Factor VIII inhibitors are produced during or after coagulation factor VIII (FVIII) therapy in hemophilia A patients. These inhibitors are usually detected by a modified Bethesda assay or an enzyme-linked immunosorbent assay (ELISA). In this study, we used the Bethesda assay to determine the incidence of FVIII inhibitors in 75 fresh plasma samples obtained from 50 hemophilia A patients, and then used ELISA and the Bethesda assay to determine the titers of these inhibitors after the samples had been frozen and thawed. The samples from the screening Bethesda assay were centrifuged and stored at -70°C in accordance with the assay guidelines. Subsequently, these samples were thawed and analyzed using ELISA and the Bethesda assay. The incidence of inhibitors in hemophilia A patients was 20.0%. Among the 35 inhibitor-positive samples identified in the screening Bethesda assay, 16 were positive in ELISA while only 4 were positive in the repeated Bethesda assay. In this study, the ELISA technique showed a higher sensitivity than the Bethesda assay in the detection of FVIII inhibitors in samples that were subjected to freezing and thawing procedures; this was because the Bethesda assay could not identify the FVIII inhibitors that were degraded after freezing and thawing.

Key Words: Hemophilia, Bethesda assay, ELISA, Factor VIII, Inhibitor
samples for the Bethesda screening assay were collected at admission, and before and after FVIII injection in each patient, as necessary. Bethesda screening assay was performed for the fresh plasma samples after which these samples were centrifuged and stored at −70°C according to the assay guidelines [3, 4]. Normal citrated plasma samples were obtained from 20 healthy donors. A modified Bethesda assay was performed by substituting the imidazole buffer in the control mixture with immunodepleted FVIII–deficient plasma (DIAGNOSTICA STAGO, Asnieres-sur-Seine, France) [5]. Equal volumes of normal pooled plasma and hemophilic patient’s plasma were incubated at 37°C for 2 hr. In comparison with the FVIII inhibition by the control samples, 1 Bethesda unit (BU)/mL of the hemophilic patient’s plasma reduced the FVIII activity by 50%. An inhibitor titre of 0.7 BU/mL or higher was considered positive, while titres lower than 0.3 BU/mL were considered negative. Inhibitor titres of 0.3–0.7 BU/mL were considered borderline titres. Solid–phase ELISA (GTI Diagnostics, Waukesha, WI, USA) was performed in accordance with the manufacturer’s instructions.

Of the 35 samples that had shown positive titres in the Bethesda screening assay, only 16 were positive in ELISA (Table 1). The Bethesda assay to determine the inhibitor titre was repeated after freezing and thawing; in the second Bethesda assay, only 4 samples (N=4) were positive (Table 1). The titres obtained in the Bethesda screening assay were between 1.3 and 2.7 BU/mL, but those obtained during the repeated assay were between 0.8 and 1.4 BU/mL.

In the repeated Bethesda assay, 12 samples showed negative results. In the initial Bethesda assay, these 12 samples were positive for FVIII inhibitors with inhibitor titres less than 1.7 BU/mL, thereby indicating titres at the borderline levels. The 2 samples with high ELISA titres (>900) were obtained from patients whose Bethesda assay titres were 1.7 and 0.6 BU/mL.

Of the 19 samples that were positive in the Bethesda screening assay but not detected in ELISA showed low titres that were around the borderline, and only 1 had a titre higher than the borderline.

Using the Bethesda screening assay, positive titres were confirmed in 10 of the 50 patients enrolled in our study: thus, the antibody incidence was 20.0%, which was higher than the overall incidence of 12.5% in Korea [6]. However, these values vary widely [7–12], because the FVIII inhibitor profile of Korean patients may be different from that of patients in other countries, and differences may exist among the Korean hemophilic patients. In addition, if a lupus–sensitive activated partial thromboplastin time (aPTT) reagent is used, the Bethesda assay will show positive titres in lupus–anticoagulant patients [13]. The Bethesda assay may also be affected by thrombin inhibitors [5, 14], although we could not assess the presence of lupus anticoagulants or thrombin inhibitors in our samples.

Of the samples obtained from hemophilia A patients in our study, none of the negative samples in the Bethesda assay were observed to be positive in ELISA. Moreover, this study showed that not all ELISA–positive samples were positive in the repeated Bethesda assay, this finding may be attributed to the presence of natural non–inhibitory antibodies or low titres of inhibitors. The ELISA–positive and the repeated Bethesda–negative results indicate that ELISA can detect low titres of inhibitors that would otherwise be undetected in the Bethesda assay. Clinically, samples with low titres of inhibitors are considered negative in the Bethesda assay, and patients with such samples often undergo inadequate therapy. Several studies have described a low–titre inhibitor known as a “transient inhibitor” which is formed spontaneously but disappears subsequently despite continuous treatment with recombinant FVIII [15]. In our study, the samples of the patients

<table>
<thead>
<tr>
<th>With fresh sample</th>
<th>With the sample after freezing and thawing</th>
<th>ELISA</th>
<th>Bethesda assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVIII inhibitor</td>
<td></td>
<td></td>
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<tr>
<td>Positive (N=35)</td>
<td>Positive (N=16)</td>
<td>Positive (N=4)</td>
<td>Negative (N=12)</td>
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<tr>
<td></td>
<td></td>
<td>Negative (N=19)</td>
<td>Negative (N=19)</td>
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<tr>
<td>Negative (N=40)</td>
<td>Negative (N=40)</td>
<td>Negative (N=40)</td>
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</table>

Table 1. Results of FVIII antibody detection in the Bethesda screening assay and ELISA.
receiving FVIII therapy were not positive in either the ELISA or Bethesda assays, and this finding is consistent with the findings of the previous studies [15, 16].

Although studies have shown that plasma samples can be frozen at −70°C without affecting the activity of FVIII inhibitors [17, 18], samples with borderline titres (between 0.3 BU/mL and 0.7 BU/mL) in the Bethesda screening assay were negative in the repeated Bethesda assay performed after thawing the sample. Moreover, the titre levels in the repeated Bethesda assay performed after thawing were lower than those in the Bethesda screening assay, thereby suggesting that antibodies in the samples undergo degradation during freezing and thawing and lead to titres below the detection level in the repeated Bethesda assay.

In this study, the ELISA technique showed a higher sensitivity than the Bethesda assay in the detection of FVIII inhibitors in samples subjected to freezing and thawing procedures. This finding is consistent with those of previous studies [1, 19]. Therefore, ELISA can be considered as a sensitive screening method for determining FVIII inhibitors in samples subjected to freezing and thawing procedures. Moreover, ELISA is an efficient technique for the detection of low titres of inhibitors.

REFERENCES


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