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CASE REPORT

Glycosylated Bence Jones Protein with Poor Thermal Reactivity in Heat Coagulation Tests

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SUMMARY

Background: **We experienced a patient with multiple myeloma whose urine contained a considerable amount of Bence Jones protein (BJP), which demonstrated poor thermal reactivity in heat coagulation test. The mechanism for this phenomenon was assessed.**
Methods: **Immunoelectrophoretic analyses reveal that a band corresponding to BJP in the urine had 2,600 Dalton by reduction after glycosidase treatment, but not after sialidase treatment. In addition, the glycosidase-treated urine tested positive in heat coagulation test.**
Conclusions: **Glycosylation of the immunoglobulin light chain, which has rarely been seen, is the cause of the unexpected behavior of this patent's BJP in heat coagulation tests.**
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KEY WORDS

Bence Jones protein, multiple myeloma, heat coagulation test, glycosylation, microheterogeneity

LIST OF ABBREVIATIONS

LMW - low molecular weight
SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IEP - immunoelectrophoresis
IEF - isoelectric focusing

INTRODUCTION

Bence Jones protein (BJP), a free monoclonal immunoglobulin light chain, is found in the urine of patients with multiple myeloma or primary macroglobulinemia. BJP has temperature-dependent structural changes which have been utilized to detect BJP in urine using a heat coagulation test (Putnam method) [1]. Briefly, the

test urine is adjusted to pH to 4.9 ± 0.1, is kept at 56°C for 15 minutes, and the turbidity is checked. Thereafter, samples are heated up to 100°C with the disappearance of turbidity defining the sample as positive for BJP.

Recently, we experienced an unexpected result in a heat coagulation test from a patient with multiple myeloma. Although a considerable amount of BJP was present in the urine, the test results were negative. Several analyses revealed that BJP glycosylation is responsible for the phenomenon.

CASE REPORT

The patient was a woman in her 50s. She was diagnosed with multiple myeloma based on the presence of serum monoclonal proteins, bone marrow plasmacytosis, and "punched-out" lesions on a skull X-ray. Her laboratory test results were as follows: 6.6 g/dL of total protein, 4.7 g/dL of albumin, 16 mg/dL of blood urea nitrogen, 0.83 mg/dL of creatinine, 2.78 g/L of IgG, 0.09 g/L of IgA, and 0.06 g/L of IgM. Serum protein electrophoresis revealed a small band in the fast γ-globulin region. Immunoelectrophoresis (IEP) and immunofixation electrophoresis (IFE) reveal the presence of λ-type BJP in the urine and serum. The protein concentration in the urine was 5.52 g/L using a pyrogallol red test. A dipstick method and sulfosalicylic acid precipitation test also gave positive reactions for BJP (1+ and 3+, respectively). BJP was detected as a single band in the β-region in agarose gel electrophoresis experiments, suggesting that most of the urinary protein was BJP. However, urine from the patient had poor thermal reactivity in the heat coagulation test as shown in Figure 1.

MATERIALS AND METHODS

Materials

The study used serum and urine samples from the patient after consent. In addition, 5 urine samples that tested positive for BJP by IEP or IFE were used as controls. In all the control samples, BJP was a major protein constituent. A buffer solution exchange of the sample collected from the patient was performed using Tris-HCl buffer (pH7.5) with a PD-10 column (GE Healthcare Inc.). Standard procedures were used for IEP and IFE using agarose plates (Helena Laboratories Corp.) and antibodies (Agilent Technologies Inc., Helena Laboratories Corp.). Sialidase (Sigma-Aldrich, Inc.) and glycosidase (PNGaseF, New England BioLabs Inc.) were used for the treatment of the samples.

Methods

Estimation of BJP molecular weight

The molecular weight of the immunoglobulin light chains in the serum and urine of the patient, as well as BJP-positive control urines were estimated using SDS-PAGE (Sodium dodecyl sulfate poly acrylamide gel electrophoresis) with a 10% - 15% gradient gel followed by immunoblotting with anti-λ light chain antibodies [2]. The patient urine was also treated with sialidase. Sialidase was added to the sample to obtain a final concentration of 2.5 U/mL and incubated at 37°C for 4 hours as previously described [3], followed by SDS-PAGE.

Isoelectric focusing

To examine the isoelectric point (pl) of BJPs, isoelectric focusing was performed for urine samples using the PhastSystem as per the manufacturer's protocols (GE Healthcare Inc.). Gels with a pI ranging from 3 to 9 were used [4].

Heat coagulation assays

The patient urine was examined for heat coagulation with control urines. Five reaction buffers with a pH from 4 to 8 were prepared and used for the tests. For each test, 250 µL of reaction buffer and 1 mL of urine were heated at 56°C for 15 minutes until turbidity was observed.

Glycosidase treatment

The patient urine was subjected to a PD10 column for buffer exchange to Tris-HCl buffer, pH 7.5. The prepared urine and the patient serum were deglycosylated according to the standard protocol. Specifically, 1 µL of 10 X glycoprotein denaturing buffer (5% SDS, 0.4 M DTT) was added to 9 µL of the sample and the mixture was heated at 100°C for 10 minutes. Subsequently, 2 µL of 10 X G7 Reaction Buffer (0.5 M Sodium Phosphate (pH 7.5), 2 µL of 10% NP-40, 5 µL of H_2O, and 1 µL of PNGaseF were added to a final volume of 20 µL. The mixture was incubated at 37°C for 1 hour for deglycosylation. The resulting materials were subjected to SDS-PAGE and heat coagulation tests. For the latter test, a 200 µL sample volume was used.

RESULTS

Estimation of molecular weight

Two bands corresponding to 30,100 and 27,500 daltons (Da) were identified in the patient serum that reacted with anti-λ light chain antibodies, whereas a 30,100 Da band was the only band in the patient urine. The molecular weight of BJP of the control urines ranged between 24,000 and 25,000 Da (data not shown). Sialidase treatment [3] of patient urine did not change the results (data not shown).

Isoelectric focusing

Patient's urine sample showed 3 main bands and 2 subbands in the range of pl 5.5 - 6.45. In contrast, each control sample had a single band that ranged from pl ≤ 5.65 to pI 7.3 (Figure 2).

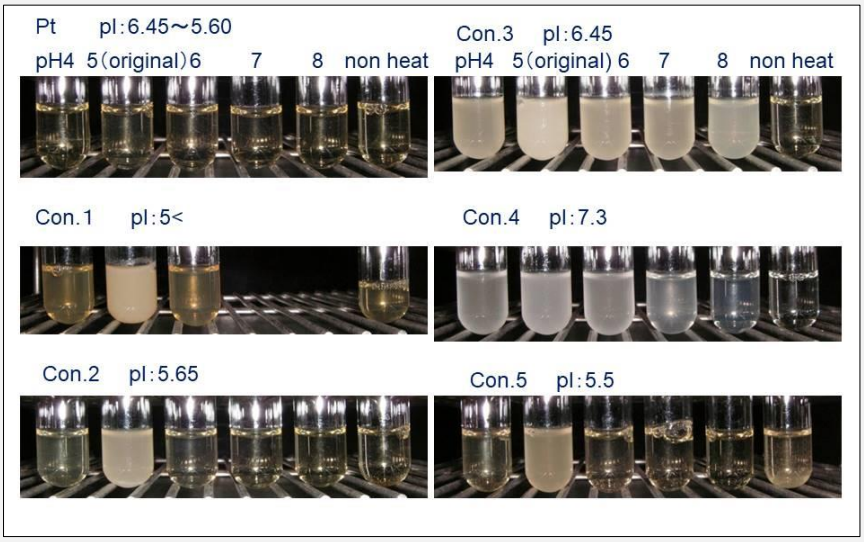

Figure 1. Heat coagulation test under various pH.

Patient sample (pt) showed no turbidity at any pH while each control (con 1 ～ 5) sample resulted positive at the optimal pH (5.0) and other pH points.

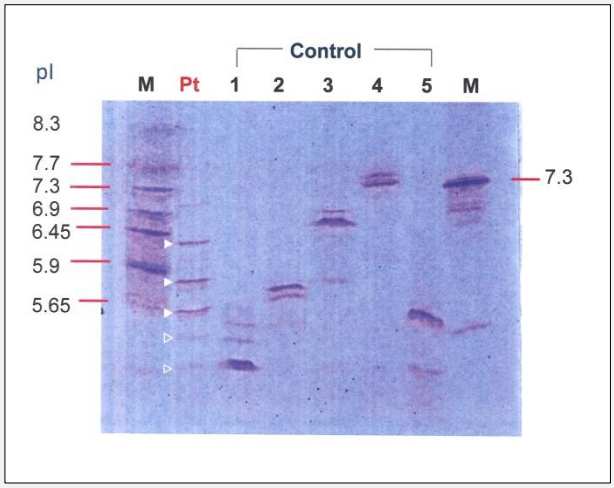

Figure 2. Isoelectric focusing of patient and control urines.

Three main bands (►) and two sub-bands (▹) were identified in the range of pI 5.5 - 6.45 in the patient urine (pt) while a single wide-band in this pI range was observed in all controls (control 1 ～ 5). M - pI marker, Pt - patient urine.

Heat coagulation properties

Control urines had the strongest thermal reactivity at approximately pH 5, in line with their respective isoelectric points. In contrast, there was no coagulation in the patient urine at or around the sample's isoelectric point (Figure 1).

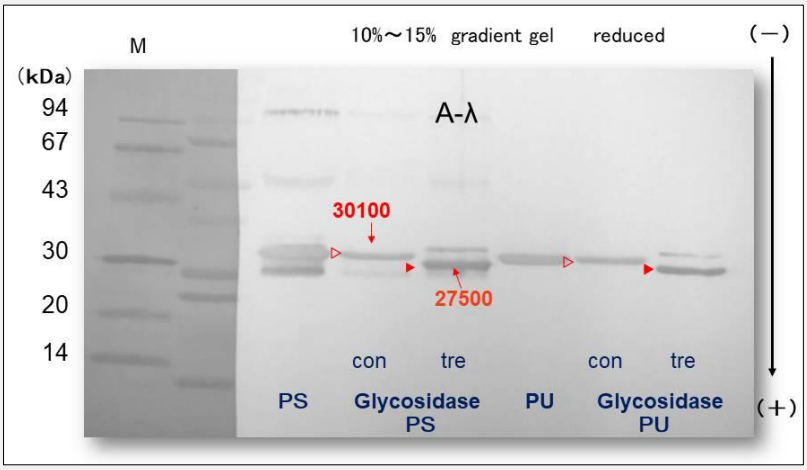

Figure 3. SDS-PAGE followed by immunoblotting with anti-λ light chain before and after glycosidase treatment.

Two bands, 30,100 and 27,500 Da, and a 30,100 Da band are seen in the serum and urine, respectively. The higher band was reduced in molecular size by 2,600 Da by glycosidase treatment.
M - Molecular weight marker, PS - patient serum, PU - patient urine, A-λ - anti-human λ light chain antibodies, con - control (non-treatment with glycosidase), tre - treatment with glycosidase.

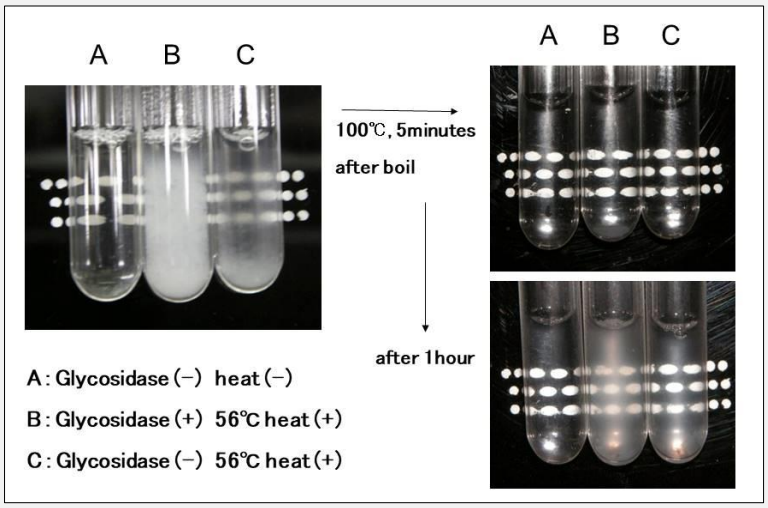

Figure 4. Thermal reactivity after glycosidase treatment.

The reaction mixture containing treated samples became turbid at 56°C, the turbidity disappeared when heated to 100°C, and appeared again 1 hour after returning to room temperature.

Effect of glycosidase treatment

Glycosidase treatment reduces the molecular weight of the main anti-λ light chain antibody reactive band in the patient's urine from 30,100 Da to 27,500 Da (Figure 3). Furthermore, unlike the untreated samples, the treated samples became turbid at 56°C in the heat coagulation

test. The turbidity disappeared when heated to 100°C and reappeared after 1 hour after room temperature (Figure 4).

DISCUSSION

In the present study, we identified λ-type BJP with weak reactivity in heat coagulation tests. Thus, we performed additional examinations to assess the cause of this discrepancy.

First, we treated the sample with sialidase to examine the effects of sialic acid at the ends of the sugar chains as sialylation causes microheterogeneity of BJP [3]. In the present case, the sialidase treatment had no effect on the BJP moiety according to IFE, indicating that sialylation was not the cause of the patient's BJP abnormalities.

Next, the isoelectric point of the patient's BJPs was examined. The BJP in these samples consisted of 3 main bands and 2 sub-bands, while the control BJPs existed as a single wide band. The presence of multiple bands that corresponded to the isoelectric points for BJP in the patient's urine and serum suggests that microheterogeneity is occurring in the light chain. With respect to thermal reactivity, control urines were less turbid at pH 4.9, although the isoelectric points of BJP ranged widely from mildly acidic to alkaline. On the other hand, the patient's urine did not exhibit thermal reactivity within the pH range close to its isoelectric points between pI 5.5 and 6.45. Finally, we surmised that the unusual conjugation of carbohydrate chains might cause microheterogeneity in the patient's BJP, causing poor thermal reactivity. This idea was confirmed in our deglycosylation experiments. Deglycosylation reduced the molecular weight of the higher weight bands in the patient BJP and promoted thermal reactivity. The enzyme employed, PNGaseF, cleaves the binding between asparagine and GlucNAc in the core of N-linked glycans bound to proteins. This unusual glycosylation altered the solubility of the patient's BJP [5]. However, the alteration of the thermal activity of BJP by N-glycosylation has not been reported.

BJP is toxic, especially in the kidney. As for a relationship between BJP pathogenesis and glycosylation, Kagimoto et al. previously examined the association between N-glycosylation of BJP and renal function [6]. They identified 5 different oligosaccharides in BJP and reported that the oligosaccharide levels negatively correlated with serum creatinine levels, suggesting that the glycosylation status of BJP may be an indicator of renal function. Of note, the renal function of this study's patient was not impaired. It would be interesting to assay if the thermal reactivity of BJPs has any relationship to renal toxicity, which was not assessed in their study [7].

IEP and IFE are the most reliable methods for detecting BJP. It is generally accepted that the heat coagulation test has poor sensitivity and reliability because proteinuria can cause false-positive reactions. Thus, the number of institutions that use the heat coagulation test has been decreasing worldwide. However, it is still being used in places where IEP and IFE are not readily available. In our institution, we perform the heat coagulation test using the Putnam method. In certain cases where the presence of BJP is suspected, based on the differences between protein and albumin levels in the urine and serum, protein electrophoresis is employed. As a result, we can identify BJP at early disease stages and report them to the clinical team. Thus, we will continue to perform the heat coagulation test as a screening method for BJP. It is important to note that the heat coagulation test can produce false-negative results even if a large amount of BJP is present in the urine.

CONCLUSION

In this study, our patient presented BJP which was negative in heat coagulation tests due to glycosylation.

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Declaration of Interest:
The authors declare no conflict of interest.

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