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Overestimation of free light chain antigen excess rate

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ABSTRACT

Background: Free light chains (FLC) are useful biomarkers for diagnosis and follow-up of plasma cell disorders. FLC quantification is encumbered by non-linearity and antigen excess (>4-fold difference between results obtained at the 2000- and 100-fold dilution).
Methods: FLC concentration was measured with Freelite® reagents on the BNII, using 100- and 2000-fold dilutions in 3645 samples. Samples displaying antigen excess were re-measured at the 2000- and/or 400-fold dilution. Carryover was evaluated by tracing samples to cuvettes and by measuring a normal sample in cuvettes that previously contained samples with a high FLC concentration.
Results: Antigen excess occurred in 0.93% of samples for κ and in 0.55% of samples for λ. In 81.5% of the cases, it could not be confirmed by a re-analysis of a 2000-fold and/or a 400-fold diluted sample. Real antigen excess was documented in 0.25% and 0.03% of the samples for κ and λ FLC, respectively. In the low concentration range (2000-fold dilution), imprecision was high. False antigen excess was reduced by batch analysis, introducing cleaning and rinsing procedures and using the 400-fold dilution. No antigen excess was detected in samples with normal FLC concentrations.
Conclusion: Falsely high results occur by imprecision in the low concentration range and/or by carryover in cuvettes.

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1. Introduction

Freelite® free light chain (FLC) is a useful laboratory tool for diagnosis and follow-up of monoclonal gammopathy and possibly also for prognosis of autoimmune diseases [1–7]. In patients with monoclonal gammopathy, important clinical decisions are based on FLC concentrations, differences in κ and λ FLC, and κ/λ FLC ratio.

FLC analysis is performed on automated platforms. The analysis, however, requires special attention due to technical and sample-related caveats. We previously reported antigen excess and non-linearity of the Freelite assay on a BNII platform [8]. Antigen excess, defined as a more than 4-fold difference between results obtained at the 2000-fold and the 100-fold dilution, was found in 5.4% of the samples for κ FLC and in 1.2% of the samples for λ FLC. Non-linearity, defined as a more than 2-fold but less than 4-fold difference between the results obtained at the 2000-fold and 100-fold dilution, was found in 6% of the samples for κ FLC and in 0.5% of the samples for λ FLC [8]. These numbers surpassed the estimates of two earlier reports [9,10] but were later confirmed in a retrospective analysis (also on BNII) showing antigen excess in 5% to 8% of the samples for κ FLC and in 1.5% to 4.5% of the samples for λ FLC [11]. In the latter study, non-linearity was detected in 5% to 6.9% of the samples for κ FLC and in 2% to 4.2% of the samples for λ FLC [11]. A multicentre study found 5% antigen excess for both κ and λ FLC [12]. The manufacturer of the Freelite assay (The Binding Site) states that samples with a normal FLC concentration and FLC ratio at the 100-fold dilution do not require antigen excess check [13].

We continuously follow the prevalence of antigen excess and non-linearity in our laboratory by systematically performing a 100-fold and 2000-fold dilution (for both FLCs). Here we report our findings on consecutive samples tested from January 2012 to November 2013. Specific aims were (i) to get a better insight in the reported high incidence of antigen excess and (ii) to check the guideline of The Binding Site that antigen excess is not indicated in samples with a normal FLC concentration and normal FLC ratio. We describe the occurrence of false antigen excess and elaborate on the analytical conditions in which this phenomenon occurs.

2. Materials and methods

Serum samples from patients suspected of or diagnosed with monoclonal gammopathy from different Belgian hospitals, including tertiary referral centres, were sent to our laboratory by regular mail or courier

Abbreviations: FLC, free light chains; κ, kappa; λ, lambda.

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service at room temperature. They were refrigerated at 2–8 °C upon arrival, analysed following centrifugation within 2 days and subsequently frozen. A total of 3645 samples were analysed between 02/01/2012 and 27/11/2013.

2.1. Quantificaton of FLC

Serum FLC concentration was measured under ISO15189 accreditation, by latex-enhanced immunoassay using Freelite® reagents (The Binding Site, Birmingham, United Kingdom) on a BNII nephelometer (Siemens Healthcare Diagnostic Products Gmbh, Marburg, Germany) according to the manufacturer's instructions for use, except for the dilution protocol. All samples were automatically diluted until a result was obtained. If not automatically performed, an additional 2000-fold dilution was programmed manually for all samples on the same day (for both FLCs). In case of antigen excess (a ≥4-fold difference between results obtained with the 2000-fold and 100-fold dilution), a second fresh 2000-fold dilution was prepared and measured on the same day. A 400-fold dilution was either performed automatically (during the initial analysis) or was done on a thawed sample (if not performed automatically). The second 2000-fold dilution and 400-fold dilution measurements were used to either confirm or refute the result obtained with the first 2000-fold dilution.

From 24/4/2013 onwards, FLC was performed as a batch analysis, after the measurement of other BNII parameters in random access mode. Prior to FLC analysis, the BNII system was cleaned with the Cleaner CSS solution (Siemens Healthcare Diagnostic Products Gmbh, Marburg, Germany) consisting of <2% of sodium hydroxide and detergent, and cuvettes were rinsed with aqua distillate. Before the manually programmed 2000-fold FLC dilutions, cuvettes were rinsed again. In this way, 1136 samples were analysed with an additional cleaning protocol and 2509 samples without.

2.2. Carryover experiment

Carryover by the tubings and pipetting was evaluated by analysing 3 times a high sample (189 mg/L κ FLC or 135 mg/L λ FLC) followed by analysing 3 times a low sample (15.4 mg/L κ FLC, 12.4 mg/L λ FLC). The percentage of carryover was calculated as follows: the difference between the third and first low sample divided by the difference between the third high sample and the third low sample.

In order to evaluate carryover by the cuvettes, signal values representing light scatter and concentrations were analysed relative to the cuvette number. We selected 5 samples with high κ FLC concentrations that were followed by normal samples for which a falsely high result was found (at the 2000-fold dilution) in the same reused cuvettes [labelled as [1–5] in Table 2]). These 5 samples with high κFLC concentrations were manually 5-fold diluted with sample diluent and then automatically 20-fold diluted. Depending on the available sample volume, the final 100-fold dilution was analysed 6 to 10 times. A normal sample was similarly 5-fold pre-diluted and automatically 20-fold diluted. Following the first analysis, cuvettes were randomly reused for the normal sample, subsequently rinsed and again used for the normal sample. Only part of the cuvettes in which the high samples had been incubated were randomly reused by the instrument for the normal sample before and after washing of the cuvette.

2.3. Statistics

Student's t-test and ROC analysis were performed with the Analyse-it software; binomial confidence interval computations can be found at www.statpages.org (accessed 28/8/2014).

3. Results and discussion

3.1. Results

Antigen excess, defined as a ≥4-fold difference between results obtained with the 2000-fold and 100-fold dilution, was found in 34 of 3645 samples (0.93%) for κ FLC and in 20 of 3645 samples (0.55%) for λ FLC over a 2-year period. In all these samples, the 2000-fold dilution was repeated and a 400-fold dilution was analysed as well. Rerunning a 2000-fold dilution confirmed antigen excess in only 12 of 3645 samples (0.33%) for κ FLC and in only 1 of 3645 samples (0.03%) for λ FLC. Analysis of the results obtained at the 400-fold dilution confirmed the presence of antigen excess for κ FLC in 9 samples (shown in Table 1), but not in 3 samples. The results for these three samples were <6.25, 20.2 and 35.8 mg/L κ FLC at the 100-fold dilution; 390, 206 and 156 mg/L at the second 2000-fold dilution; and <25 mg/L, 62.1 mg/L and 65 mg/L at the 400-fold dilution. Table 1 summarises the 9 samples with antigen excess confirmed at the 2000-fold and 400-fold dilution, whereas Table 2 summarises the samples for which the antigen excess was not confirmed at either the 2000-fold or the 400-fold dilution. If the three samples with discordant results between the 2000-fold and 400-fold dilution are considered as false, non-confirmed antigen excess, then the lowest κ FLC concentration at a 100-fold dilution in samples with confirmed antigen excess was 28.8 mg/L. The unique underestimated λ FLC concentration was 57 mg/L. In the unconfirmed antigen excess samples, κ FLC concentrations ranged from <6.33 mg/L to 103 mg/L (Table 2) and λ FLC concentrations from <5.09 mg/L to 101 mg/L (Table 3). In these samples, the increments in scattered light intensity (delta bit signal values) of the first 2000-fold dilution analysis were significantly higher than the ones of the repeat 2000-fold dilution analysis ($p < 0.0001$ paired t-test). No difference was seen for the start values.

We were interested to find out whether sample carryover might play a role in the falsely elevated delta bit values. Therefore, we studied the data from the last previously analysed sample that used the same cuvette as the sample that produced the falsely high 2000-fold dilution results. The delta bit signal values for the earlier analysed samples varied between 57 and 16383 bits (pooled for κ and λ FLC).

Preceding samples that used the same cuvette as the samples with the falsely elevated κ FLC had κ FLC concentrations <10 mg/L (4 samples), ranging from 10 mg/L to 100 mg/L (5 samples), ranging from 100 to 1000 mg/L (1 sample), ranging from 1000 mg/L to 10000 mg/L (3 samples) or ranging from 10000 mg/L to 100000 mg/L (6 samples). For 3 samples, the previous analyte was not FLC; for 2 samples, no data were available; and for another sample, the cuvette was not used before. Preceding sera for κ FLC analysis were diluted at 20-fold ($n = 2$), 100-fold ($n = 13$) or 400-fold ($n = 4$).

For λ FLC, similar results were obtained. Preceding samples that used the same cuvette as the samples with the falsely elevated λ FLC had λ FLC concentrations ranging from 100 mg/L to 1000 mg/L (4 samples), or ranging from 1000 mg/L to 10000 mg/L (3 samples). For 8 samples, the previous analyte was not λ FLC; for 2 samples, no data were available; and for 2 samples, the cuvette was not used before.

Preceding sera for λ FLC analysis were diluted 20-fold ($n = 6$) or 100-fold ($n = 1$).

Subsequently, we set up an experiment to evaluate carryover by tubings, pipettes or reused cuvettes. Carryover by tubings or pipetting was absent (−0.5% for κ FLC and 0.1% for λ FLC). The effect of re-using the cuvettes was evaluated by measuring a sample with a normal FLC level in a cuvette that previously contained a sample with either a normal FLC level or a high FLC level, with or without an extra rinsing step. Samples with a high FLC level were selected based on possible carryover in routine analysis (Table 4). The concentration of the normal sample re-measured in the same cuvette as used for the first measurement was 1.42 mg/L κ FLC. This increased up to 13.3 mg/L and even 60 mg/L in cuvettes that previously contained samples with high FLC

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