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Validation of the chromogenic Bethesda assay for factor VIII inhibitors in hemophilia a patients receiving Emicizumab

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Dear Editors:

Development of antibodies interfering with the function of factor VIII (FVIII) replacement products is one of the most significant complications in the treatment of hemophilia A (HA). Laboratory testing for such antibodies, called inhibitors, is an important part of hemophilia care and is conducted both to identify the cause of treatment failure and as routine screening to detect early antibody appearance. Treatment of HA patients who develop inhibitors is often carried out by giving repeated doses of FVIII to induce immune tolerance and allow use of FVIII or by use of by-passing agents that act by facilitating coagulation without the need for FVIII. The newest by-passing product, emicizumab (Hemlibra®), is a bispecific antibody that mimics the function of FVIII by bringing factor IXa and factor X (FX) together to produce the Xa complex.[1,2] Emicizumab, which is long acting and given subcutaneously, is now widely available for use in patients both with and without inhibitors to avoid frequent use of intravenous FVIII replacement.

Monitoring of patients treated with emicizumab presents challenges for the clinical laboratory, because the drug interferes with the FVIII one-stage assay (OSA).[1-3] Emicizumab reacts in a FVIII chromogenic substrate assay (CSA) only if human-derived FX is used; it does not react with the bovine FX used in some FVIII CSA.[3] This differential species reactivity has been exploited to allow measurement of FVIII and FVIII inhibitors in the presence of emicizumab by using such a CSA.[4]

The traditional assays for FVIII inhibitors based on a OSA include a modified Nijmegen-Bethesda assay (NBA) that uses preanalytical heat treatment of patient plasma to remove infused or endogenous FVIII without destroying the antibodies to be measured[5]; however, emicizumab, itself an antibody, cannot be removed by this method.[1,2] We[6] and others (as reviewed in Miller[7]) have previously demonstrated improved performance of a chromogenic Bethesda assay (CBA) over the NBA due to its insensitivity to nonspecific inhibitors of

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AUTHOR CONTRIBUTIONS

C. H. Miller planned the study, analyzed results, and wrote the manuscript. B. Boylan and J. Driggers performed research, analyzed results, and wrote the manuscript. AB Payne analyzed results and wrote the manuscript. C. J. Bean wrote the manuscript.

CONFLICT OF INTEREST

The authors state that they have no real or potential conflicting interests.

coagulation in patients treated with FVIII products. The aim of this study was to describe the performance characteristics of the CBA for FVIII inhibitor measurement in patients receiving emicizumab.

Data were analyzed from 800 specimens collected from subjects with congenital HA enrolled in the Registry for Bleeding Disorders Surveillance of the Community Counts program[8] at US Hemophilia Treatment Centers who were receiving emicizumab as their primary treatment product. Participants were not required to give informed consent for surveillance specimens. Data on previous inhibitor history collected from the enrolling sites on forms submitted with each specimen were available for 781 specimens from 648 patients.

Specimens were collected as previously described[5] into 3.2% sodium citrate in a ratio of 1:9 with blood and centrifuged at 1600 × *g* for 20 minutes at 4°C, followed by a second centrifugation of separated plasma under the same conditions in polypropylene tubes. The separated plasma was shipped to CDC overnight on cold packs or frozen on dry ice and there aliquoted and stored in polypropylene tubes at −70°C.

The chromogenic Bethesda assay (CBA) was performed as previously described,[6] including heating of patient plasma to 56°C for 30 minutes and centrifugation prior to testing. One chromogenic Bethesda unit (CBU) was defined as the amount of inhibitor per milliliter (mL) of patient plasma which inactivates 50% of the FVIII activity in 1 mL of pooled normal plasma (PNP) during a 2-hour incubation at 37°C. FVIII activity was measured using a CSA (Siemens Factor VIII Chromogenic Assay, Siemens, Marburg, Germany) performed on a STAR Evolution (Diagnostica Stago). Plasma diluted 1:31 in imidazole buffer (Siemens) was incubated with bovine FX, bovine factor IXa, bovine thrombin, $CaCl_2$, and phospholipid for 90 seconds at 37°C. Factor Xa substrate with a thrombin inhibitor and a stopping buffer was added. The change in absorbance per minute was read at 405 nanometers. Antibodies binding to FVIII were measured by fluorescence immunoassay, as previously described.[9] Results were 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 subjects.

Group frequencies were compared by Fisher's exact test with significance set at $P < .05$ using GraphPad Prism 8.3 (GraphPad Software Inc).

The threshold for positivity for the CBA was evaluated by two methods previously used for the NBA: examination of CBA results in patients with and without history of inhibitor[5] and comparison of CBA results with the presence of anti-FVIII IgG_4 antibodies.[10] Anti-FVIII antibodies of IgG_4 subclass have been shown to be most closely linked to the presence of a functional inhibitor, other subclasses also appearing in inhibitor-negative patients.[9,11] Figure 1 shows distributions of CBA results from patients with and without previous history of inhibitor. Among 378 negative history specimens, 3 (0.8%) were above 0.4 CBU. One of the three had 7 CBU and was positive for anti-FVIII IgG_4 antibodies; two with 0.6 and 0.7 CBU were IgG_4 antibody-negative, indicating a false positive rate of 2 in 378 (0.5%) among those with negative history of inhibitor. Measurement of anti-FVIII IgG_4 among CBA-positive specimens showed 244 of 250 (97.6%) to be positive. The group of 91 specimens with

0.5-1.9 CBU did not differ significantly from the 159 with ≥2.0 CBU with 96.7 and 98.1% of specimens positive for anti-FVIII IgG_4, respectively ($P = .67$). A rate of 6 of 250 (2.4%) lacking IgG_4 is significantly lower than the 11.5% reported for 122 specimens positive in the NBA ($P = .0008$).[9] Of 375 CBA-negative specimens from patients with negative history of inhibitor, 17 (4.5%) were anti-FVIII IgG_4 positive. This was not significantly different from the 6.0% IgG_4 antibody positivity rate previously reported for 369 NBA-negative patients not receiving emicizumab ($P = .41$) or the 1.8% seen in 56 healthy controls ($P = .49$).[9] These may represent antibodies not interfering with FVIII function or errors in identifying patients with a previous immune response. Among specimens from patients known to be previously positive for inhibitor that were currently CBA-negative, 45 of 156 (28.8%) were IgG_4-positive; these were excluded from Figure 2. When the percent of specimens positive for anti-FVIII IgG_4 was plotted by CBU (Figure 2), antibody positivity was 90%-100% when CBU was ≥0.5. The comparable range of antibody positivity of the NBA was 67%-97%.[10] The threshold for positivity for the CBA based on anti-FVIII IgG_4 positivity thus appeared to be the same as that previously reported for the NBA when using the CDC-modified methods including preanalytical heat inactivation of patient plasma.[10]

Limit of detection (LOD) of the CBA was estimated using the classical method,[12] with blank specimens prepared from equal parts PNP and FVIII-deficient plasma and low-level specimens of 1.0 CBU prepared by diluting high-titer inhibitor plasma (George King Biomedical) in imidazole buffer (Siemens) tested on two different days and with three different sets of reagents for a total of 30 tests each. Nonparametric methods were used to calculate limit of the blank (LOB) as the 95th percentile rank for negative specimens. LOD was calculated as the LOB plus the standard deviation (SD) of the low-level specimens corrected to the 95th percentile of the normal distribution. SD of the low-level specimens was 0.081, and LOB was 0 CBU. The LOD of the CBA, which is reported to one decimal place, was calculated to be 0.1 CBU. In addition, the CBA was performed on specimens from 10 healthy subjects with no history of coagulation disorders. Results showed mean 0.0050, SD 0.0158, and range 0-0.05 CBU. LOD (mean + 3SD) was 0.052 CBU. The best estimate of the LOD of the CBA, therefore, is 0.1 CBU. This is lower than the 0.2 calculated for the NBA,[10] suggesting that the CBA is slightly more sensitive than the NBA.

Significant assay discrepancies occur between the NBA and the CBA for specimens with positive results less than 2.0 NBU,[6] levels which may represent developing or transient inhibitors or a declining titer in a patient previously positive. Specimens in that range are tested without dilution and may be more susceptible to nonspecific inhibitors of OSA.[7] We have previously suggested that positive results <2.0 in the NBA be considered to be in a “gray zone," requiring confirmation by measurement in the CBA and/or detection of anti-FVIII antibodies.[7] The present study shows that this gray zone is not necessary when the CBA is used as the primary test, allowing a greater degree of confidence in low-titer positive results when tests are performed with the CBA than with the NBA. Because the CBA is a more accurate indicator than the NBA of the presence of anti-FVIII antibodies, its use for patients treated with FVIII products as well as those receiving emicizumab should be considered.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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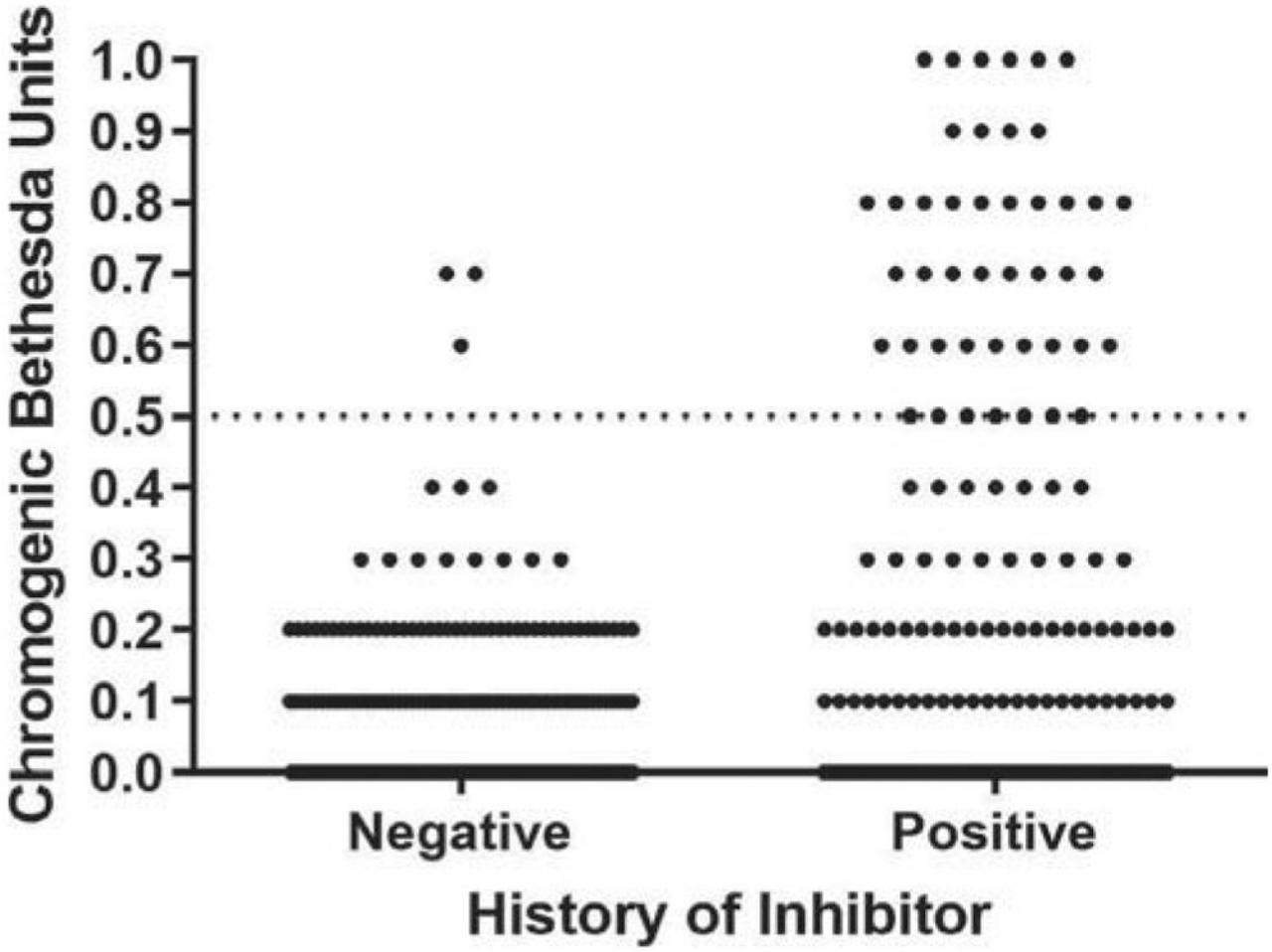

FIGURE 1.

Comparison of results between groups of hemophilia A patients with negative and positive history of factor VIII inhibitor by chromogenic Bethesda assay in patients receiving emicizumab. The dotted line represents the threshold for positivity previously established for the modified NBA[5,10]

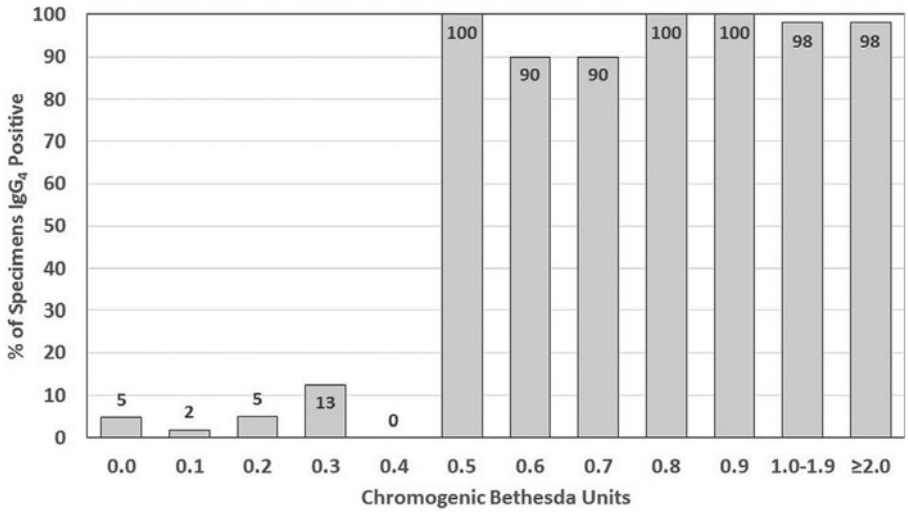

FIGURE 2.

Frequency of antifactor VIII IgG_4antibody positivity by chromogenic Bethesda units among subjects receiving emicizumab