A Detailed Comparison of the Performance of the Standard versus the Nijmegen Modification of the Bethesda Assay in Detecting Factor VIII:C Inhibitors in the Haemophilia A Population of Canada

Alan R. Giles¹, ², Bert Verbruggen³, Georges E. Rivard², Jerome Teitel², Irwin Walker² and the Association of Hemophilia Centre Directors of Canada

On behalf of Factor VIII/IX Subcommittee of Scientific and Standardization Committee of International Society on Thrombosis and Haemostasis

From the ¹AHCDC Factor VIII Inhibitor Reference Laboratory Kingston General Hospital, Kingston, Ontario, Canada, ²the Association of Hemophilia Centre Directors of Canada (AHCDC), Toronto, ON, Canada; ³the Central Laboratory for Hematology of Academic Hospital St. Radboud, Nijmegen, The Netherlands

Summary

The Bethesda assay is widely used to monitor the development and progress of Factor VIII:C inhibitors. Factor VIII stability in the substrate plasma (normal pool) is compromised by pH shift and reduction in protein concentration. Preliminary study, by Verbruggen and colleagues (8), suggested a reduction in spuriously positive assay results may result from buffering the normal pool plasma substrate with imidazole to pH 7.4 and substituting Factor VIII deficient plasma for imidazole buffer in the control incubation mix. These laboratory findings have now been confirmed by the performance of both the standard and the modified Bethesda assays in parallel on 877 patient samples screened during the Factor VIII:C Inhibitor Surveillance Program instituted following the conversion of all Canadian haemophilia A patients to recombinant Factor VIII. Although this study does not address the question of the clinical significance of spurious positive assays, these laboratory findings do support the conclusions of Verbruggen and the modified assay has recently been endorsed by the Factor VIII/IX Subcommittee of the SSC.

Introduction

The development of homologous antibodies to Factor VIII:C (F VIII:C) in the treatment of patients with haemophilia A (Factor VIII deficiency) remains as one of the major challenges in the treatment of this condition (1). The actual incidence reported varies widely from as low as 2.4 (2) to as high as 52% (3). The explanation for this discrepancy is clearly multifactorial, reflecting amongst other things known changes in prevalence during the natural history of the disorder in any one individual (4) and the clearly heterogeneous nature of the group of inhibitors that compromise F VIII function and/or survival following its infusion into a deficient individual (5). The latter clearly has a major influence with regard to the choices of methodological approach, any one of which may be more or less appropriate for the detection of a particular class or type of inhibitor. Despite this, however, there is clear need to standardize the methods used wherever possible in order to minimize variations in procedure as contributing factors to discrepant observations. Recognition of this led to a consensus of North American haemophilia treaters to recommend the adoption of an assay where the methodological approach and the F VIII inhibitory units assigned were standardized (6). Subsequently, the Bethesda assay has achieved international recognition and is now the most frequently used approach for this purpose throughout the world. Nonetheless, as acknowledged by its original proponents, it is clearly a compromise approach given that no one assay can be expected to capture all clinically significant inhibitors to F VIII:C (6). Despite this reservation, however, the introduction of new therapies, such as recombinant F VIII (rF VIII) and the prospect of gene therapy in the not too distant future, emphasizes the need for a consensual approach to be adopted in evaluating these treatment strategies with regard to any potential risk for the development of inhibitors. In monitoring the introduction of rF VIII, the Bethesda assay has been used in most studies as the gold standard for this purpose (7). Verbruggen and co-workers, however, have presented theoretical and clinical data suggesting that the use of the original method may give rise to spuriously positive results due to its failure to accommodate shifts in pH.
and protein concentration which lead to loss of F VIII:C activity unrelated to the presence of inhibitory activity (8).

Late in 1993, the Canadian Blood Agency agreed to fund the conversion of all Canadian haemophilia A patients from plasma derived to rF VIII. Consequent to this, the Association of Hemophilia Centre Directors of Canada developed a surveillance program to monitor F VIII inhibitor development before and after this took place. In recognition of the possible influence of methodological differences in the performance of F VIII inhibitor assays in laboratories associated with the 24 Canadian regional haemophilia programs, all directors agreed to send patient samples, obtained before and for two years after conversion to rF VIII, to a national reference centre established under the supervision of one of the authors (ARG). Initially and by consensus amongst all the directors, the standard Bethesda assay was adopted and used exclusively during the first year of surveillance. Subsequently, however, with the sponsorship of the F VIII Subcommittee of the Scientific & Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH), it was agreed that all samples received by the reference centre would be assayed in parallel by the standard and Nijmegen modified Bethesda assay in order to provide comparative data from a large group of haemophiliacs all receiving the same replacement therapy and with all plasma samples collected by a standardized protocol and analyzed by a single laboratory. This study was not intended to examine the clinical significance of these findings, although when considered in the light of the data of Verbruggen and co-workers (8), does suggest that the modifications described by them should be adopted in all future surveillance programs where the Bethesda assay is used.

Materials and Methods

Note: Detailed protocols on blood collection/plasma sample preparation and assay performance are available from the principal author (ARG) at the address/E-mail (preferred) given above.

Plasma Preparation

A standard protocol was followed by all participating clinics. Blood was drawn by a two syringe technique via 19 gauge butterfly needle. The first draw (5 ml) was discarded and the second (10 ml) used to fill a vacutainer (Becton & Dickinson, NJ, USA) containing sodium citrate 0.11 M (3.2%) - 9 vol. blood to 5 ml was discarded and the second (10 ml) used to fill a vacutainer (Becton & Dickinson, NJ, USA) containing sodium citrate 0.11 M (3.2%) - 9 vol. blood to 5 ml was discarded and the second (10 ml) used to fill a vacutainer (Becton & Dickinson, NJ, USA) containing sodium citrate 0.11 M (3.2%) - 9 vol. blood to 5 ml was discarded and the second (10 ml) used to fill a vacutainer (Becton & Dickinson, NJ, USA) containing sodium citrate 0.11 M (3.2%) - 9 vol. blood to 5 ml was discarded and the second (10 ml) used to fill a vacutainer (Becton & Dickinson, NJ, USA) containing sodium citrate 0.11 M (3.2%) - 9 vol. blood to.

PPP sample was screened (APTT/PT/TT) prior to pooling to provide a given lot of normal pool plasma. Lots were collected approximately every two months. After pooling, each lot was divided into two and one buffered by adding solid imidazole (Sigma, St. Louis, MO, USA) to a concentration of 0.1M with adjustment to pH 7.4 by the slow addition of 1N HCl with constant stirring at 4° C. Plasma was removed and aliquoted in 1.0 ml vol. into capped plastic tubes. All specimens were frozen either by placing on dry ice or in a low temperature freezer (-60° C or lower) within 2 h of venesection. All specimens were shipped on dry ice to the reference centre for assay.

Normal pool plasma was prepared by an identical protocol following collection of blood from 20 normal (10 male/10 female) volunteers. Each individual PPP sample was screened (APTT/PT/TT) prior to pooling to provide a given lot of normal pool plasma. Lots were collected approximately every two months. After pooling, each lot was divided into two and one buffered by adding solid imidazole (Sigma, St. Louis, MO, USA) to a concentration of 0.1M with adjustment to pH 7.4 by the slow addition of 1N HCl with constant stirring at 4° C. Plasma was removed and aliquoted in 1.0 ml vol. into capped plastic tubes. All specimens were frozen either by placing on dry ice or in a low temperature freezer (-60° C or lower) within 2 h of venesection. All specimens were shipped on dry ice to the reference centre for assay.

Factor VIII:C Assay

F VIII:C assay was measured in a one stage APTT-based clotting assay (9) on a Coag-a-mate X2 (Organon Teknika Canada, ON, Canada). APTT reagent and immunodepleted F VIII deficient plasma was obtained from Biopool Canada (Biopool, Burlington, ON, Canada).

Bethesda Assay Protocol

One sample from each patient was assayed for evidence of F VIII inhibitory activity by both the standard or classical Bethesda assay (CB) as described by Kasper and co-workers (6) and the Nijmegen modified Bethesda (NB) as described by Verbruggen and co-workers (8). In each case, the assays were performed concurrently. In the CB 1 vol of patient plasma was incubated with 1 vol of unbuffered normal pool plasma for 2 h at 37° C in a waterbath. At the same time, 1 vol of unbuffered normal pool plasma was incubated with imidazole buffer (0.05 M, pH 7.3) as a control mixture. In the case of the NB, 1 vol of patient plasma was incubated with 1 vol of imidazole buffered (pH 7.4) normal pool plasma for 2 h at 37° C together with a control mixture of 1 vol of imidazole buffered normal pool plasma (pH 7.4) with 1 vol of immunodepleted F VIII deficient plasma. After the 2 h incubation, in stopped tubes, each mixture was assayed for residual F VIII activity. The percentage residual activity of the test versus the control mixture was determined and plotted logarithmically against units of inhibitor activity on an arithmetic scale. One unit of Bethesda inhibitor activity was determined as previously described by Kasper and co-workers as that resulting in a 50% loss of F VIII activity after 2 h incubation at 37° C (6). Where the percent residual found was between 25-75% Bethesda units the result was determined using the standard curve as previously described (6). Where the loss of activity was >75%, the assay was repeated with appropriate dilution with F VIII deficient plasma and the result calculated to account for this.

Patients

All patients surveyed were registered with one of the 24 comprehensive regional haemophilia programs serving the total haemophilia population in Canada. The basis for the surveillance program, ie. to provide early warning of increased incidence of F VIII:C inhibitors should this occur following the use of rF VIII (rF VIII), was explained to each patient and/or their guardian. The blood samples obtained were collected during regular six-monthly and annual reviews at the clinic which they attended. The investigations were part of the normal battery of tests performed at that time. The details of the program were reviewed by the ethics review board at each of the institutions involved. In the majority of cases it was decided that a signed, informed consent was not required due to the investigations being performed being part of normal management procedures. In the minority, the recommendation to obtain written, informed consent from each patient or their guardian was followed. All patients reviewed below were converted from plasma-derived F VIII to rF VIII (Kogenate - Bayer, West Haven, CT, USA) during the period of the survey. As the majority of the patients had undergone conversion prior to the initiation of this comparative study, only a minority of pre-conversion samples were available for comparative assay purposes.

Survey of Laboratory Protocols for F VIII Inhibitor Development

The majority of laboratories continued to perform F VIII inhibitor assays using their own in-house method and reagents. A detailed survey was performed to identify methodological differences both between and within the assay systems used. Particular emphasis was placed on the use of commercial (usually lyophilized), normal and F VIII-deficient plasma preparations in the assay. The normal practice of buffering plasmas prior to lyophilization was considered particularly relevant in view of the proposed influence that this procedure could have on subsequent F VIII inhibitor assay performance.

Statistical Analysis

Where appropriate, statistical analysis was performed by unpaired Student’s t-test.

Results

Eight hundred and seventy-seven Canadian haemophilia A patients have been converted from plasma-derived to recombinant F VIII (rF VIII) since October 1993. Plasma samples from all these patients...
were obtained prior to conversion and assayed by the standard or classical Bethesda assay (CB). Of these, 626 patients have been followed for one year and 326 for two years. Prior to evaluating the results obtained, 19 laboratories, serving the 24 haemophilia programs and normally performing the Bethesda assay, were surveyed as to the cut-off used in Bethesda units (BU) that would be reported as compatible with the presence of an inhibitor to F VIII:C. The recommendations ranged from minimal (>0) to >1.9 BU. The majority (N = 9) reported values of 0.5 BU or greater. By consensus therefore, this value has been used in reporting the outcome of the surveillance program. Preliminary analysis suggests that there has been no increase in the incidence of inhibitor development one and two years following conversion to rF VIII (10). A full report will be prepared and submitted after all patients have been followed for at least two years.

Comencing in October 1995, all samples were assayed by CB and the Nijmegen modification (NB). Eight hundred and seventy-seven assays have been run in parallel, of which 233 were performed on samples obtained from individuals prior to conversion from plasma-derived to rF VIII with the balance performed on post-conversion samples. Using the consensual but arbitrary cut-off of 0.5 BU or greater, 47 samples demonstrated the presence of an inhibitor using the classical method compared with 35 using the Nijmegen modified procedure. The distribution of results are shown in Table 1. Only one specimen was negative by CB (0.4 BU) and positive by NB (0.5 BU). More frequently, the relationship was reversed with 13 specimens being judged positive by CB (Range: 0.5-0.8 BU) and negative by NB (Range: 0-0.2 BU). As measured by these criteria, the incidence of inhibitors in the rF VIII Canadian haemophilia population was 5% as assessed by CB and 4% by NB (Table 2). In those individuals judged to be positive for inhibitor presence, the mean inhibitor titre was 15.48 ± 34.57 (SD) BU with a range of 0.5-165 BU by the classical method. Using the Nijmegen modified procedure, the mean titre was 33.44 ± 69.72 (SD) with a range of 0.5-275 BU. The differences were not significant (P <0.05 - unpaired Student’s t-tests).

Although the cut-off adopted in the Canadian surveillance program is in line with that used in many other reported studies, it does not necessarily reflect universal practice. Table 3 provides the distribution of all the assay results obtained, including those in the “grey zone”, from minimal detectable inhibitory activity (>0 BU) to <0.5. The trend for the Nijmegen modification to reduce the number of positive assays was also observed even in the “grey zone”. Thirty-one samples exhibited some inhibitory activity (>0-<0.5 BU) by CB but were completely negative by NB. In eleven specimens this relationship was reversed.

Survey of Bethesda Assay Protocols Used by Participating Canadian Haemophilia Centre Laboratories

Nineteen of the participating laboratories followed the classical Bethesda assay in broad detail but a number of methodological variables and differences in reporting practices were identified. Full details of this survey will be reported elsewhere but of particular relevance to this report was the finding that nine laboratories used commercial lyophilized normal pool plasma in their assay systems. Cross-checking with the manufacturers confirmed that all were buffered prior to lyophilization but in no case was imidazole used for this purpose.

Discussion

These data support the preliminary findings of Verbruggen and coworkers in support of their prediction based, on study of F VIII stabil-
ity under varying conditions of pH and protein concentration, that failure to control these parameters during the course of performing the Bethesda assay may lead to the reporting of spuriously positive results (8). Although the Canadian F VIII Inhibitor Surveillance Program had arbitrarily elected a cut-off of 0.5 BU or greater, the data suggest that the conclusion reached was independent of the value chosen. As would be expected on theoretical grounds, the modified method appeared to be mainly influential in reducing the number of low titre inhibitors reported with the classical method, where F VIII was not protected from shifts in pH and protein concentration. There was also a trend towards higher recorded titres with the modified method in samples demonstrating inhibitory activity in the upper range of the titres observed in this study. The latter did not reach statistical significance, however, when compared to the results obtained with the un-modified assay. The significance of the low titre “grey zone” (>0-<0.5 BU) results with the modified assay can only be determined by carefully controlled F VIII recovery/survival study, treatment outcome observations and follow up study with both laboratory and clinical monitoring. The primary objective of this study was to perform a detailed parallel comparison of the two assays’ performance. Consequently, these data cannot address these issues but such follow-up studies are clearly warranted.

The finding that a number of laboratories using commercially available lyophilized and therefore buffered normal pooled plasma have, albeit inadvertently, adopted one of the two recommended Nijmegen modifications is of interest. This may well explain why the impact of the recommendations made have been questioned by some laboratories’ experience in evaluating the modified assay against the standard procedure. It is entirely possible that the benefits observed in this study may have been blunted by partial adoption of the recommendations made through the use of commercial plasmas. Although none of the preparations available commercially are buffered with imidazole, there is no reason to suspect that other buffering systems cannot be substituted. This should, however, be confirmed by further study.

The use, in the Nijmegen modification, of F VIII deficient plasma in place of imidazole buffer, both as the substitute for patient (test) plasma in the control mixture and as a diluent of the patient sample if the assay result falls outside the permissible range, deserves additional comment. The commercial F VIII deficient plasma used in these studies was immunodepleted. Use of chemically depleted F VIII deficient plasma could theoretically give spuriously results due to the presence of F VIII which, although functionless, could still provide the antigenic sites for F VIII antibodies in the test plasma. This theoretical consideration has been confirmed by personal observation (BV) and more recently by a detailed Canadian multicentre comparative assay study (11). Thus, in general, chemically depleted F VIII deficient plasma should not be used as a diluent in any F VIII inhibitor assay procedure including those described here. The use of F VIII deficient plasma as a substitute for buffer solutions also has significant cost implications. Theoretically, the objective of maintaining protein concentrations in the control mixture and following test sample dilution could be achieved using an albumin solution or other protein substitutes as a cheaper alternative. This possibility is currently under evaluation.

It should be emphasized that in proposing this approach to the laboratory monitoring of F VIII inhibitor development, Kasper and co-workers clearly recognized its limitations but recommended it as a standardized and manageable compromise for the monitoring of the majority of haemophilia A patients and comparing the results obtained from one laboratory to the next (6). The recommendations of the Nijmegen group are in line with this approach of standardizing and optimizing the assay procedure in order to facilitate inter-laboratory comparisons that are essential for both monitoring the safety of F VIII replacement therapy with regard to inhibitor development and also in evaluating the efficacy of procedures designed to ablate or moderate established F VIII:C inhibitors in congenital or acquired F VIII:C deficiency. Thus, based on the findings reported, the recommendation that the standard or classical Bethesda assay should be modified to include buffering of the substrate normal pool plasma and the use of F VIII deficient plasma in place of imidazole buffer in the control mixture, as described under Methods, was recommended to the Subcommittee on F VIII and F IX of the Scientific & Standardization Committee of the International Society of Thrombosis and Haemostasis at their meetings in Barcelona and Dublin in June, 1996. This was accepted by consensus for recommendation to the membership and other interested parties.

Acknowledgments

The substantial contribution to the technical and administrative performance of these studies by Ms Louise Dwyre, Cindy Johnston, Kathy Blazer and Caroline Hensman is gratefully acknowledged, as is the contribution of Ms Barbara Saunders in the preparation of the manuscript. The willing participation of all our haemophilia A patients and the co-operation of the members of the Association of Hemophilia Centre Directors of Canada and the Hemophilia Program Nurse Co-ordinators in collecting the plasma samples was essential for the success of the program. Financial support was provided in part by the Canadian Blood Agency, Bayer Canada, BioPool Canada, Immuno Canada and Organon Teknika Canada. ARG is a Distinguished Research Professor of the Heart & Stroke Foundation of Ontario.

References