Association of anti-idiotypic antibodies with immune tolerance induction for the treatment of hemophilia A with inhibitors

Background and Objectives. Hemophilia A patients with inhibitors can be treated effectively with immune tolerance induction therapy (ITI). One of the underlying mechanisms of ITI is conceived to be a neutralizing activity of anti-idiotypic antibodies on inhibitors. The goal of the present study was to develop an uncomplicated method for assessing anti-idiotypic antibodies and to prove the advent of anti-idiotypic antibodies in ITI.

Design and Methods. We studied a total of 26 plasma samples obtained from 9 hemophilic inhibitor patients who were treated with ITI. The samples were investigated with a novel method for detecting anti-idiotypic antibodies based on a liquid phase blocking immunoprecipitation.

Results. Plasma anti-factor VIII (FVIII) antibody titer was reduced by adding plasma from patients who had received completely successful ITI. This anti-FVIII antibody-neutralization activity of the plasma was impaired by treating the plasma with protein G beads. In addition, treating inhibitor plasma from patients in whom ITI had been unsuccessful with FVIII affinity beads resulted in the development of the anti-FVIII antibody-neutralization activity. Furthermore, the anti-FVIII antibody-neutralization activity of anti-FVIII antibody-depleted plasma obtained in a late period of ITI on inhibitor plasmas obtained during ITI increased over time.

Interpretation and Conclusions. Our results suggest that; (i) plasma from patients in whom ITI was completely successful contained an anti-FVIII antibody-neutralization factor; (ii) the anti-FVIII antibody-neutralization factor was in the IgG fraction (i.e., the factor would be anti-idiotypic antibodies), and (iii) anti-idiotypic antibodies existed even in plasma from patients in whom ITI was unsuccessful. Our observations support the notion that the mechanism of ITI is associated with the development of anti-idiotypic antibodies.

Key words: hemophilia A, antiidiotypic antibody, immune tolerance induction therapy, affinity maturation.

The development of factor VIII (FVIII) inhibitors remains a serious clinical complication arising from the treatment of hemophilia A patients. A recent meta-analysis revealed that the average cumulative incidence of FVIII inhibitors is 15-52%. Once an inhibitor develops, treatment for bleeding episodes is difficult, particularly in high responder patients. The main goal of the treatment of patients with hemophilia A who have inhibitors is to eradicate the inhibitor. For this purpose, induction of immune tolerance (ITI) to FVIII therapy is the most promising strategy. In 1977, Brackmann and Gormsen reported a curative ITI protocol for inhibitor patients. The protocol, known as the Bonn Protocol, is based on administration of high doses of FVIII (150 IU/kg twice a day). In 1999, Oldenburg et al. reported the outcome of 60 hemophilia A inhibitor patients treated according to the Bonn Protocol. Successful immune tolerance was achieved in 52 patients (86.7%), while the therapy failed in eight patients (13.3%). Furthermore, several studies have been performed using a modified dosage scheme and the outcome of the low-dose ITI protocol was reported to achieve a similar success rate to that of the Bonn Protocol. However, the mechanism by which immune tolerance is induced towards the inhibitor antibodies in hemophilia patients with inhibitors is controversial. Broadly speaking, tolerance has been hypothesized to be induced by several mechanisms: clonal deletion, clonal anergy, clonal ignorance, receptor editing, suppressor cells, and anti-idiotypic antibody. Clonal deletion is the removal of immune response cells through programmed cell death or apoptosis. In clonal anergy, immune response cells are alive but
fail to respond to antigen challenge. In these conditions, a cellular immune response can no longer exist. Clonal ignorance is a state in which immune response cells are alive and able to respond to antigen challenge, but do not see antigen because it is observed in immunologically privileged sites including the central nervous system, eye, and testis. In receptor editing, as an immature B cell grows and divides, its antibodies slowly mutate and change structure so that they no longer bind the antigen and would not be eliminated or inactivated. Furthermore, the antigen may induce suppressor T cells that can suppress immune responses of both B and T cells. Although all the possible mechanisms could result in successful ITI, some investigators suggest that anergy induced by cross-linking surface immunoglobulins such as anti-idiotypic antibodies can achieve immune tolerance.12 The presence of anti-idiotypic antibodies reacting with FVIII:C inhibitors in intravenous immunoglobulin preparations has been reported since 1984.14-20 These preparations are used as a treatment for spontaneous FVIII:C inhibitors, i.e. acquired hemophilia.21 Furthermore, it has been suggested that anti-idiotypic antibodies play a role in the down-regulation of anti-FVIII antibody production as well as inhibiting the binding of antibodies to FVIII in the ITI.22 The method used in the previous studies to detect the anti-idiotypic antibodies did reveal their presence, but has the drawbacks that it needs complicated and time-consuming column operations and is based on a solid phase immunoassay. The fluctuation of production of anti-idiotypic antibodies during the course of ITI therapy remains unclear. The aim of the present study was to establish a simple method for assessing anti-idiotypic antibodies and to elucidate the role of such antibodies in ITI. We present the possibility that an idiotypic network is associated with ITI.

Design and Methods

Patients

In this study, we investigated a total of 26 plasma samples obtained from 9 hemophilic inhibitor patients who were treated with ITI using highly purified FVIII concentrate derived from human plasma. This work was approved by the institutional review board of Nara Medical University. All plasma samples were obtained from the patients who had given informed consent.

Bethesda and immunoprecipitation assays

Plasma samples were analyzed by the Bethesda assay23 for inhibitory antibodies and/or through the more sensitive immunoprecipitation (IP) assay for all anti-FVIII antibodies. The latter assays were performed as previously described.24 In brief, 7 µg of FVIII in 20 µL of 0.2 M sodium acetate buffer (pH 6.8) were 125I-labeled with IODO-Gen Pre-Coated Iodination Tube (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. Specific radioactivities ranged from 3.4 to 4.5 µCi/µg. Duplicates of 50 µL inhibitor plasma dilutions in 20 mM Tris, 0.15 M NaCl, pH 7.4 (TBS), and 1% bovine serum albumin (BSA) were incubated with 10 µL 125I-labeled FVIII (0.75 nM, final concentration) at 4°C overnight with agitation, and for 2 more hours after addition of 100 µL TBS dilution buffer and 50 µL of a suspension of protein G-Sepharose beads (Amersham Biosciences, Piscataway, NJ, USA). After washing the beads three times with TBS-0.05% Tween 20, bound radioactivity was determined in a gamma counter (ARC-380, Aloka, Tokyo, Japan). Background radioactivity without antibody was 1% to 2%, and maximal binding with antibody was 60% to 70%. Results were expressed as immunoprecipitation units per milliliter (IPU/mL), being calculated as: 1 - (bound/total radioactivity - background) × plasma dilution × 16.7 (to convert to IPU/mL).

Detection of anti-FVIII antibody-neutralization activities in patients' plasma

The assays used in this study are based on a novel liquid phase blocking IP method, and specific neutralization of a defined IP titer of anti-FVIII antibody was detected using anti-FVIII antibody-neutralization factors (i.e., presumably anti-idiotypic antibodies) in the test sample. After the test plasma had been allowed to react with inhibitor plasma (i.e., positive BU titer plasma), the mixture of the test plasma and inhibitor plasma was transferred to a tube containing protein G-Sepharose beads for the IP assay. The presence of anti-FVIII antibody-neutralization factors in the test plasma would result in the formation of immune complexes, such as complexes of probable anti-FVIII antibodies-anti-idiotypic antibodies, and consequently reduce the amount of free anti-FVIII antibodies trapped by the protein G-Sepharose beads. After washing the beads and adding radiolabeled FVIII, radioactivity (i.e., IP titer) was measured: a reduction was observed when anti-FVIII antibody-neutralization factors were present.

Firstly, in order to detect anti-FVIII antibody-neutralization factors in a patient's plasma, plasma from a patient who had undergone successful ITI was mixed with an equal volume of plasma from the same patient showing positive inhibitors prior to ITI (Figure 1A). The mixture was incubated at 4°C with agitation overnight, and anti-FVIII antibody titer of the mixture was measured by the IP assay. In all mixing studies, inhibitor plasma samples that showed a value of more than 10
IPU/mL were adjusted to give values of less than 10 IPU/mL.

Secondly, to confirm that the anti-FVIII antibody-neutralization factors in the patient’s plasma were in immunoglobulins (IgGs), sample plasma pretreated with protein G-Sepharose beads (IgG-depleted plasma) or buffer A as control (30 mM CaCl₂, in 10 mM Tris-HCl, pH 7.2) was mixed with an equal volume of the same plasma without the protein G-Sepharose bead treatment (Figure 1B). The protein G-Sepharose beads for the elimination of IgGs were washed three times with one volume of buffer A containing 0.1% BSA, followed by 3 washes with one volume of buffer A without BSA. Fifty microliters of the patient's plasma were mixed with an equal volume of protein G-Sepharose beads (50% slurry) and incubated at 4°C with agitation overnight. Samples were centrifuged at 250×g for 3 min, and the supernatant was recovered as IgG-depleted sample plasma. The mixture of 50 µL of patient’s plasma and 25 µL of buffer A was also incubated at 4°C with agitation overnight. This solution was used as a reference. The solutions were mixed with equal volume of the same patient’s plasma and incubated at 4°C with agitation for 3 hours. Anti-FVIII antibody titer was measured by the IP assay.

Thirdly, to elucidate whether anti-FVIII antibody-neutralization antibodies existed in the patient’s plasma, sample plasma pretreated with FVIII-Sepharose (i.e., anti-FVIII antibody-depleted plasma) or buffer A (control) was mixed with an equal volume of plasma from the same patient without the FVIII-Sepharose treatment (Figure 1C). Sample plasmas were collected before ITI (patient D) and during ITI from the patients who received incompletely successful ITI (patients E, F, and G), and unsuccessful ITI (patients H and I). FVIII-Sepharose for elimination of anti-FVIII antibody was prepared. FVIII (3 mg) was coupled to 1 g CNBr-activated Sepharose 4B (Amersham Biosciences) in coupling buffer (0.1 M NaHCO₃ containing 0.5 M NaCl, pH 8.3) at 4°C for 18 hours. Prior to use, the FVIII-Sepharose beads were washed three times with one volume of buffer A containing 0.1% BSA, followed by 3 washes with one volume of buffer A without BSA. Fifty µL of the patient’s plasma were mixed with an equal volume of FVIII-Sepharose (50% slurry) and incubated at 4°C overnight with agitation. Samples were centrifuged at 250×g for 3 min, and the supernatant was recovered as anti-FVIII antibody-depleted sample plasma. The mixture of 50 µL of patient’s plasma and 25 µL of buffer A was also incubated at 4°C with agitation overnight. This solution was used as a reference. The solutions were mixed with equal volume of the same patient’s plasma and incubated at 4°C with agitation for 3 hours. Anti-FVIII antibody titer was measured by the IP assay.

### Table 1: Inhibitor titer and outcomes of ITI in 9 inhibitor patients with hemophilia A.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>BU/mL</th>
<th>IPU/mL</th>
<th>Outcomes</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Before</td>
<td>2</td>
<td>52.5</td>
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<tr>
<td></td>
<td>After</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Before</td>
<td>1</td>
<td>85.6</td>
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<tr>
<td></td>
<td>After</td>
<td>6</td>
<td>50.4</td>
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<tr>
<td>C</td>
<td>Before</td>
<td>28</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>2</td>
<td>40.5</td>
</tr>
<tr>
<td>D</td>
<td>Before</td>
<td>0</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0</td>
<td>6.4</td>
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<tr>
<td>E</td>
<td>Before</td>
<td>12</td>
<td>1644</td>
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<td></td>
<td>After</td>
<td>234</td>
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<td>8</td>
<td>2</td>
<td>148</td>
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<tr>
<td></td>
<td>After</td>
<td>0</td>
<td>3.6</td>
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<tr>
<td>G</td>
<td>1</td>
<td>284</td>
<td>7890</td>
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<td></td>
<td>After</td>
<td>756</td>
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<td>7.6</td>
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<td>2</td>
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<td>After</td>
<td>2</td>
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Lastly, anti-FVIII antibody-depleted plasma obtained 2 and 48 weeks after the start of ITI or buffer A (control) was mixed with plasma obtained at four sampling points (week 2, 4, 8 and 48) during ITI from a patient who underwent incompletely successful ITI (Patient F) (Figure 1D). Week 48 corresponds to the completion of ITI in this patient. After the mixture had been incubated at 4°C with agitation overnight, anti-FVIII antibody titer was measured by the IP assay.

Results

Inhibitory antibody and anti-FVIII antibody titer of patients

We investigated a total of 26 plasma samples from 9 hemophilic inhibitor patients who were treated with ITI (Table 1). Depending on BU and IP titers, patients were categorized into 3 groups, those in whom ITI was completely successful, whose IP and BU titers were undetectable in plasma after ITI, those in whom ITI was incompletely successful whose BU titer was undetectable but IP titer was still detected after ITI, and those in whom ITI was unsuccessful whose BU titer was detectable after ITI. The ITI was completely successful in three patients (A, B, and C), incompletely successful in four (D, E, F, and G) and unsuccessful in two (H and I).

Presence of anti-FVIII antibody-neutralization factor in the plasma from patients with completely successful ITI

Addition of plasma after ITI from patients with completely successful ITI to plasma taken from the same patients during ITI reduced IP titer in a dose-dependent manner (Figure 2A). On the other hand, the addition of plasma after ITI from patients with incompletely successful ITI to plasma taken from the same patients during ITI produced a higher IP titer than that...
following addition of buffer A (control) (Figure 2B). This indicates that plasma from patients in whom ITI was completely successful contained some anti-FVIII antibody-neutralization factors.

**Identification of anti-FVIII antibody-neutralization factor as IgGs**

The effects of IgG-depleted plasma on IP titer of untreated plasma were investigated using plasma samples from the 3 patients in whom ITI had been completely successful. While plasma without protein G-Sepharose treatment reduced binding of antibodies to FVIII, IgG-depleted plasma did not influence it (Figure 3). Elimination of IgGs resulted in the loss of anti-FVIII antibody-neutralization effects, indicating that anti-FVIII antibody-neutralization factor would fall within IgGs. This implies that the plasma from patients with completely successful ITI contained IgGs against anti-FVIII antibodies, i.e. anti-idiotypic antibodies.

**Anti-idiotypic antibodies existing in plasma during ITI**

We also assessed the effects of anti-FVIII antibody-depleted plasma on IP titer of untreated plasma. All anti-FVIII antibody-depleted plasma samples reduced binding of antibodies to FVIII, while plasma without FVIII-Sepharose treatment did not influence, or even increased binding of antibodies to FVIII (Figure 4). These
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Results imply that anti-idiotypic antibodies existed in plasma before and during ITI from patients in whom ITI was incompletely successful or unsuccessful.

**Alterations of anti-idiotypic antibodies during ITI**

Addition of anti-FVIII antibody-depleted plasma reduced IP titer in all cases (Figure 5), whereas addition of week 2 or week 48 untreated plasma increased IP titer compared to that of each control to which buffer A had been added. Anti-FVIII antibody-depleted week 2 plasma maximally reduced IP titer of week 4 plasma. Meanwhile, anti-FVIII antibody-depleted week 48 plasma reduced IP titer of week 2, 4, 8, and 48 plasma samples, in a time sequence.

**Discussion**

Idiotype refers to the assembly of antigenic determinants which are located in the variable regions of antibodies or antigen-specific receptors of T cells. Each idiotype contains the determinants that are specific to the antibody molecule or T-cell receptor, which is called idiotope. Corresponding anti-idiotypic antibodies can develop against an immunogenic idiotope. Idiotypes and anti-idiotypic antibodies are natural components of immune responses, and exert a regulatory role which maintains the homeostasis of the immune system. These interactions between idiotypes and anti-idiotypic antibodies constitute an idiotypic network. Immunological tolerance might be found in the subtle equilibrium
between anti-FVIII and corresponding anti-idiotypic antibodies. Since normal individuals produce both anti-FVIII and corresponding anti-idiotypic antibodies,26,27 the cause of the tolerance to FVIII is not limited to deletion of self-reactive B and T cells.27 It might thus be fruitful to investigate whether an idiotypic network plays a role in establishing and maintaining tolerance.

The immunoprecipitation method used in the present study, originally used to measure levels of all anti-FVIII antibodies in plasma samples, is highly sensitive and is independent of inhibitor activity measurable by the Bethesda method. By testing plasma samples from patients in clinical trials, 5% to 10% of patients without a detectable inhibitor by the Bethesda method have a significant immune response which ranges from 3-fold to 100-fold above the background.28,29 It has been reported that the half-life of transfused FVIII is reduced as the anti-FVIII antibody concentration detected by an ELISA, which is as sensitive as the IP assay, increases in non-inhibitor patients with hemophilia A.30 This study has a limitation, but indicates that some of the inhibitor-negative patients may have symptom exacerbation as a result of a rise of anti-FVIII antibodies.

In the present study, in order to elucidate whether plasma from patients after ITI contained anti-idiotypic antibodies, simple mixing studies were performed using the immunoprecipitation method. In the previous studies the detection method generally used was a solid-phase ELISA. We developed a novel liquid phase blocking IP method, which should provide more reliable results. Anti-FVIII antibody-neutralization activity was observed in plasma from patients in whom ITI was completely successful, but not in plasma from those in whom ITI was incompletely successful or unsuccessful, suggesting the presence of anti-FVIII antibody-neutralization factor in plasma from the completely successfully ITI treated patients. The second assays revealed that IgG-depleted plasma from patients with completely successful ITI contained no anti-FVIII antibody-neutralization factor. Furthermore, the third assays proved the presence of anti-idiotypic antibodies even in plasma samples that showed positive inhibitor titer. These results suggest that anti-FVIII antibody-neutralization factor is an IgG; i.e., the anti-FVIII antibody-neutralization factor would be anti-idiotypic antibodies against anti-FVIII antibodies, and anti-idiotypic antibodies exist at any time in any hemophilic patients who receive FVIII replacement therapy.

The last mixing studies using one patient's plasma samples taken at different time points provided the possibility to examine the affinity maturation of IgG anti-idiotypic antibodies. Anti-FVIII antibody-depleted plasma obtained during an early period of ITI (week 2 plasma) reduced IP titer in plasma samples in a time independent manner. On the other hand, the anti-FVIII antibody-neutralization activity of anti-FVIII antibody-free plasma obtained at completion of ITI increased over time. These results indicate that neutralization activity of anti-idiotypic antibody increases over the course of ITI. Affinity maturation of IgG antibodies in adaptive immune responses is a well-accepted mechanism to improve effector functions of IgG within 2 weeks to several months after antigen encounter. Idiotype suppression function is a driving force for diversification and maturation of the antigen-induced response.31 Further studies will be necessary to clarify the role of affinity maturation of anti-idiotypic antibodies in ITI.

Our observations suggest that, in patients whose IP titer was suppressed by ITI, anti-idiotypic antibodies shifted the immune system toward the steady-state equilibrium that prevented alloimmunity in hemophilia A patients with inhibitors. The present study thereby contributes to a better understanding of the association of anti-idiotypic antibodies with ITI.

YS was responsible for the conception and design of the study, for acquisition, analysis and interpretation of data and for drafting the article. IT, KF and KY were responsible for acquisition, analysis and interpretation of data. MS and AY were responsible for conception of the study, enrolling patients and interpretation of data. All authors drafted or critically revised the manuscript, and all authors approved the final version of the manuscript. The authors reported no potential conflict of interest.


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