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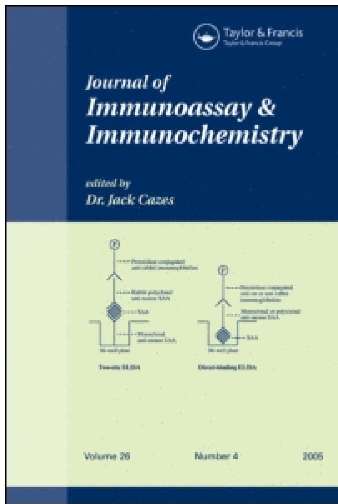

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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Proposed Reference Material for Human Free Immunoglobulin Light Chain Measurement

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To cite this Article Nakano Ph.D., Takanari , Miyazaki, Shuichi , Shinoda, Yuichi , Inoue, Ikuo , Katayama, Shigehiro , Komoda, Tsugikazu and Nagata, Atsuo(2006) 'Proposed Reference Material for Human Free Immunoglobulin Light Chain Measurement', Journal of Immunoassay and Immunochemistry, 27: 2, 129 — 137

To link to this Article: DOI: 10.1080/15321810600573069

URL: http://dx.doi.org/10.1080/15321810600573069

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Journal of Immunoassay & Immunochemistry, 27: 129–137, 2006
Copyright © Taylor & Francis Group, LLC
ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810600573069

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Abstract: Human free immunoglobulin light chain (FLC) kappa and lambda are useful clinical markers for light chain myeloma and AL amyloidosis. With the recent development of specific and reliable FLC immunoassays, the quantitative measurement of FLCs will be widely used in clinical practice. However, researchers have used various calibrators, mainly monoclonal FLCs; thus, no standardization has been performed among the assay methods. This prompted us to purify intact FLCs from the pooled urine specimens of healthy volunteers as the first reference materials for FLC assays. After precipitation with ammonium sulfate, FLCs were purified by the

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following steps of chromatography; cation exchange, gel filtration, and antibody-assisted immunoaffinity. SDS-PAGE and Western blotting analyses showed that the purity of FLC kappa and FLC lambda was more than 98%. These purified FLCs did not contain the other immunological types of light chains. These intact and purified FLCs are suitable as the first reference materials to standardize FLC assays.

Keywords: Free immunoglobulin light chain, Purification, Reference material, Immunoassay

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INTRODUCTION

The measurement of human free immunoglobulin light chain (FLC) is useful in the diagnosis of light chain myeloma and AL amyloidosis.[1–3] The analytical difficulties of FLCs had limited its applications, but recently we and other researchers have developed specific and reliable FLC assays.[4–6] These achievements facilitate the clinical use of FLC measurements, however, these assay methods have not been standardized due to the lack of available reference material.

For assay calibrators, researchers have used monoclonal FLCs, or Bence Jones protein,[2] because these are easier to purify than intact FLCs in specimens. The problem of monoclonal FLCs as assay standards is that each one has respective peculiar structures, resulting in considerable differences in their antigenecities among the origins. In fact, the estimated normal serum reference values for FLCs were widely varied among the reports.[5]

Immunoglobulin light chains in specimens are usually polyclonal:[2] each light chain is different in its amino acid sequence in its variable region. Thus, there may be no perfect reference material that reacts in parallel with FLCs in all specimens. One compromising idea for this is the use of polyclonal FLCs that are similar with FLCs in all specimens except for monoclonal origin. Further, polyclonal FLCs appear to contain all the light chain sub-types.

The purification of intact polyclonal FLCs from serum is difficult, because serum contains more thousands fold amounts of whole immunoglobulin than FLCs.[7] We thought that urine is a good source for intact FLCs, because it usually contains a few milligrams of FLCs per liter with less amounts of whole immunoglobulins and other serum proteins. In the present study, we tried to purify FLCs from urine for the primary reference material in FLC assays.

EXPERIMENTAL

Free Light Chain Immunoassays

The concentrations of FLC kappa (κ) and FLC lambda (λ) were determined by the two enzyme immunoassays described previously with a slight modification.[3,6] Briefly, four assay standards with $20\,\mu g/L$ to $320\,\mu g/L$ of

FLC κ and 17.5 μg/L to 560 μg/L of FLC λ, respectively, and diluted specimens were applied to anti-FLC specific mAb 4E6 or mAb 2G7-coated 96-well plates in duplicate, incubated for 2 h, and washed. Then, the plates were incubated with horseradish peroxidase (HRP)-conjugated mAb 2G5 F(ab')$_2$ fragment and mAb 4D12 F(ab')$_2$ fragment for 30 min and washed again. The bound conjugates were detected by a reaction with *o*-phenylenediamine. In this study, purified FLC κ and FLC λ from urine were used as assay calibrators (see below).

Antibodies and Antibody-Conjugated Affinity Column

Anti-free and bound light chain κ and anti-free and bound light chain λ antisera were purchased from Dakopatts (Stockholm, Sweden). Anti-free and bound light chain κ monoclonal antibody (mAb) 2G5 and anti-free and bound light chain λ mAb 4D12 were obtained from our mAb library. These two mAbs were coupled with CNBr-activated sepharose 4B (Amersham Biosciences, NJ) at a protein concentration of 10 mg per milliliter of the gel.

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Purification of Free Light Chains

FLCs in 4 L pooled urine obtained from healthy subjects were purified by stepwise chromatography as follows. Proteins in the urine were precipitated with ammonium sulphate at 80% saturation. After the centrifugation at 10,000 $\times$ *g*, the precipitate was dialyzed against 10 mmol/L acetate buffer, pH4.8. Then, the soluble fraction was recovered and applied to the cation exchange chromatography using SP sepharose (Amersham Biosciences). The FLCs absorbed in SP sepharose were eluted with the acetate buffer containing 0.25 mol/L NaCl at a flow rate of 1 mL/min. The FLC fractions were then pooled and concentrated with Ultrafree 10 K NMWL membrane (Millipore Corp., Bedford, MA) and applied to Ultrogel® AcA44 gel filtration chromatography (BioSepra, SA, France) using phosphate-buffered saline as an eluent. FLC κ and FLC λ were finally purified by mAb 2G5- and mAb 4D12-conjugated affinity column chromatography, respectively. The conjugated FLC κ or FLC λ was eluted with 0.1 mol/L glycine-HCl buffer, pH2.7, and neutralized with 1/10 volume of 1 mol/L Tris-HCl buffer, pH8.0, in the fraction tube. We determined the protein concentrations of the purified FLCs using the Bradford protein assay method with bovine γ globulin as a standard (Protein standard I, Bio-Rad laboratories, Herules, CA).

SDS-PAGE and Western Blotting Analysis

Partially purified fractions and purified FLCs were loaded under reducing conditions, respectively, onto 10% polyacrylamide gels using a Mini Protean II

electrophoresis cell (Bio-Rad) and the gels were stained with 0.1% Coomassie brilliant blue. The purity of FLCs was determined by the densitometry analysis (Image J version 1.31, available at http://rsb.info.nih.gov/ij/) of the electrophoresed patterns. Western blotting was performed as described in a previous report.[6] Briefly, electrophoresed proteins were transferred onto nitrocellulose membrane and incubated with the indicated antibodies for 1 h. The remaining antibodies were detected using HRP-labeled species-specific anti-IgG antisera with HRP Color Development Reagent (Bio-Rad).

Specimens

To estimate normal reference values, we obtained 97 sera and 29 urine specimens from volunteers who agreed to our study.

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RESULTS

Purification of Free Light Chains from Urine

The protein precipitin of 4 L urine with ammonium sulfate contained 374.4 μg of FLC κ and 527.7 μg of FLC λ (Table 1). The FLCs in the precipitin were absorbed to SP sepharose at pH 4.8 and, then, most of the FLCs were eluted with the same buffer containing 0.25 mol/L of NaCl (Fig. 1A). In this step, the recovery of FLCs was >70% and the protein content was reduced to one-fiftieth. The major protein (around 100 kDa) and a part of albumin were removed by the SP-sepharose chromatography (Fig. 2A). The following gel filtration raised the purity more than 700-fold from the pooled fractions after SP sepharose chromatography. Finally, FLC κ and FLC λ were separated and purified by antibody-assisted affinity column chromatography (Fig. 1C and 1D).

The purity of both preparations were found to be >98% by SDS-PAGE. FLC assays and Western-blotting analysis with anti-light chain κ and anti-light chain λ antibodies showed that both peparations were free from the other light chain and whole immunoglobulins (Fig. 2B). Finally, we obtained 180.6 μg of purified FLC κ and 228.1 μg of purified FLC λ with total recovery of 46.0% and 60.3%, respectively.

Free Light Chains in Urine and Serum

Using purified FLCs as standards, we determined the concentrations of FLC κ and FLC λ in urine and serum samples. The mean concentrations of FLC κ were 2.1 ± 2.8 (SD) mg/L in urine and 43.5 ± 12.0 mg/L in serum, and those of FLC λ were 2.8 ± 3.5 and 55.2 ± 17.9 mg/L, respectively. The

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Table 1. Summary of purification of free light chains in urine

Purification step	Total protein (g)	Free light chain κ Amount (μg)	Free light chain κ Recovery (%)[a]	Free light chain κ Specificity (%)[b]	Free light chain λ Amount (μg)	Free light chain λ Recovery (%)	Free light chain λ Specificity (%)
Ammonium sulphate saturation							
Precipitate	127.37	374.3	95.3	0.0003	527.7	94.1	0.0002
Supernatant	0.026	18.5		0.071	26.1		0.05
SP sepharose	2.56	279.1	71.1	0.011	393.5	80.2	0.008
AcA44 gel filtration	0.0033	257.3	65.5	7.9	362.8	74.3	5.6
Anti-κ mAb affinity column	1.83×10^{-4}	180.6	46.0	98.7	254.7	70.1	
Anti-λ mAb affinity column	2.25×10^{-4}				228.1	60.3	98.6

Free light chain κ and λ were purified from four-litter pooled urine.
[a]Percentage of recovery at each purification step was calculated by the following formula: The amount of FLC/total FLC contents in the urine.
[b]Percentage of specificity was calculated by the following formula: The amount of FLC/total protein amount $\times$ 100.

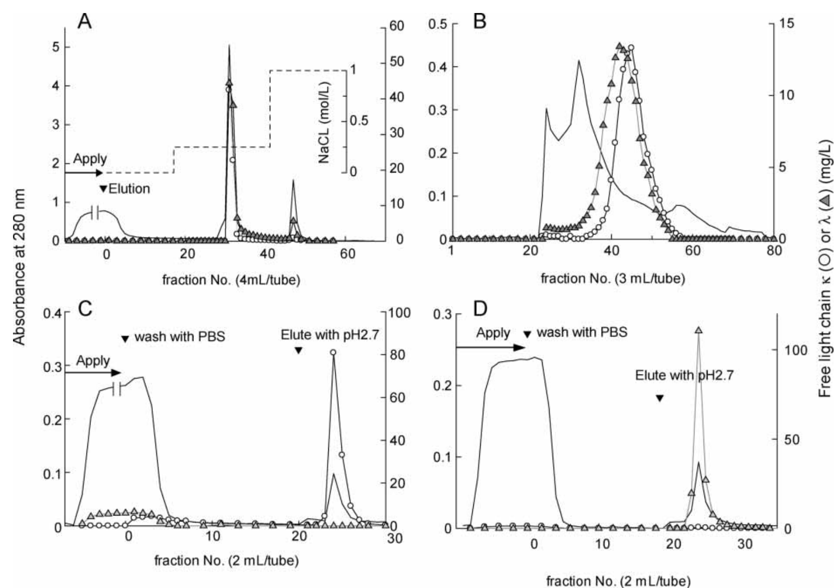

Figure 1. Purification of urinary FLCs. A. The protein precipitin of pooled urine was applied to SP sepharose column (ϕ16 mm × 50 mm) with flow rate at 1 mL/min. The FLCs absorbed to the column were eluted with the starting buffer containing 0.25 mmol/L of NaCl solution. B. The fractions #31–#37 from SP sepharose column chromatography was then applied to the gel filtration (ϕ16 mm × 960 mm) with flow rate at 0.1 mL/min. Fractions #37–#52 were recovered. C. Antibody-assisted affinity column chromatography for κ light chain (ϕ9 mm × 20 mm). Pure FLC κ was obtained by eluting with 0.1 mol/L glycine-HCl buffer, pH2.7. D. The pass-through fraction of chromatography C was applied to the affinity column for λ light chain (ϕ9 mm × 20 mm). Pure FLC λ was also obtained in the same manner.

means of the urinary and serum FLC κ/λ ratio were 0.82 ± 0.29 and 0.90 ± 0.23, respectively.

DISCUSSION

In human FLC assays, no reference material is available to standardize the measured values. In the present study, we tried to purify intact polyclonal FLC κ and FLC λ from pooled urine to obtain the first reference material. Intact FLCs in the urine were purified by ion exchange, gel filtration, and antibody assisted immunoaffinity chromatography. The purity of the resulting FLC κ and FLC λ was more than 98%. In addition, the resulting FLCs were not contaminated with the other immunological type of light chain.

Using the purified FLCs as calibrators, the mean normal values of urinary FLC κ and FLC λ were determined as 2.1 mg/L and 2.8 mg/L, respectively,

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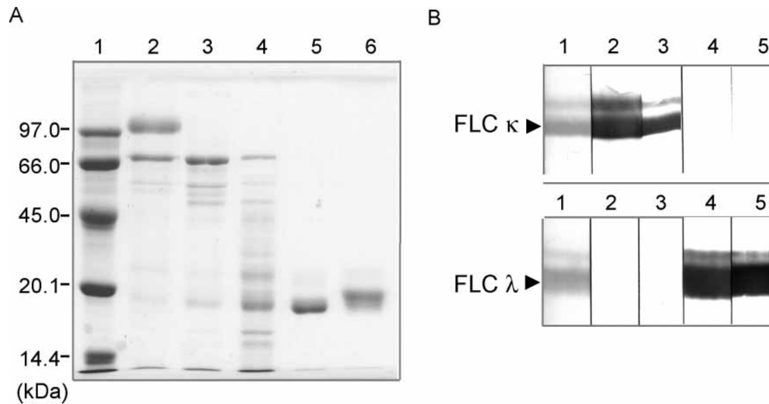

Figure 2. A. SDS-PAGE analysis of purified FLCs. Lane1, protein size marker; lane 2, ten μg of protein precipitation of urine with ammonium sulphate; lane 3, ten μg of free light chain fraction from SP sepharose; lane 4, ten μg of free light chain fraction from AcA44 gel filtration; lane 5, two μg of purified free light chain κ; lane 6, two μg of purified free light chain λ. B. Western blotting analysis of purified free light chains. Lane 1, stained with amid black; lane 2, anti-light chain κ mAb; lane 3, anti-light chain κ antiserum; lane 4, anti-light chain λ mAb; lane 5, anti-light chain λ antiserum.

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which is approximately consistent with the previously reported values; from 1.8 mg/L to 5.5 mg/L for FLC κ and from 0.75 mg/L to 3.17 mg/L for FLCλ. On the other hand, the normal amounts of FLCs in serum were varied about 10-fold among the studies; from 1.5 mg/L to 14 mg/L for FLC κ and from 0.5 mg/L to 10 mg/L for FLCλ.[5] Our FLC assays with newly purified FLCs as standards showed greater values than those earlier studies.

In the studies for FLC measurements, the standardization has been lacking. Different calibrators have been used in respective assays.[8–10] Those were mostly monoclonal FLCs that considerably differ in their structure and antigenecity to each other.[2,11] The mean normal serum values for FLCs varied 10- to 20-fold among the reports.[5] This inconsistent data is probably due to the different calibrators. Another possibility is the different degrees of crossreactivity with light chains in whole immunoglobulins.[7] To compare this discrepancy, an internationally available reference material will be useful.

Carr-Smith et al.[12] obtained a free form of light chain by reducing and acetylating purified polyclonal IgG. The purified FLCs were polyclonal origin, but those seem to differ in the glycosylation, the folding of the polypeptide and polymerization from intact FLCs.[2] In the present study, we purified intact urinary FLCs from polyclonal origin that seemed reliable as standard materials, because no chemical treatment was done on these purified FLCs and, therefore, native antigenecities would be preserved.

The discrepancy in normal FLC values in serum might due to the different method of sample preparation and calibrators. In addition, serum whole immunoglobulins might interfere and could affect the measured values. Therefore, we strongly believe that reliable reference material is needed to standardize FLC assays.

In conclusion, we propose that the purified FLC materials from urine specimens may be useful as the first reference material to standardize various FLC assays in the future.

ABBREVIATIONS

FLC, free immunoglobulin light chain; κ, kappa; λ, lambda; mAb, monoclonal antibody; HRP, horseradish peroxidase.

ACKNOWLEDGMENTS

The authors thank Mrs. Katsue Sekine and Miss Saori Hinuma for the technical assistance and our colleagues for their comments and advice on this work.

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Received June 1, 2005
Accepted June 28, 2005
Manuscript 3179

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