0273-2289/00/83/0085/$12.50

Does Catalytic Activity of Bence–Jones Proteins Contribute to the Pathogenesis of Multiple Myeloma?

HYOGO SINOHARA*,[1] AND KINJI MATSUURA[2,3]

[1]Life Science Institute and [2]Department of Biochemistry, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511, Japan; [3]The University of Texas Houston, Medical School, Department of Pathology and Laboratory Medicine, Houston, Texas 77225, USA

Abstract

Some Bence–Jones proteins have been found to be capable of hydrolyzing DNA, chromogenic amide substrates, such as benzoylarginine *p*-nitroanilide, and natural oligopeptides, such as arginine vasopressin. Patients who excrete Bence–Jones protein with the DNA-nicking activity have shown moderately severe symptoms. When incubated with LLC-PK_1 (porcine kidney proximal tubule) cells, some Bence Jones proteins penetrated the cytoplasm, and entered the nucleus with little or no degradation of epitopes. Intranuclear Bence Jones proteins ultimately induced DNA fragmentation *in situ* and cell death. This cytocidal activity was not directly associated with the DNA-nicking activity, since Bence Jones proteins with no detectable DNase activity also produced cell death. These results, however, suggest that the biological activities of Bence Jones proteins described here makes a significant contribution to the development and/or deterioration of multiple myeloma.

Index Entries: Bence Jones proteins; cytotoxicity; DNase activity; $LLCLK_1$ cell line; peptidase activity; nuclear import.

Introduction

In 1974, Erhan and Greller *(1)* raised the possibility that immunoglobulins may possess proteinase activity, since the spatial arrangement of Ser-27a, His-93, and Asp-1 (Kabat numbering system) of light chains is very similar to that of the catalytic triad of chymotrypsin. Two decades passed, however, before this prediction was verified by Paul et al. *(2–5)* and Matsuura et al. *(6,7)*, who showed that most Bence Jones proteins were capa-

*Author to whom all correspondence and reprint requests should be addressed.

ble of detectable cleavage of one or more chromogenic amidase substrates and oligopeptides. Recent evidence indicates that some autoantibodies can hydrolyze their own antigens. For example, human autoantibodies to vasoactive intestinal peptide (VIP) *(4)*, thyroglobulin *(8)*, and DNA *(9)* cleave their respective autoantigens. Furthermore, some antibodies produced by entirely natural mechanisms and light chains derived from these catalytic antibodies have also proven to be capable of cleaving their antigens, such as thyroglobulin *(8)* and HIV gp120 *(11)*, and some physiologically relevant peptides such as Arg-vasopressin *(10)* and VIP *(4)*. In most cases studied, these light chains had much greater catalytic potential than their parent antibodies themselves *(2,3)*. In recent studies, we showed that—of 18 Bence Jones proteins examined—four had DNA-nicking activity, and that patients excreting DNase-active Bence Jones proteins showed moderately severe symptoms *(12)*. These results are reviewed in the present article, which also includes some unpublished results obtained more recently with the cultured LLC-PK_1 (porcine kidney proximal tubule) cells.

Amidolytic Activity of Bence Jones Proteins

Matsuura et al. *(6)* showed that five monoclonal Bence Jones proteins studied were all capable of cleaving the synthetic chromogenic substrates for trypsin, such as carbobenzoxy-Ile-Glu-Gly-Arg *p*-nitroanilide. Paul et al. *(5)* also showed that—of 21 Bence Jones proteins studied—16 hydrolyzed peptide methylcoumarinamides, such as *t*-butyloxycarbonyl-Ile-Glu-Gly-Arg methylcoumarinamide. In both cases, Bence Jones proteins preferred basic amino acids—such as arginine and lysine—to other amino acids.

Peptidolytic Activity of Bence Jones Proteins

All four Bence Jones proteins studied were capable of hydrolyzing the arginylglycinamide bond of vasopressin, but not the other bonds *(10)*. Under the same conditions, virtually no cleavage was observed with desmopressin (*L*-Arg is replaced by *D*-Arg) and oxytocin (Arg is replaced by Leu). A recombinant light chain of autoantibody to VIP also cleaved the C-terminal side of basic amino acids of VIP, mainly at two adjacent bonds, Lys-Lys and Lys-Tyr *(2,5)*. On the other hand, a light chain of anti-VIP antibody that had been subjected to denaturation followed by renaturation displayed a different cleavage pattern—hydrolysis occurred between Gln-Met, Met-Ala, and Ala-Val, besides Lys-Lys. The K_m value of the denatured/renatured protein was about 10-fold elevated. These results suggest that a Bence Jones protein exists in several conformationally distinct states whose catalytic property differs from conformation to conformation, and that the pathological significance of catalysis by Bence Jones proteins may be very diverse.

DNase Activity of Bence Jones Proteins

Shuster et al. *(9)* showed that some autoantibodies to DNA in patients with systemic lupus erythematosus had DNA-nicking activity. We recently

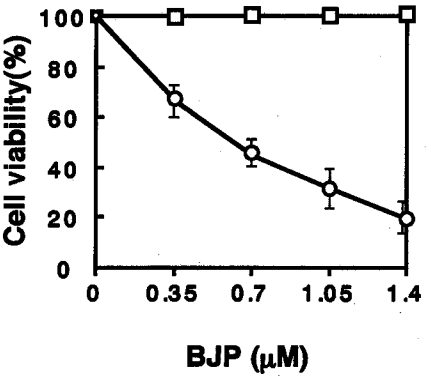

Fig. 1. Cytotoxic activity of Bence Jones proteins (BJPs). After incubation of LLC-PK_1 cells with Bence Jones protein at final concentrations of 0, 0.25, 0.5, 0.75, 1.0, and 1.5 μM, the cell viability was determined by the method of Mosmann *(14)*. Cytotoxic BJP (○); noncytotoxic BJP (□). Vertical bars indicate standard deviation.

found *(12)* that 4 of 18 monoclonal Bence Jones proteins had DNase activity, and that all the patients excreting Bence Jones protein with DNase activity showed moderately severe symptoms—particularly renal failure associated with shortened survival. These results suggested that the DNase activity might be directly related to the progressive deterioration of clinical status. However, the degree of DNase activity did not correlate with that of cytotoxicity in vitro, suggesting the multifactorial nature of disease status.

Cytotoxic Activity of Bence Jones Proteins

In healthy subjects, *Ig* light chains are produced in excess over the heavy chains, and most light chains are filtered through the glomerulus, reabsorbed by renal proximal tubular epithelial cells, and degraded into amino acids *(7)*. Since some Bence Jones proteins are known to be toxic to renal tubule cells *(7,13)*, LLC-PK_1 cells derived from pig-kidney proximal tubule were used for the assay of cytotoxicity according to Mosmann's tetrazolium method *(14)*. This method is based on the observation that only living cells reduce significant amounts of tetrazolium to form insoluble dark-blue formazan. Five of 18 Bence Jones proteins examined caused cell death in a concentration-dependent manner (*see* Fig. 1). However, the remaining 13 Bence Jones proteins had no detectable cytotoxic effects at all.

Incorporation of Bence Jones Protein into LLC-PK_1 Cells

When LLC-PK_1 cells were incubated with Bence Jones proteins for 10 h followed by staining with fluorescein isothiocyanate-labeled antikappa-chain goat antibody as described *(15)*, positive staining was seen in the nuclei of cells incubated with cytotoxic (*see* Fig. 2B), but not noncytotoxic (*see* Fig. 2A) Bence Jones proteins. To further substantiate this result,

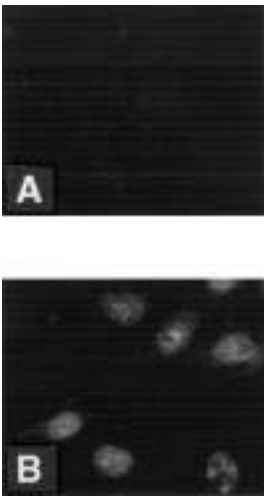

Fig. 2. Immunofluorescent staining of intracellular Bence Jones proteins (BJPs). After incubation of LLC-PK_1 cells with 1.0 μM nontoxic **(A)** and toxic BJP **(B)** as described in the legend for Fig. 1, the cells were stained with fluorescein isothiocyanate-labeled antihuman-kappa-chain goat IgG, essentially as described *(15)*.

the cells treated with cytotoxic Bence Jones proteins were lysed and the lysate was subjected to electrophoresis followed by immunoblotting with antikappa chain antibody. An immunoreactive band corresponding to the molecular mass of Bence Jones protein was detected (unpublished results). By contrast, no positive band was detected in the lysate that had been treated with noncytotoxic Bence Jones proteins. These results suggest that a subgroup of Bence Jones proteins was taken up by LLC-PK_1 cells with little or no degradation of epitopes, whereas the majority of Bence Jones proteins were degraded without access to the nuclei.

Cytochemical Detection of Dead Cells

Hoechst 33342 dye stains the nuclei of live cells, whereas propidium iodide costains dead cells *(16)*. After incubation of LLC-PK_1 cells with Bence Jones protein followed by double staining with Hoechst 33342 and propidium iodide as described *(16)*, a large number of dead cells were seen in cultures incubated with cytotoxic Bence Jones proteins (*see* Fig. 3B). In marked contrast, a few dead cells were seen in cultures incubated with noncytotoxic Bence Jones proteins (*see* Fig. 3A). These results conform with those obtained by Mosmann's tetrazolium method (*see* Fig. 1).

Cytochemical Detection of Biotin-Labeled DNA F ragmentation

It is known that TUNEL-positive staining is indicative of DNA fragmentation, which was found in histologically-defined apoptotic cells as well as in morphologically intact cells that are destined to undergo cell

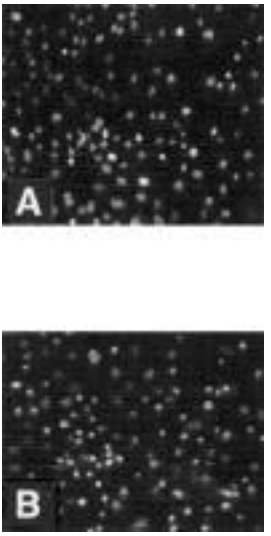

Fig. 3. Cytocidal activity of Bence Jones proteins (BJPs). After incubation of LLC-PK_1 cells with 1.0 μM nontoxic **(A)** and toxic BJP **(B)** as described in the legend for Fig. 1, the cells were doubly stained with Hoechst 33342 (greenish blue) and propidium iodide (reddish yellow), essentially as described previously *(16)*.

death *(16,17)*. As shown in Fig. 4B, TUNEL-positive staining was detected as green fluorescent signals on the nuclei of LLC-PK_1 cells that had been treated with cytotoxic Bence Jones proteins. When biotinylated dUTP was omitted from the reaction process, no green signals were detected (data not shown). On the other hand, TUNEL staining was negative for cells treated with noncytotoxic Bence Jones proteins (*see* Fig. 4A).

Relationship of Cytotoxicity to Catalytic Activities

As described above, the majority of Bence Jones proteins had weak but detectable amidase activity *(2,5–7)*. On the other hand, about one-fifth of Bence Jones proteins examined had DNA-hydrolyzing *(12)* and cytocidal activities (*see* Fig. 1), although the two activities did not coincide exactly. The preparations with relatively high amidase activity showed a tendency to gain access to the nucleus, while 3 out of 5 Bence Jones proteins with relatively high DNA-nicking activity reached the nucleus (*see* Fig. 5). One of the Bence Jones proteins with highest DNA-nicking activity failed to reach the nucleus or to induce cell death. These results suggest that the mechanism underlying the import of Bence Jones proteins to the nucleus is not directly related to that responsible for the catalytic activity.

Discussion

Among the various symptoms associated with multiple myeloma, kidney dysfunction is the first clinical symptom in about two-thirds of the

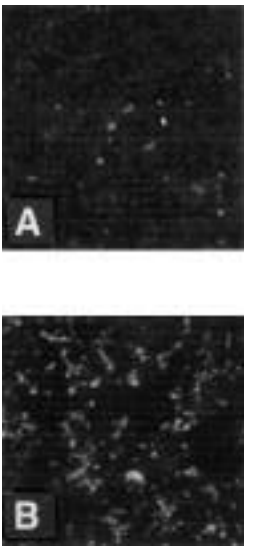

Fig. 4. Cytochemical detection of DNA fragmentation induced by Bence Jones proteins (BJPs). After incubation of LLC-PK_1 cells with 1.0 μM nontoxic **(A)** and toxic BJP **(B)** as described in the legend for Fig. 1, the cells were subjected to TUNEL staining as described *(17,18)*.

cases, and a major cause of their death *(7,13,19)*. Bence Jones proteinuria was detected in 49 *(20)* to 77% *(21)* of the patients who had the test, and is thought to be responsible for severe renal damage in a substantial portion of myeloma patients *(7,13,19)*. However, little is presently known of the molecular mechanism by which Bence Jones proteins interfere with renal function. We have introduced the possibility that the catalytic activity of Bence Jones protein may contribute to the pathogenesis *(7)*. The present results show that a subset of Bence Jones protein entered LLC-PK_1 cells, gained access to the nucleus, and caused DNA fragmentation, ultimately causing cell death. This lends new support to the previous hypothesis *(7)* that the catalytic activity of Bence Jones protein plays a role in the renal failure of multiple myeloma. However, the catalytic potential of individual Bence Jones proteins differs greatly not only in the degree of enzymic activity, but also in the substrate specificity. Thus, the extent to which individual Bence Jones protein contributes to the etiology may be very diverse, ranging from practically null to significant levels *(7)*. If Bence Jones protein is taken up by the cell and has contact with protein(s) or DNA, slow cleavage of a single peptide or nucleotide bond may be enough to lead to the gradual loss of function, resulting in cell death.

Madaio and colleagues *(15,22)* showed that some lupus anti-DNA autoantibodies and their fragments F(ab′) entered the cell and gained access to the nucleus in a time-dependent manner. This suggests that the antigen-binding region is primarily responsible for both cell entry and nuclear

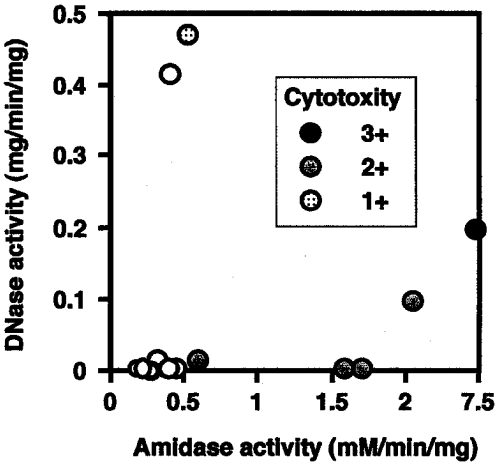

Fig. 5. Relationship between amidase, DNase, and in vitro cytotoxicity of Bence Jones proteins. Cytotoxicity: 3+, 25% or less viable after 1 h incubation with 1 μM Bence Jones protein; 2+, about 50% viable; 1+, about 75% viable.

localization. The present results obtained with Bence Jones protein are in general agreement with these findings, previously obtained for anti-DNA autoantibodies. In the past few years, enormous progress has been made in understanding nuclear protein import *(22–26)*. Nuclear proteins are translocated efficiently and precisely from the cytoplasm to the nucleus through the nuclear-pore complex present in the nuclear envelope. The pore complex contains an aqueous diffusion channel that can accommodate the active transport of large molecules. The latter process is conferred by several nuclear localization signals. Among them, two signals are relatively well-characterized: 1) single basic type consisting of short stretch of highly basic amino-acid residues such as PKKKRKV and 2) bipartite basic type consisting of two stretches of basic amino-acid residues separated by a spacer of random amino-acid residues such as KRPAAIKKAGQAKKKK. It is possible that Bence Jones protein coincidentally has a similar sequence in its CDR, since the human immune system can provide with 10^{10}–10^{12} different CDRs *(2)*. Foster et al. *(26)* showed that the nuclear localization-like motifs were present in CDR3 of nuclear localizing anti-DNA lupus antibodies.

As shown in Figs. 2–4, some Bence Jones proteins with DNase activity are imported into nuclei and cause cell death. It seems unlikely, however, that the DNA fragmentation seen in the cultured cells was caused primarily by DNase associated with some Bence Jones proteins, since cell death was also caused by a Bence Jones protein without DNase activity (*see* Fig. 5). At present, internucleosomal cleavage of chromosomal DNA is considered to be catalyzed by constitutive endonuclease, DNase I, or DNase gamma *(27,28)*. Thus, the intranuclear import of Bence Jones pro-

tein may trigger the mechanism associated with cell death. Further elucidation of these phenomena will lead to a better understanding of the pathogenesis of multiple myeloma and also to the elucidation of the nuclear import of proteins and apoptosis.

References

1. Erhan, S. and Greller, L. D. (1962), *Nature* **251,** 353–355.
2. Paul, S. (1996), *Mol. Biotechnol.* **5,** 197–207.
3. Paul, S. (1998), in *Contemporary Immunology: Autoimmune Reactions*, Paul, S., ed., Humana Press Inc., Totowa, NJ, pp. 221–234.
4. Sun, M., Mody, B., Eklund, S. H., and Paul, S. (1991), *J. Biol. Chem.* **266,** 15,571–15,574.
5. Paul, S., Li, L., Kalaga, R., Wilkins-Stevens, P., Stevens, F. J., and Solomon, A. (1995), *J. Biol. Chem.* **270,** 15,257–15,261.
6. Matsuura, K., Yamamoto, K., and Sinohara, H. (1994), *Biochem. Biophys. Res. Commun.* **204,** 57–62.
7. Sinohara, H. and Matsuura, K. (1998), in *Contemporary Immunology: Autoimmune Reactions*, Paul, S., ed., Humana Press Inc., Totowa, NJ, pp. 235–247.
8. Li, L., Paul, S., Tyutyulkova, S., Kazatchkine, M., and Kzveri, S. (1995), *J. Immunol.* **154,** 3328–3332.
9. Shuster, A. M., Gololobov, G. V., Kvashuk, O. A., Bogomolova, A. E., Smirnov, I. V., and Gabibov, A. G. (1992), *Science* **256,** 665–667.
10. Matsuura, K. and Sinohara, H. (1996), *Biol. Chem.* **377,** 587–589.
11. Uda, T., Hifumi, E., Okamoto, Y., Yan, Z., and Ishimaru, M. (1998), *Clin. Sci.* **2,** 429–433.
12. Matsuura, K., Ikoma, S., Yoshida, K., and Sinohara, H. (1998), *Biochem. Biophys. Res. Commun.* **243,** 719–721.
13. DeFronzo, R. A., Cooke, C. R., Wright, J. R., and Humphrey, R. L. (1978), *Medicine*, **57,** 151–166.
14. Mosmann, T. (1983), *J. Immunol. Methods* **65,** 55–63.
15. Yanase, K., Smith, R. M., Cizman, B., Foster, M. H., Peachey, L. D., Jarett, L., and Madaio, M. P. (1994), *Lab. Investig.* **71,** 52–60.
16. Signhal, P. C., Sharma, P., Sanwal, V., Prasad, A., Kapasi, A., Ranjan, R., Franki, N., Reddy, K., and Gibbons, N. (1998), *Kidney Int.* **53,** 350–357.
17. Gavrieli, Y., Sherman, Y., and Ben-Sasson, A. (1992), *J. Cell Biol.* **19,** 493–501.
18. Mori, C., Nakamura, N., Okamoto, Y., Osawa, M., and Shiota, K. (1994), *Anat. Embryol.* **190,** 21–28.
19. Selby, P. and Gore, M. (1995), in *Oxford Textbook of Oncology*, Peckham M, Pinego, H., and Veronesi, U., eds., Oxford University Press, Oxford, England, vol. 1, pp. 1852–1878.
20. Kyle, R. A. (1975), *Mayo Clin. Proc.* **50,** 29–40.
21. Bernstein, S. P. and Humes, H. D. (1982), *Arch. Intern. Med.* **142,** 2083–2086.
22. Madaio, M. P., Yanase, K., Foster, M. H., Smith, R. M., Emmons, T. K., Fabbi, M., Puccetti, A., and Jarett, L. (1997), *Ann. NY Acad. Sci.* **815,** 263–266.
23. Schlenstedt, G. (1996), *FEBS Lett.* **389,** 75–79.
24. Yoneda, Y. (1997), *J. Biochem.* 121, 811–817.
25. Gorlich, D. (1997), *Curr. Opin. Cell Biol.* **9,** 412–419.
26. Foster, M. H., Kieber-Emmons, T., Ohliger, M., and Madaio, M. P. (1994), *Immunol. Res.* **13,** 172–185.
27. Kerr, J. F. R. and Harmon, B. V. (1991), in *Apoptosis: The Molecular Basis of Cell Death*, Tomei, L. D., and Cope, F. O., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 5–29.
28. Shiokawa, D., Ohyama, H., Yamada, T., and Tanuma, S. (1997), *Biochem. J.* **326,** 675–681.

Discussion

Kozyr: I have similar results to yours using IgG fractions. What cell lines did you use, and what was the L-chain concentration required for cytotoxicity?

Sinohara: We did not do many experiments varying the concentrations. But 1 μ*M* L chain is good enough to be a cytotoxic. We used the cell lines LLC-PK, which derive from pig-kidney proximal tubules. The most prominent effect of the Bence Jones proteins in vivo is on the tubules, which is the reason we use tubular cells.

Kozyr: Have you studied the effect of complement?

Sinohara: No, we have not.

Vijayalakshmi: In the correlations between cytotoxicity and the catalytic activities, in both the cases, did you use purified proteins?

Sinohara: We used several methods involving ammonium sulfate, hydrophobic interaction chromatography, and gel filtration to purify the light chains. Both types of experiments were done with purified proteins.

Unidentified: How do you classify the Bence Jones into the nontoxic and toxic groups?

Sinohara: This was by using tissue culture assay to incubate the cells with Bence Jones proteins; then we determined cytotoxicity by MTT assay. If the cell number was reduced, we concluded this to be a cytotoxic effect.

Kohler: I am curious as to whether the proteins are cannibalistic. Do they digest themselves, and if so, can this reaction generate amyloid fibrils?

Sinohara: This is an important question. Sometimes, the degradation of Bence Jones is evident. We have tried many times, but we did not draw any conclusions because of possible contaminants.

Paul: We screened about 35 light chains for self-cleavage as well as cleavage of other light chains. We found a minority of the light chains to display autolytic activity. We do not know whether aggregation of light chains is increased upon cleavage. Of interest to you, Heinz, is that the cleavage site appears to be at the junction of the VL and CL domains. So a public idiotope may be the target if you express this phenomenon in the language of idiotypy.

Neuberger: Can I just clarify—you're saying that a Bence Jones protein can get into cells and then go on to the nucleus—that it has nuclear localization sequences. If you soak the Bence Jones proteins in known nuclear proteins, does the protein then get transported from the cytosol and targeted to the nucleus?

Sinohara: We haven't done that experiment, but it is an important point.

Gololobov: Does the DNase activity of the light chains require magnesium ions? All other DNases need magnesium for catalytic activity.

Sinohara: Magnesium is present in the assay, but we do not know if it is required.

Gololobov: Have you done the reaction in the presence of EDTA?

Sinohara: No, we haven't done that.