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ORIGINAL ARTICLE *Inhibitors*

A flow cytometry evaluation of anti-FVIII antibodies: correlation with ELISA and Bethesda assay

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Summary. In this study, we describe a flow cytometry (FC) system for detecting antibodies to factor VIII (FVIII) and compare its results with those of enzyme-linked immunosorbent assay (ELISA) that detects both inhibitory (I-Ab) and non-inhibitory (NI-Ab) antibodies and the Nijmegen modification of the Bethesda method, detecting I-Ab. FC was set up in our laboratory. Recombinant FVIII (rFVIII) was coupled to microspheres (FVIII-m) and reacted with different plasma dilutions. Microspheres without rFVIII were used as control (control-m). Captured anti-FVIII antibodies were detected using anti-human IgG. Plasma samples from the following patients with severe haemophilia A (SHA) patients were evaluated: 17 P (patients without I-Ab, <0.5 BU mL^{-1}); 13 PI (patients with I-Ab, 1.1–8200 BU mL^{-1}). Of these 13, two PI were referred during immune tolerance induction (ITI), and plasmas from 12 healthy donors (HD) were evaluated. Semi-quantitative results were given as an index (the highest mean fluorescence intensity ratio between FVIII-m and control-m multiplied by the inverse of the corresponding plasma dilution). Both plasma and serum were suitable for the test. FC agreed with the Bethesda method (r = 0.8; P = 0.0001). FC and ELISA had 80% of coincidence. Four of 17 patients (23.5%) had NI-Ab by FC, and two of them developed high levels of I-Ab later on. This test provides a useful alternative for measuring FVIII antibodies supplementing Bethesda assay. FC is fast and easy to perform. No more than 200 μL of plasma or serum is required especially making it useful for paediatric patients.

Keywords: flow cytometry, FVIII antibodies, haemophilia A, microspheres

Introduction

Haemophilia A patients receiving replacement therapy can develop an inhibitory antibody (I-Ab) response to transfused FVIII and this is the major complication impairing the efficacy of treatment. Their prevalence varies from 20% to 30% of severe haemophilia A (SHA) patients [1]. Development of inhibitors is associated with an increase in treatment cost and serious clinical complications. Immune tolerance induction (ITI) therapy is the only strategy that has been proven to successfully eradicate FVIII inhibitors.

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Inhibitory-Ab are routinely detected by the Bethesda method with the Nijmegen modification [2] which is carried out in plasma and measures the capacity of antibodies to neutralize the procoagulant activity of FVIII, expressed in Bethesda units (BU). A positive Bethesda assay registers levels above 0.5 BU mL^{-1}. Low responders have I-Ab <5 BU mL^{-1} and do not develop an increase in I-Ab levels after further exposure to FVIII [3]. High responders have I-Ab titres >5 BU mL^{-1}. Quantification of low-titre I-Ab by the Bethesda assay can be difficult, because of its lack of sensitivity. This method may be affected by the presence of thrombin inhibitors, heparin or lupus anticoagulants [4]. Moreover, it requires relatively large citrated plasma volumes and additional technical time when multiple dilutions are necessary. Non-inhibitory antibodies (NI-Ab) interacting with non-functional sites of FVIII are not detectable by this assay. The clinical relevance of antibodies unrecognized by the Bethesda assay has not been well elucidated. They may prevent the interaction between FVIII and other molecules of the coagulation cascade or

form immune complexes [5]. It has been suggested that NI-Ab may contribute to disease immunopathogenesis by influencing clearance of FVIII from blood circulation [6] but more studies in this area are needed.

A number of published reports have utilized enzyme-linked immunosorbent assay (ELISA) to detect I-Ab and NI-Ab [6–10]. These NI-Ab may be found in haemophilia A patients without I-Ab (15–38%). Natural anti-FVIII antibodies, which ranged between 0.4 and 2 BU mL^{-1} by Bethesda and generally low levels by ELISA, were also detected in plasma from approximately 10–20% of healthy donors (HD), [6,7,11–13]. Results of the ELISA method generally agreed with that of the Bethesda functional method, particularly when I-Ab were present at high titres. However, detection and quantification of low level anti-FVIII antibodies have been controversial and requires a more sensitive technique. The suggestions given in the last World Congress of Haemophilia (2008-Istanbul) were to employ both the Bethesda assay and ELISA to monitor the immune response to FVIII in the haemophilia A patients.

We took advantage of flow cytometry techniques to design a flow cytometric (FC) system to evaluate anti-FVIII antibodies in haemophilic patients in comparison with both the Bethesda with Nijmegen modification and the ELISA-based assays. Flow cytometers have the capacity to detect fluorescence emissions with high sensitivity. As long as the early 1980s, many reports in the field of immunological monitoring have been published which used a combination between flow cytometry and microparticles [14–17]. We used microspheres as solid support for FVIII and measured the level of anti-FVIII IgG employing a semiquantitative assay by flow cytometry. A good relationship between FC assay, Bethesda levels and ELISA results were established. The FC assay offers some advantages, as it is sensitive; requires smaller sample volumes and can detect both I-Ab and NI-Ab to FVIII.

Material and methods

Patients

Plasma and serum samples were collected from SHA patients at least 7 days after the last infusion of FVIII concentrate. None of the samples was haemolysed, lipemic or heat inactivated. Thirty patients referred to our centre were studied: 17 (P1–P17) without inhibitors (P), 13 showing a detectable I-Ab by Bethesda assay (1.1–8200 BU mL^{-1}) (PI 18–PI 30), two PI during ITI and 12 HD were included. Patients under ITI received 200 IU kg^{-1} of anti-haemophilic factor VIII once a day during 18 months.

All haemophiliacs were negative for HIV infection. The study was approved by the Ethics Committee of Academia Nacional de Medicina of Buenos Aires.

Adsorbing rFVIII to microspheres

Forty-five microlitres of a 2.5% suspension of 2 μm microspheres (Polysciences, Inc., Warrington, PA, USA) was washed five times in 0.1 M borate buffer pH 8.5 (BB) centrifuging 5 min at 15 000 *g* and 8°C. One ml of dialysed rFVIII (1000 U, kindly provided by Baxter, Glendale, CA, USA) was added to pellet and incubated overnight at room temperature with gentle end-to-end mixing (FVIII-m). A tube processed in parallel but incubated with BB was used as control (control-m). After centrifugation, pellets were resuspended in BB with 30% bovine serum albumin (BSA) to block unbound sites, incubated 30 min at room temperature with gentle mixing and washed. This step was repeated again. Finally, microspheres were stored at 4°C in 1 mL of phosphate buffered saline (PBS) pH 7.4 containing 1% BSA, 0.1% sodium azide (PBS-C) and 5% glycerol, previously filtered using a filter of 0.45 μm of pore. Preparations were stable at least for 1 year. rFVIII binding to microspheres was checked by adding to 2.5 μL of each FVIII-m and control-m, 5 μL of a 1/500 dilution of biotinylated sheep IgG anti-human factor VIII (Affinity Biologicals Inc., Ancaster, Ontario, Canada). Following incubation for 30 min at 4°C, microspheres were washed and revealed with 5 μL of a 1/5 dilution of phycoerythrin-conjugated streptavidin (Vector, Ontario, Canada). After incubating during 30 min at 4°C, microspheres were pelleted and resuspended in FACSflow (Becton Dickinson, San Jose, CA, USA). Flow cytometric analysis was performed on FACScan cytometer (Becton Dickinson) equipped with a 488 nm argon LASER and CellQuest software. We used logarithmic amplification in all parameters recorded. Beads were selected by gating (R1) according to size (FSC) and side (SSC) scatter profiles. A threshold of 200 was set up on SSC parameter to eliminate unwanted events. Ten thousand gated events were acquired for each determination. Mean fluorescence intensity (MFI) of FL-2 emission was recorded. MFI ratio between MFI of FVIII-m and MFI of control-m was calculated each time after the analysis. Variability including all determinations was given as a coefficient of variation calculated as:

$$\text{CV\%} = \frac{\text{Standard deviation of the mean} \times 100}{\text{Mean}}$$

Flow cytometry anti-FVIII assay

Plasma or serum dilutions (1/4–1/2000) in PBS-C were reacted with 2.5 μL of both FVIII-m and control-m in a final volume of 50 μL during 2 h at 4°C. After washing, captured antibodies were detected using 5 μL of biotinylated goat F(ab′)2 fragment anti-human IgG (Immunotech, Marseille, France) diluted 1/50 and incubated for 30 min at 4°C. Following another wash, 5 μL of a

1/5 dilution of phycoerythrin-conjugated streptavidin (Vector) was added and incubated again under the same conditions. Finally, 1 mL of PBS-C was added. Microspheres were pelleted by centrifugation at 700 *g* during 5 min and resuspended in 250 μL of FACSflow (Becton Dickinson). Each sample was acquired as described above. MFI of FL-2 emission was recorded. For semiquantitative results an index was calculated multiplying the highest MFI ratio by the inverse of the corresponding plasma dilution. Values above mean + 3 SDs obtained with samples from 12 HDs, for each dilution, were considered as positive. To verify the specificity, an assay was performed by incubating 50 μL of 1/50 diluted plasma with 50 μL of rFVIII or 50 μL of PBS-C for 30 min at 37°C. Then, 47.5 μL of each solution was added to 2.5 μL of FVIII-m and processed for anti-FVIII antibody detection as described earlier.

Bethesda assay

Inhibitory-Ab were measured using the Nijmegen modification of the Bethesda method, as previously described [2]. We defined a positive test by the Bethesda assay as a result >0.5 BU mL^{-1}.

ELISA

Solid-phase ELISA kit from GTI Diagnostics was employed for the anti-FVIII assay following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney and Spearman correlation tests in GraphPad PRISM software. A value of $P < 0.05$ was considered significant difference between groups.

Results

Recombinant FVIII coupling to microspheres

Recombinant FVIII coupling was verified each time after preparing the microspheres and monthly during storage time. Figure 1 shows an example. Flow cytometry analysis of MFI of FVIII-m (350) and control-m (7.83) using anti-human FVIII demonstrated the binding of FVIII to microspheres. In this example, MFI ratio (FVIII-m/control-m) was 44.69. The %CV for all determinations was 24%, $n = 7$.

Anti-FVIII titre determination using FC

To detect anti-FVIII antibodies, 30 plasma samples from haemophilic patients (17 P and 13 PI) and 12 from HD were evaluated. Indices were obtained as described in Material and Methods. A ratio for each dilution was calculated. An example of a patient (PI 28) with 133 BU mL^{-1} is given in Fig. 2. The maximum ratio value was obtained for 1/100 dilution (443.34/8.06 = 55) and the index was 5500. All samples had one maximum response dilution. To evaluate the possible interference of

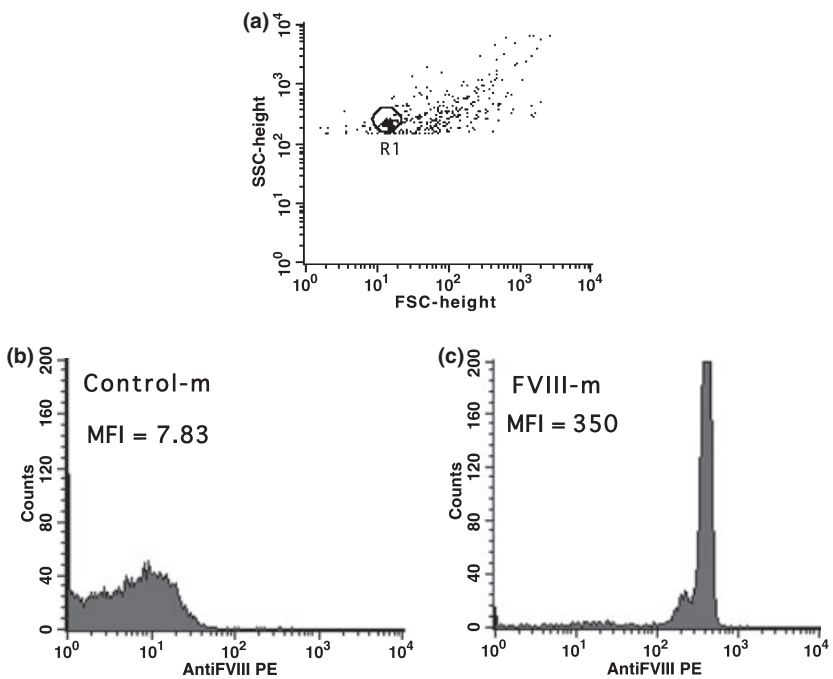

Fig. 1. Fcator VIII binding to microspheres. (a) Location (R1) of 2 μm microspheres on a FSC–SSC dot plot with logarithmic amplification. (b and c) Histograms displaying MFI of microspheres (control-m and FVIII-m) after incubation with biotinylated anti-human FVIII followed by phycoerythrin-conjugated streptavidin. MFI, mean fluorescence intensity.

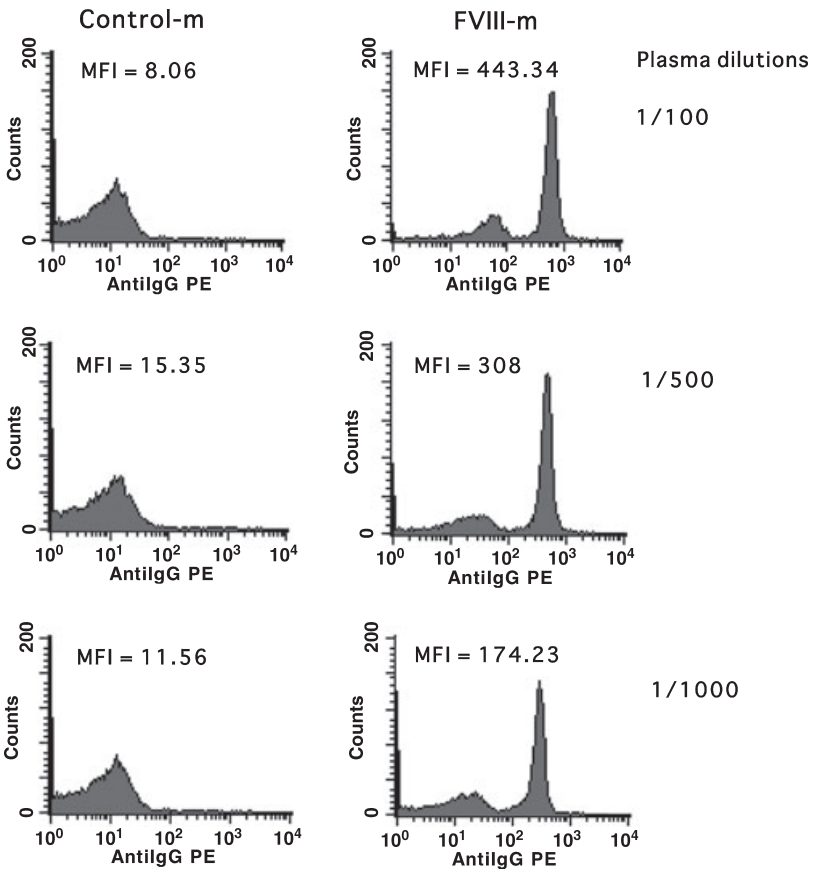

Fig. 2. Anti-FVIII antibodies measured using flow cytometry. Representative example corresponding to patients with inhibitory-Ab (PI) (133 BU mL^{-1}) that shows anti-FVIII antibodies index calculation. Control-m (without FVIII) and FVIII-m (FVIII-coupled) microspheres were incubated (2 h, 4°C) with different dilutions of plasma (1/4–1/2000) and stained with biotinylated anti-human IgG followed by phycoerythrin-conjugated streptavidin. An index was calculated as the highest ratio between MFI of FVIII-m and control-m, multiplied by the inverse of the corresponding dilution (443.34/8.06 × 100). Three serial dilutions are shown. MFI, mean fluorescence intensity.

the complement in the binding, three plasma samples from PI were heat inactivated. There was no difference in the assay results whether samples were heat inactivated or not (data not shown). Intra-assay variation was determined as %CV from triplicates of three samples processed the same day (%CV = 11.63, 15 and 22). To establish inter-assay variability, three assays were performed on three different occasions using the same batch of microspheres (%CV = 14, 17 and 18).

Anti-FVIII antibodies detected using FC in haemophilia A patients and healthy donors

Figure 3 shows indices comparing three groups included in this study. Mean value in PI was significantly higher than those of P ($P < 0.0001$) and HD ($P = 0.0005$). No significant difference was found between P and HD ($P = 0.84$). According to cut-off values obtained from HD for each dilution (mean ± 3 SD: 8.34; 18.28; 62.01; 127.40 and 219.36 for 1/4; 1/10; 1/20; 1/50 and 1/100 respectively), 13 of 13 PI (100%) and four of 17 P (23.5%) were above the cut-off level. However, FC indices from these four P were weakly positive: P5: 254.51 (1/100); P7: 134.29 (1/4), P8: 118.48 (1/20) and P13: 82.73 (1/20). P5 and P7 developed high Bethesda titres (430 and 90 BU mL^{-1} respectively) later on. P5 could be studied again using FC when its Bethesda titre was 430 BU mL^{-1} yielding a FC index of 4142. Unfortunately, the P7 sample corresponding to the Bethesda titre of 90 BU mL^{-1} was not available for FC evaluation. An additional non-haemophilic individual (not included in the HD group) showed FC indices of 1153 and 530 from two different plasma samples that were obtained 8 months apart.

Correlation between Bethesda and FC assays

We found a strong correlation coefficient between Bethesda titres and FC indices ($r = 0.80$, $P < 0.0001$) including P and PI plasmas (Fig. 4). When we compared BU mL^{-1} with the corresponding FC index calculated from one-fourth and 1/100 PI plasma dilutions, we found $r = 0.025$ ($P = 0.458$) and $r = 0.77$ ($P < 0.0001$) respectively suggesting that higher dilutions are better indicators of the level of antibodies in PI with high titres. However, it was not the case for patients having BU mL^{-1} in the range between 0.5 and 10 as we detected maximum MFI in dilutions lower than 1/100 (1/4, 1/20, 1/50).

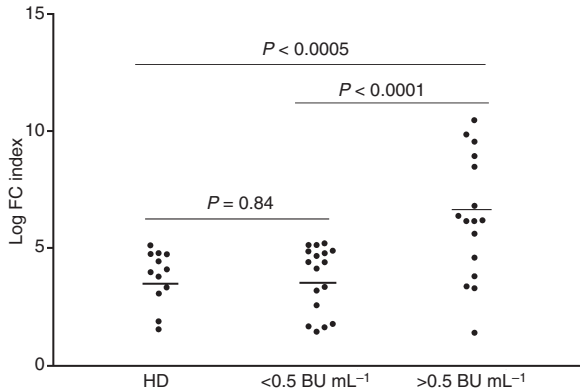

Fig. 3. Comparison of flow cytometry values for anti-FVIII antibodies. The three groups were included: haemophilia A patients with inhibitors (>0.5 BU mL^{-1}), without inhibitors (<0.5 BU mL^{-1}) and healthy donors (HD). Serial dilutions (1/4–1/2000) of plasmas were incubated (2 h, 4°C) with control-m (without FVIII) and FVIII-m (coupled with FVIII)) microspheres and revealed with biotinylated anti-human IgG followed by phycoerythrin-conjugated streptavidin. Flow cytometry indices were calculated as the highest ratio between MFI of FVIII-m and control-m, multiplied by the inverse of the corresponding dilution. Black lines represent mean values. Statistical significances are indicated. MFI, mean fluorescence intensity.

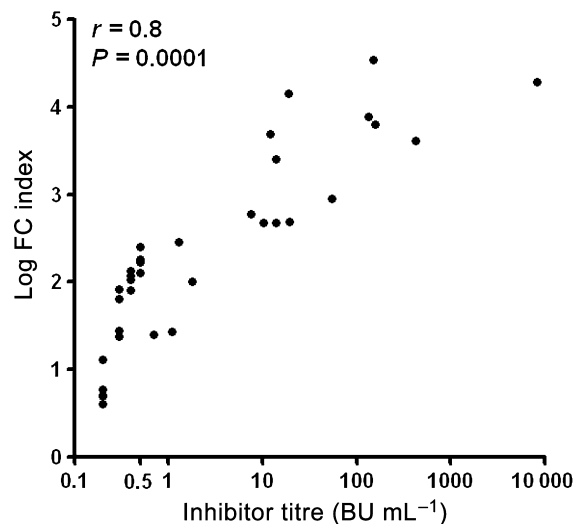

Fig. 4. Correlation between flow cytometry (FC) results with Bethesda assay. The 30 haemophilia A patients comprised 13 with a positive Bethesda titre and 17 without detectable inhibitors. Control-m (without FVIII) and FVIII-m (coupled with FVIII) microspheres were incubated (2 h, 4°C) with different dilutions (1/4–1/2000) of plasmas and stained with biotinylated anti-human IgG followed by phycoerythrin-conjugated streptavidin. Indices by FC were calculated as the highest ratio between MFI of FVIII-m and control-m, multiplied by the inverse of the corresponding dilution. Results <0.5 BU mL^{-1} have been plotted between 0.1 and 0.5 for clarity of the illustration. MFI, mean fluorescence intensity.

Specificity of FC assay

Four plasma samples from PI were employed for specificity assays. Bethesda titres and FC indices each were 1.3; 12; 19; 430 BU mL^{-1}, and 284.69; 3836; 14 203; 4142, in P19, P23, P25 and P5 (second evaluation), respectively. Histograms displayed in Fig. 5 show the results. A marked inhibition of MFI was observed for the four cases after pre-incubation with rFVIII.

Anti-FVIII antibodies in sera

To evaluate if serum samples may be utilized in case that citrated plasma is not available, the FC test was performed in four matched plasma and serum samples with different levels of I-Ab (P7: <0.5 BU mL^{-1}; P19: 1.3 BU mL^{-1}; P26: 19.2 BU mL^{-1} and P29: 151 BU mL^{-1}). FC indices were similar indicating that both plasma and serum are suitable to be evaluated by FC (data not shown).

Comparison between ELISA and FC results

We tested all plasmas included here using ELISA following manufacturer's instructions. This assay provides a qualitative screening as the results are expressed as positive or negative using a one-fourth plasma dilution. To compare ELISA with FC, we incubated FVIII-m and Control-m with samples at similar concentration (1/4). Twenty-one of 30 patients (70%) presented concordant measurements. The majority (14 of 15) of P without detectable anti-FVIII antibodies by ELISA were also negative by FC. However, when we compared both techniques, but calculating the FC indices as described in Material and Methods at optimal plasma dilution (not at the 1/4 dilution required for the ELISA assay), we found concordance in 24 samples (80%) (Table 1).

In Table 2, we summarized results from the 30 patients included in this study. All PI were positive not only by Bethesda but also by FC and ELISA. On the other hand, most patients in the P group (11 of 17) were negative by the three methods. The remaining six (P: 4, 5, 6, 7, 8 and 13) were weakly positive by one test, either FC or ELISA.

FC index in two patients during ITI

A longitudinal analysis of IgG variation and the I-Ab level was performed in two SHA patients during ITI. The results are shown in Fig. 6. Plasma samples were obtained between 24 months before ITI and 24 and 27 months after the treatment. FC index profile followed those of inhibitor measurements. Tolerance was induced in patient 32 whose antibodies decreased until undetectable levels. In patient 31, the successful of ITI was partial and the remaining antibodies were found by both techniques.

Discussion

Administration of exogenous FVIII as replacement treatment in SHA patients results, in 15–30% of the

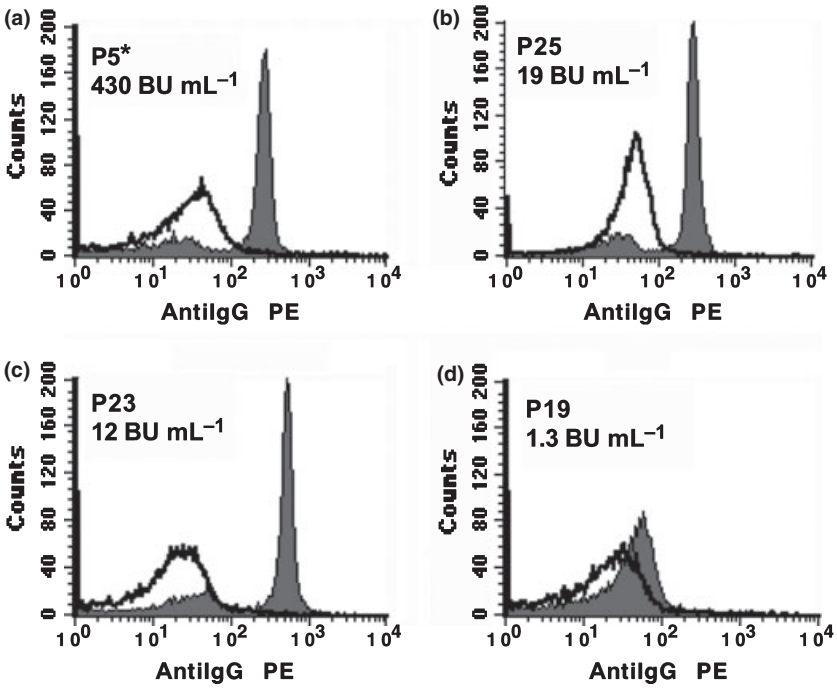

Fig. 5. Inhibition of anti-FVIII antibodies binding to FVIII coupled to microspheres. Plasmas (50 μL) from (a) P5 (*second evaluation), (b) P25, (c) P23 and (d) P19 diluted 1/50 were pre-incubated with 50 μL of soluble recombinant factor VIII (rFVIII, empty histograms) or buffer (filled histograms) for 30 min at 37°C. Final solutions (47.5 μL) were added to 2.5 μL of FVIII-m and processed for anti-FVIII antibody detection according to Material and Methods description. Titre by Bethesda assay corresponding to each sample is indicated.

Table 1. Distribution of 30 haemophilia A patients according to flow cytometry and enzyme-linked immunosorbent assay results.

	ELISA+	ELISA−
FC+	13	4
FC−	2	11

ELISA, enzyme-linked immunosorbent assay; FC, flow cytometry.

cases, in the development of anti-FVIII antibodies that neutralize the procoagulant activity of the therapeutically administered FVIII. Antibodies with inhibitory properties (I-Ab) towards FVIII activity are measured using the Bethesda assay that has been the laboratory standard test since 1975. The Nijmegen modification of the Bethesda assay has been recommended by the Scientific Subcommittee on Factor VIII and Factor IX of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, as a gold standard for measuring I-Ab [18].

Some groups have developed immunoassays that can detect both types of antibodies, I-Ab and NI-Ab. A number of published reports have utilized ELISA, which provides an alternative way to examine the immune response to FVIII [6–10]. Anti-FVIII antibody detection by ELISA generally agreed with that of the Bethesda method.

In this study, we developed a semiquantitative immune assay based on coupling FVIII to microspheres as solid support. After incubating with serial dilutions of plasma or serum, antibodies to FVIII can be detected with anti-human IgG conjugated to a fluorochrome, recording the fluorescence using a flow cytometer. Non-specific binding of proteins, present in the sample, to the microspheres was avoided by calculating ratios between MFI of FVIII-m and control-m, giving more reliability to the results. The specificity was confirmed as MFI was decreased after pre-incubation of I-Ab positive samples with soluble rFVIII.

We found a good correlation between Bethesda and FC assays. We did not expect a complete correlation because Bethesda test only detects functional inhibitors. However, 13 of 13 PI were also positive by FC. One non-haemophilic donor, not included in the HD group, showed FC indices of 1153 and 530 in two serial samples, respectively. This was not a surprising finding because the existence of natural antibodies in 20% of normal individuals has been reported [11–13].

As this technology provides a sensitive tool, suitable to evaluate especially low-responder patients (<5 BU mL^{-1}), we paid special attention to patients with negative or low I-Ab by Bethesda. The FC test was positive in four of 17 P (23.5%). The fact that four of 17 P showed positive results by FC was taken as evidence for antibodies against non-functional epitopes. Nevertheless, these P presented a weak response and none had clinical manifestation of the anti-FVIII presence. Surprisingly, two of them (P5 and P7) developed high I-Ab later on, indicating that the observed increase of MFI in FVIII-m respect to control-m was not an artefact. However, the low level of anti-FVIII antibodies in P5 and P7 at the moment of the study could represent true I-Ab that might have been undetected by the Bethesda assay because of its low sensitivity. Although the limited number of patients investigated at the moment does not enable us to draw definitive conclusions, these results suggest that NI-Ab should be taken

Table 2. Bethesda, enzyme-linked immunosorbent assay and flow cytometer results in the 30 haemophilia A patients included in the study.

Patient	BU mL^{-1}	ELISA*	FC Indices
1	<0.5	–	–
2	<0.5	–	–
3	<0.5	–	–
4	<0.5	+	–
5	<0.5	–	254.41**
6	<0.5	+	–
7	<0.5	–	134.29**
8	<0.5	–	118.48
9	<0.5	–	–
10	<0.5	–	–
11	<0.5	–	–
12	<0.5	–	–
13	<0.5	–	82.73
14	<0.5	–	–
15	<0.5	–	–
16	<0.5	–	–
17	<0.5	–	–
18	1.1	+	27.78
19	1.3	+	284.69
20	1.8	+	100.37
21	7.5	+	599
22	10.3	+	480.18
23	12	+	3836
24	14	+	471
25	19	+	14 203
26	19.2	+	623
27	55	+	893
28	133	+	5500
29	151	+	35 362
30	8200	+	19 262

ELISA, enzyme-linked immunosorbent assay; FC, flow cytometry.
*According to kit's instructions, OD values equal to or greater than 2× the value obtained for the mean of negative controls are regarded as positive results.
**P5 and P7 developed a high level of inhibitors later on.

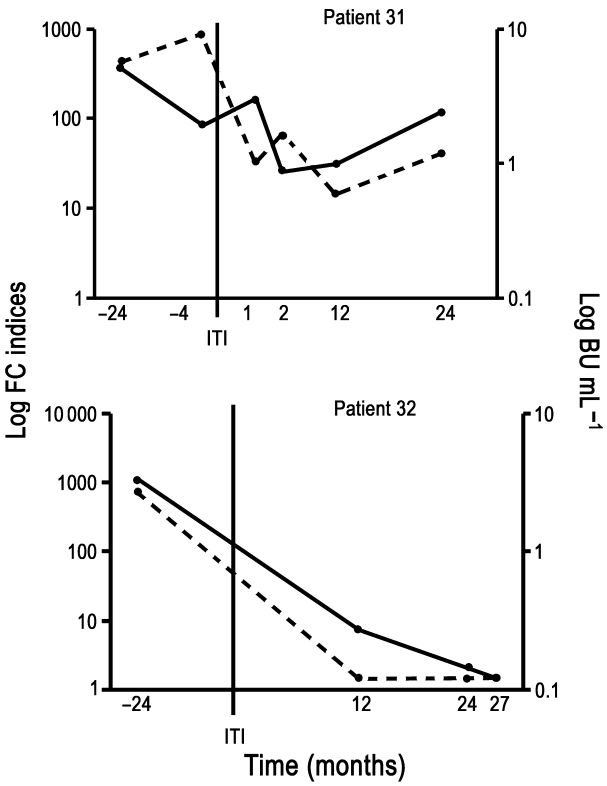

Fig. 6. Longitudinal analysis of anti-FVIII antibodies in two patients during the immune tolerance induction (ITI). Flow cytometry (FC) indices (solid line) and BU mL^{-1} (dotted line) were performed in plasma samples obtained before and after ITI in the months indicated. Control-m (without FVIII) and FVIII-m (coupled with FVIII) microspheres were incubated (2 h, 4°C) with different dilutions (1/4–1/2000) of plasmas and stained with biotinylated anti-human IgG followed by phycoerythrin-conjugated streptavidin. Indices by FC were calculated as the highest ratio between MFI of FVIII-m and control-m, multiplied by the inverse of the corresponding dilution. MFI, mean fluorescence intensity.

into consideration in the management of SHA patients, not only because they can accelerate the clearance of transfused FVIII, but also because its presence would help to alert the future development of I-Ab.

Moreau *et al.* [12] have observed I-Ab in plasma from non-responder patients with haemophilia A and healthy individuals and suggested that I-Ab originate from the expansion of pre-existing natural anti-FVIII B cell clones. This underlines the importance of evaluating anti-FVIII antibodies by both Bethesda and an immunoassay such as ELISA or FC.

In this regard, we here show the follow up of two patients during the ITI comparing the BU mL^{-1} and the FC measurements. With the limitations related to the analysis of only two patients, it appears that the FC method may prove to be useful to monitor the outcome of ITI.

A concordance of 70% between ELISA and FC was obtained using one-fourth dilution as the ELISA manufacturer suggests. However, when we compared both techniques but considering the FC indices calculated taking into account all serial dilutions, as described in Material and Methods, the percentage was increased to 80%. It means that one-fourth is not the optimal dilution by FC when PI with high level of anti-FVIII is evaluated. In this regard, Sahud *et al.* [8] have reported stronger correlation between ELISA and Bethesda assay performing a 1/400 comparing to one-fourth dilution on 27 samples that had a wide range of I-Ab titres.

Microspheres have been employed in previous reports to measure anti-FVIII antibodies [19,20]. These systems are good tools to detect non-functional antibodies, but sometimes they are not available in common laboratories. Quantification of antibodies in human plasma has been the aim of Krudys-Amblo *et al.* [20]. They introduced an affinity-purified anti-FVIII antibody from a haemophilia A patient plasma as calibrator. As a result of the limited amount of human calibrator, they validated a mouse anti-FVIII antibody as a suitable surrogate calibrator. As the immune response to FVIII of the patients is polyclonal and different IgG subclasses are involved [21], that type of calibrator may not provide the optimal standard.

Efforts have been made to find a way to predict the development of I-Ab. Ter Avest *et al.* [22] designed a score that stratifies untreated patients with severe

haemophilia according to their risk of developing inhibitor antibodies. This first approach together with improving methods to detect anti-FVIII antibodies will contribute to a better management of haemophilic patients. Similarly, it will be interesting to include FC-based inhibitor assay techniques to predict inhibitor development or to monitor ITI.

Conclusions

This assay provides an alternative for measuring FVIII antibodies. The method is a new semiquantitative immune test that is easy and rapid to carry out, as it can be completed in 4–6 h. Beyond its sensitivity and specificity, only small sample volumes are required making this test suitable to be used in paediatric patients. An additional advantage is that sera may be utilized, if citrated plasma is not available.

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Disclosures

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

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