antibody response by IgG subclass, and assesses the clinical relevance of antibody subtype by evaluating the extent of correlation between FLI results and those obtained using the NBA.

Materials and Methods

Subjects

The study includes 491 plasma samples from 371HA patients (median/mean age 13/18.5 years) enrolled in the Hemophilia Inhibitor Research Study (22). 20.5% of patients (n=76) and 24.8% of samples (n=122) were NBA positive. Inhibitor measurements were performed using a modified version(23) of the NBA(9). The investigational review boards of the CDC and each participating site approved the protocol, and all participants or parents of minor children gave informed consent. Control samples were obtained from 56 paid healthy donors.

Fluorescence immunoassay

The α VIII-FLI is a modified version of our previously described method(18). Briefly, plasma samples diluted 1:30 in phosphate buffered saline (PBS) containing 1% dried milk (PBSM) were incubated with SeroMAP beads (Luminex Corporation, Austin, TX) coupled to Kogenate FS (Bayer Healthcare, Tarrytown, NY). Anti-FVIII antibodies were detected using serial incubations with biotinylated anti-human Ig (anti-IgG1, A-10650; anti-IgG2, 05-3540; anti-IgG3, MH1532; anti-IgG4, A-10663; anti-IgM, H15015; Life Technologies, Carlsbad, CA) and R-phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) using a Bio-Plex 200 suspension array system (Bio-Rad Laboratories, Hercules, CA). Results are expressed as median fluorescence intensity (MFI). The threshold for positivity was set at two standard deviations above the mean MFI of the results obtained for healthy donors.

Statistical Analyses

Comparisons of FLI and NBA results on individual plasma samples were made using GraphPad Prism (GraphPad Software, San Diego, CA) to generate Spearman's correlation coefficient and two-tailed p values. Fisher's exact test was used to evaluate differences in categorical data.

Results and Discussion

Characterization of anti-factor VIII antibodies in the plasma of hemophilia A patients

HA patient plasma samples were examined for the presence of anti-FVIII IgGs 1-4 and IgM using an α FVIII-FLI (Table 1 and Figure 1). IgG subclass specific analysis of plasma samples showed that 40.5%, 17.3%, 6.1%, and 26.5% of the 491 patient samples were positive for anti-FVIII IgG₁, IgG₂, IgG₃, and IgG₄, respectively, compared to 5.4% (IgG₁ and IgG₂) or 1.8% (IgG₃ and IgG₄) of healthy donors (IgG₁ and IgG₄ $p < 0.0001$; IgG₂ $p = 0.02$; IgG₃ $p = 0.353$). Evaluation of the IgG subclass specific FLI results segregated by NBA status revealed that NBA-positive specimens had significantly higher rates of positivity than NBA-negative samples for anti-FVIII IgG₁, IgG₂, IgG₃, and IgG₄ ($p < 0.0001$) (Table 1; Figure 1). Rates of anti-FVIII IgM positivity were not significantly different in patients (3.9%) compared to healthy donors (7.1%) ($p = 0.285$).

In order to assess the relative importance of each subclass of anti-FVIII IgG in patients with FVIII inhibitors, we analyzed the IgG subclass specific FLI results to determine the composition of the FVIII antibody response in NBA positive samples. The results show that 98.4% of the NBA positive samples had positive FLI titers for one or more subclasses of anti-FVIII IgG, including 13.9% that were positive for a single subclass of anti-FVIII IgG, 84.4% that contained multiple subclasses of anti-FVIII IgG, and the remaining 1.6% had no FLI detectable anti-FVIII antibodies (Table 2). All of the 120 NBA-positive samples that also tested positive by FLI contained anti-FVIII IgG1 and/or IgG₄, and 101 (84.2%) were positive for both IgG₁ and IgG₄. Both of the NBA positive/FLI negative results were on samples with low titer inhibitors (0.7 and 0.8 NBU), and one of these samples was previously reported to be a false positive due to its negative result by CBA(18).

Linear correlations were calculated according to Spearman to evaluate the relationship between titers obtained from the α FVIII-FLIs and NBA. The α FVIII-FLIs for IgG₁ and IgG₄, which were positive in 92.6% and 88.5% of samples, respectively, exhibited a strong positive correlation with NBA titers ($r(\text{IgG}_1) = 0.5438$, $r(\text{IgG}_4) = 0.5766$; $p < 0.0001$). Correlations between FLI and NBA results were weak, yet significant for anti-FVIII IgG₂ ($r = 0.3411$; $p < 0.0001$) and IgG₃ ($r = 0.2829$; $p < 0.0001$), while anti-FVIII IgM did not exhibit a quantitative correlation with NBA results (Table 1).

Anti-FVIII IgG composition in serial samples from individual hemophilia A patients

Sixteen patients exhibited a change in NBA inhibitor status over the course of specimen collection. Seven of those patients (patients 1-7) had negative NBA titers on their initial study specimen, but later developed a positive NBA reaction following FVIII infusion therapy for the indicated exposure days (Table 3). Examination of FLI results on plasma

samples from these seven patients revealed that five of them harbored one or more classes of anti-FVIII Igs in samples prior to developing an inhibitor detectable by the NBA (Table 3, patients 1-5). All of these five patients were positive for anti-FVIII IgG₁ prior to their conversion from NBA negative to NBA positive; one was also positive for anti-FVIII IgG₄ (patient 5) and one for IgM (patient 4). Analysis of the FLI results on 201 samples from all 81 patients who contributed multiple specimens (data not shown) showed that 5 of 15 (33.3%) patients with a negative inhibitor history and a positive IgG₁ result later developed an inhibitor, compared to 2 of 33 (6.1%) patients with a negative inhibitor history without anti-FVIII IgG₁ antibodies (p=0.0239). Patients 8-16 (Table 3) all have a history of inhibitors and are of interest due to the transitory nature of their NBA positivity. It is important to note that while overall the FLI results for anti-FVIII IgG₁, IgG₂, IgG₃, and IgG₄ displayed significant positive correlations with the NBA, FLI and NBA results on serial samples from individual patients did not necessarily change proportionally with time. The lack of intra-patient consistency is probably attributable to the differing role of kinetics in the two assays, and may also reflect changes in the patient's immune response over time.

Positive FLI results on samples with a corresponding negative NBA result were present in a low percentage of samples tested for anti-FVIII IgG₂₋₄, occurring in 3-9%, while disparities for anti-FVIII IgG₁ were more common, with positive FLI results occurring in 23.3% of NBA negative samples. These discrepant results may be caused by the presence of anti-FVIII antibodies that are of insufficient titer to render an inhibitory effect on coagulation in the NBA, the presence of anti-FVIII antibodies that recognize epitopes that are insignificant to the functional integrity of the FVIII molecule, or non-specific or indirect antibody binding to the FVIII coupled beads. Our data on serial samples drawn from 81 patients support the first hypothesis. While it is important to note that patients harboring non-neutralizing antibodies may never progress to developing an inhibitor, one-third of 15 patients who had a negative inhibitor history and were positive for IgG₁ converted from NBA negative to NBA positive over the course of the sample collection, compared to only 6.1% of patients with a negative inhibitor history without anti-FVIII IgG₁. These findings, although preliminary, suggest that NBA-negative patients with IgG₁ anti-FVIII antibodies are more likely to develop inhibitors detectable by the NBA than patients without such antibodies, and that these patients may merit closer scrutiny (e.g., patients undergoing surgical procedures) or more frequent follow-up testing (e.g., patients receiving initial FVIII infusions) to facilitate prompt clinical intervention.

The identification of anti-FVIII antibodies in HA patients is an important clinical development, but results presented here and by others have shown that the mere presence of antibodies does not always correlate with the clinical manifestations of FVIII inhibition (11;12;16-19;24;25). Identifying the underlying features that distinguish cases of benign and/or transient anti-FVIII antibodies from those that are clinically relevant anti-FVIII inhibitors is an important area of research. Although it remains unclear why the presence of certain antibody subclasses may be predictive of a worse clinical outcome, the data presented herein support those from a recently published study by Whelan et al.(12) in which the authors used an ELISA to show that anti FVIII IgG₁ and IgG₄ were present in 19 of 20 inhibitor positive HA patients. They also found that IgG₄ was completely absent in 77 non-inhibitor patients and 600 healthy individuals, and that anti-FVIII IgG₁ was present in

19% and 6% of non-inhibitor HA patients and healthy individuals, respectively(12). Whelan et al. hypothesized that their data could indicate the presence of variations in immune regulatory pathways in the different study cohorts. Previous studies that examined the potential link between SNPs in immune response genes and a predisposition to inhibitor development(26-30), and the results from the current study with a larger patient population using different methodology support this hypothesis. In addition, our data illustrate that IgG₄ may be present in a low percentage of patients lacking inhibitors as measured by the NBA including 2.5% (7 of 283) of patients with a negative inhibitor history(data not shown) and that anti-FVIII IgG₁ production may be an early checkpoint in inhibitor development. Taken together, these data provide a rationale for future clinical studies designed to monitor the dynamics of HA patients' anti-FVIII antibody profiles in order to assess their value as predictors of future development of clinically relevant inhibitors and to determine the usefulness of the α FVIII FLI as a supplement to traditional inhibitor testing methods.

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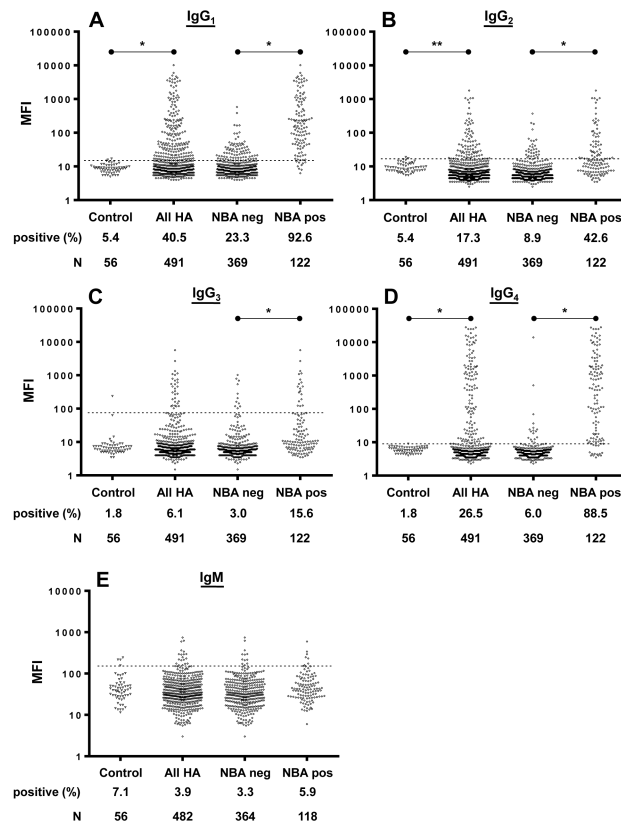


Figure 1. *Fluorescence immunoassay (FLI) results for anti-FVIII antibodies in plasma from hemophilia A (HA) patients and healthy controls. Individual data points represent plasma samples assayed for anti-FVIII IgG₁ (A), IgG₂ (B), IgG₃ (C), or IgG₄ (D), or IgM (E). Results are displayed on a log-scale for control plasmas from healthy donors, all HA patient samples, and the subsets of HA patient samples with negative or positive Nijmegen-Bethesda (NBA) results for each Ig measured. The dashed line, which represents the assay’s positive threshold, is two standard deviations above the mean median fluorescence intensity (MFI) of 56 control samples from healthy donors. The number of samples (n) and the percentage of the samples that tested positive are as indicated. * p<0.0001; **p=0.02*

Table 1

Summary of positive fluorescence immunoassay (FLI) results for anti-FVIII antibodies segregated by Ig subclass

	n	% Positive for Anti-Factor VIII by FLI				
		IgG ₁	IgG ₂	IgG ₃	IgG ₄	IgM*
Healthy donors	56	5.4	5.4	1.8	1.8	7.1
All HA specimens	491	40.5	17.3	6.1	26.5	3.9
NBA-negative HA specimens	369	23.3	8.9	3	6	3.3
NBA-positive HA specimens	122	92.6	42.6	15.6	88.5	5.9
Correlation of FLI and NBA		0.5438 p<0.0001	0.3411 p<0.0001	0.2829 p<0.0001	0.5766 p<0.0001	0.0643 p=0.1589

*n=482 HA specimens, 364 NBA-negative, and 118 NBA-positive

Table 2

Fluorescence immunoassay (FLI) results on 122 NBA-positive specimens

FLI Result	NBA-Positive Samples	Number of FLI-Positive Samples			
	Percent (n)	IgG ₁	IgG ₂	IgG ₃	IgG ₄
Negative	1.6 (2)	0	0	0	0
Positive for one subclass of IgG	13.9 (17)	10	0	0	7
Positive for two subclasses of IgG	40.2 (49)	49	1	1	47
Positive for three subclasses of IgG	32.0 (39)	39	37	2	39
Positive for four subclasses of IgG	12.3 (15)	15	15	15	15

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Anti-factor VIII fluorescence immunoassay (FLI) results on serial plasma draws from hemophilia A patients who exhibited a change in Nijmegen-Bethesda assay (NBA) status over the course of sample collection. A grey background highlights positive results.

Table 3

Pt.	severity	Draw date	Median fluorescence intensity units (MFI)					NBU	Exposure days
			IgG1	IgG2	IgG3	IgG4	IgM		
1	mild	12/5/07	5.5	4.5	5	6	11.5	0.1	0-20
		9/10/08	11.5	7	7	6	84.3	0	
		9/16/09	25.5	5.5	6	5	17	0	
		4/21/10	1093.3	7	191.5	8	38	1.7	0-20
		6/9/10	4646.8	46	332.3	85	60.8	1.3	
9/21/10	386.5	7.5	20.5	96.8	29	1.8			
2	severe	8/9/10	22	5	4.5	3.3	39.5	0.1	0-20
		10/11/10	4111.8	42.3	612	1921	34	3.2	21-50
		11/18/10	827	10	28.8	1109	83.5	18.7	
		2/8/11	3352	43.5	44	1277	25.5	7.2	
		3/9/11	234.5	7.5	10.3	262	25	1.4	
3	severe	10/1/08	75.3	5	8	4.5	90.5	0	0-20
		9/22/09	441.3	15.5	8.3	1592	85.3	13.6	0-20
4	severe	7/23/08	37.5	8.3	5.8	6.5	746.5	0.2	0-20
		7/8/09	16.8	5.8	5.8	4	69	0	
		6/2/10	240.5	9	8	792.3	173.8	3.9	21-50
5	severe	8/6/08	33	6	3.5	9	25.5	0.3	21-100
		8/12/09	48.5	12.8	6.5	14.5	53.8	1.4	>150
		8/14/09	11	6	3.5	10.5	46	1.4	
		6/30/10	6	6.8	4	3.5	58.8	0	
6	mild	3/3/10	10.5	4.3	6	4.5	109.3	0.1	0-20
		5/27/10	504.8	11.8	73.5	12.3	597.5	1.4	0-20
		6/14/10	3914.5	111.3	746.8	114.5	103	1.7	

No history of inhibitor

Pt.	severity	Draw date	IgG ₁	IgG ₂	IgG ₃	IgG ₄	IgM	NBU	Exposure days
		11/14/12	7.5	5.5	5	4.5	70.8	0.1	
		2/5/07	7	4.5	3.5	4	ND	0	101-150
7	severe	6/18/08	34	6.8	4.8	1193.8	39	6.5	101-150
		6/17/09	51.5	7	6.8	1276.5	248.8	3.8	
		7/5/06	249.5	12	13	8548.3	32	19.3	ND
8	severe	7/23/08	7	5.5	5	5	42	0.2	
		3/15/06	10	5.3	4	11.5	231	0.5	ND
9	severe	5/7/08	14.5	6.3	6.5	18.5	110.3	0	
		5/6/09	9	8	5.8	7	93.8	0	
		9/5/07	157.3	11	6.8	27	26.5	1.1	ND
10	severe	9/5/12	41	6.3	5.5	27.3	16	0.4	
		6/17/08	35.8	5.8	6.5	39.5	42.5	0.5	ND
11	severe	6/17/09	38.5	10.5	8.5	21	15.8	0.3	
		6/16/10	19.5	4.5	5.5	22.5	25	0.3	
		4/12/06	15.5	4	4.5	4	54.8	0.5	ND
12	severe	4/23/08	16.5	6	4	6	15.5	0.4	
		4/29/09	8	8.5	5	4	46.5	0	
		12/15/08	66.3	96.5	12	542	37	0.8	ND
13	mild	3/4/09	10.8	58.3	6.5	14	40.5	0	
		11/16/07	85.5	10	5.8	1527	15	24.6	ND
14	severe	9/25/09	14.5	5	4	9.8	37	0.3	
		6/2/10	337.5	92.3	398.5	145.8	95.5	3.3	
15	severe	2/6/08	240.8	55.5	1341.5	85.3	41.5	3.9	ND
		4/8/09	16	6.5	14.5	69	23.5	0.2	
16	severe	10/10/07	48	25	31	510	20.8	0.3	ND

Previous history of inhibitor

Pt.	severity	Draw date	Median fluorescence intensity units (MFI)					NBU	Exposure days
			IgG ₁	IgG ₂	IgG ₃	IgG ₄	IgM		
		12/5/08	13.3	9	7	207.5	26.5	0.6	
Threshold for Positivity			14.6*	16.1*	75.5*	8.3*	153.6*	0.5	

ND- No data collected

* Mean + 2sd of 56 healthy donors