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Some Bence–Jones proteins enter cultured renal tubular cells, reach nuclei and induce cell death

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SUMMARY

Eighteen monoclonal Bence–Jones proteins (BJPs) were examined for their effects on cultured LLC-PK_1 (porcine kidney proximal tubule) cells as well as for their amidase and DNase activities. Five proteins were found to enter the cell and to gain access to the nucleus without degradation of epitopes. Intranuclear BJPs ultimately induced DNA fragmentation and cell death. BJPs with relatively high amidase activity were cytotoxic. On the other hand, three of four BJPs with DNase activity had a cytocidal effect on cultured cells; the remaining BJP, which had a relatively high DNase activity but a very low amidase activity, failed to enter the cell and was not cytotoxic *in vitro*. These results suggest that catalytic and cytotoxic activities of some BJPs may make a significant contribution, in a substantial proportion of myeloma patients, to the development and/or deterioration of the disease.

INTRODUCTION

There is increasing evidence that some autoantibodies can hydrolyse their own antigens.[1,2] For example, human vasoactive intestinal peptide (VIP),[3] thyroglobulin[4] and DNA[5] were shown to be cleaved by some of their respective autoantibodies. Catalytic autoantibodies themselves were much less active than their light chains,[1–3] and most Bence–Jones proteins (BJPs) were capable of detectable cleavage of one or more chromogenic amidase substrates.[3,6–8] Although the amidolytic activities of BJPs were generally very weak, several lines of evidence indicate that this was not the result of proteinase contamination.[1–9] The catalytic activities reside in the region of immunoglobulins that includes the complementarity-determining regions (CDRs), and thus differ greatly among individual immunoglobulins.[1,2,9] These results suggest that catalytic antibodies may be a relatively standard component of natural immune responses[1] and that the catalytic activity of natural immunoglobulins or BJPs, albeit very weak, may affect clinical processes of patients with autoimmune diseases (who produce immunoglobulins with a high catalytic potential) or with multiple myeloma (who excrete massive levels of BJP).[8] In a previous study, we showed that four out of 18 BJPs examined had DNA-nicking activity and that patients excreting DNase-active BJPs showed somewhat severe clinical symptoms.[10] Recently, it was shown that when added to cultured LLC-PK_1 (porcine kidney proximal tubule) cells, some anti-DNA antibodies traverse the cytoplasm and enter the nucleus in a time- and temperature-dependent manner[11] and that when injected into normal mice, some anti-DNA monoclonal antibodies (mAbs) produced intracellular immunoglobulin deposits in multiple organs.[12] It remains to be determined, however, whether BJPs behave in a manner similar to these intact autoantibodies. The present study was undertaken to clarify this issue. It is shown that some, but not all, BJPs actually enter the LLC-PK_1 cell and reach the nucleus in a manner similar to anti-DNA antibodies, and that the intranuclear BJPs ultimately induce DNA fragmentation and cell death.

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Abbreviations: BJP, Bence–Jones protein; CDR, complementarity-determining region; DMEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labelling; VIP, vasoactive intestinal peptide.

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MATERIALS AND METHODS

Materials

Monoclonal BJPs were purified to homogeneity from the urine of patients with multiple myeloma, as described previously.[6] Virtually all preparations had detectable amidase activity[6] whereas only four of 18 monoclonal BJPs examined had DNA-nicking activity.[10]

Determination of enzymatic activities
The amidase[6] and DNA-nicking[10] activities of BJPs were determined as described previously.

Cell growth
LLC-PK$_1$ cells (CRL 1392; American Type Culture Collection, Rockville, MD) were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). LLC-PK$_1$ cells were cultured in Dulbecco's modified Eagles's minimal essential medium (DMEM) supplemented with 5% fetal calf serum (FCS; Gibco, Rockville, MD) and subcultured every 5–7 days.

Determination of cell viability after incubation with BJP
Cell viability was determined according to the method of Mosmann[13] with some modifications. LLC-PK$_1$ cells were distributed in 96-well plates, at a density of 5×10^4 cells/0·1 ml/well, and incubated at 37° for 24 hr. The medium was then replaced with FCS-free DMEM containing BJPs at final concentrations of 0, 0·25, 0·50, 0·75, 1·0 and 1·5 μM, and incubation was continued for 24 hr. To each well, 20 μl of 0·5% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Indianapolis, IN), in phosphate-buffered saline (PBS), was added and incubation was continued for a further 4 hr at 37°. Under these conditions, only living cells reduce significant amounts of MTT to form insoluble formazan. After incubation, 0·04 N HCl in isopropanol and 3% sodium dodecyl sulphate (SDS) solution (0·1 ml of each) were added to each well to ensure that dark blue MTT formazan was dissolved. After incubation for a few minutes at room temperature, the absorbance was read at 590 nm using a microplate reader. The cell survival rate was expressed as a percentage of the control that contained no BJP.

Dual staining of cells
LLC-PK$_1$ cells were incubated with BJPs, essentially as described above, and then double stained with Hoechst 33342 (Calbiochem, La Jolla, CA) and propidium iodide (PI; Calbiochem), as previously described by Singhal *et al.*[14] Under these conditions, PI is taken up only by damaged cells, while Hoechst 33342 stains both living and dead cells.[14]

Immunofluorescence staining of intracellular BJPs
LLC-PK$_1$ cells were placed onto cover glass and allowed to proliferate overnight. They were then washed with FCS-free DMEM, and incubated for 10 hr with DMEM in the presence of different concentrations of BJP, essentially as described above. After incubation, the slides were washed three times with PBS at 4°. The cells were fixed in acetone at −20° for 15 min, washed once with 0·1% Triton-X-100 in PBS, three times with PBS alone and then blocked by incubation with 3% bovine serum albumin (BSA) in PBS for 20 min. After washing with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-labelled anti-human κ- or λ-chain goat immunoglobulin G (IgG) (Boehringer Mannheim Biochemicals, Indianapolis, IN), for 2 hr at 4°, and then washed three times with PBS. The slides were mounted and examined using an Olympus fluorescence microscope (Olympus Co., Tokyo, Japan).

Detection of DNA fragmentation in situ
Fragmented DNA was detected *in situ* by a modification[15] of the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labelling (TUNEL) method[16] using the MEBSTAIN apoptosis kit (Medical & Biological Laboratories Co., Nagoya, Japan). Biotinylated dUTP was omitted in control staining.

Extraction of DNA from cells and electrophoretic detection of the DNA ladder
DNA extraction and detection of the DNA ladder were carried out essentially as described by Sellins & Cohen.[17]

Radiolabelling of monoclonal BJPs and binding assay
BJP was labelled with ^{125}I by the chemical oxidation method,[18] using the IODO-BEADS iodination kit (Pierce, Rockford, IL) according to the manufacturer's instructions. LLC-PK$_1$ cells were placed onto cover glass and allowed to proliferate until confluent. They were then washed three times with FCS-free DMEM, and incubated at 4° for 2 hr with DMEM containing ^{125}I-labelled BJP (1×10^4 counts per minute [c.p.m.]). BSA and non-labelled BJP at 10-, 100-, and 1000-fold concentrations were used as non-specific binding controls. After washing with FCS-free DMEM, cells were lysed with 1 N NaOH, and radioactivity was measured in a scintillation counter.

RESULTS

Cytotoxic effect of BJPs

While studying the effect of BJPs on LLC-PK$_1$ cells, we observed that BJPs sometimes induced cell death. We therefore studied the effects of individual BJPs on cell viability. Of 18 BJPs examined, five induced gradual cell death in a concentration-dependent manner (Fig. 1). The remaining 13 BJPs had virtually no effect on cell viability.

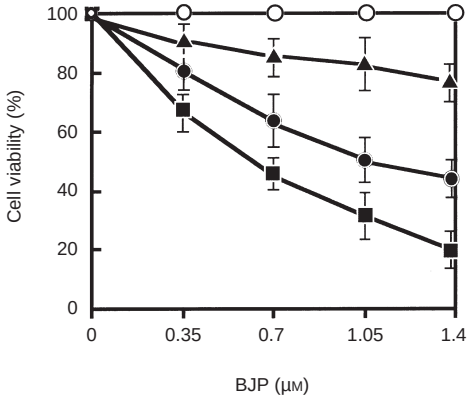

Figure 1. Effects of Bence–Jones proteins (BJPs) on cell viability. LLC-PK$_1$ cells were preincubated with different concentrations of BJP, and the cell viability was determined as described in the text. Amidase and DNase activities of individual BJPs are shown in Fig. 7, and the same symbols are used for the same BJPs in both figures: ■, patient c in Fig. 7; ●, patients d–f in Fig. 7; ▲, patient a in Fig. 7; ○, patient b and all other BJPs that are not indicated by alphabetical characters in Fig. 7. Vertical bars indicate standard deviations of the mean values.

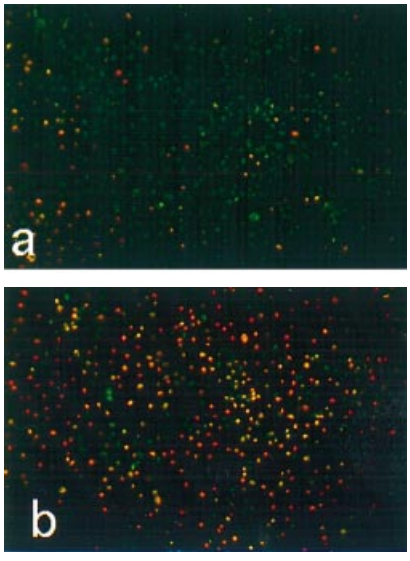

Figure 2. Cytocidal effect of Bence–Jones proteins (BJPs). After incubation of LLC-PK$_1$ cells with 1·0 μM of non-cytotoxic (a) or cytotoxic (b) BJP for 1 hr as described in the text, the cells were double stained with Hoechst 33342 (greenish blue) and propidium iodide (reddish yellow), essentially as described previously.[14]

Cytochemical detection of cell death

After incubation of LLC-PK$_1$ cells with BJPs followed by double staining with Hoechst 33342 and PI, very few dead cells were observed in cultures incubated with non-cytotoxic BJPs (Fig. 2a). In marked contrast, many dead cells were seen in cultures incubated with cytotoxic BJPs (Fig. 2b). The results are in good agreement with those obtained using Mosmann's tetrazolium method (Fig. 1), namely, the greater the cytotoxicity of BJP, the higher the proportion of cells stained with PI.

Incorporation of BJPs into LLC-PK$_1$ cells

When immunofluorescence staining for BJPs was performed on LLC-PK$_1$ cells treated with BJPs, there appeared to be some cytoplasmic staining as well as the brighter nuclear staining (Fig. 3b). By contrast, all the non-cytotoxic BJPs were negative for this staining (Fig. 3a). To further substantiate this result, the cells treated with cytotoxic BJP were lysed and the lysate was subjected to electrophoresis followed by immunoblotting with anti-κ chain antiserum, as described previously.[19] An immunoreactive band with molecular mass corresponding to that of the original BJP was detected (data not shown). By contrast, no positive band was detected in the lysate that had been treated with non-cytotoxic BJP. These results indicate that a subgroup of BJPs was taken up by LLC-PK$_1$ cells with little or no degradation of epitopes, whereas the majority of BJPs were degraded without access to the nuclei.

Binding of BJPs to the surface of LLC-PK$_1$ cells

The above results indicate that there are two subgroups of BJP: one reaches the nucleus while the other does not. In order to clarify whether or not the specific surface receptor is involved with this intracellular transport, competitive binding experiments were carried out between the two subgroups. As shown in Fig. 4(a), binding of cytotoxic BJP (■ in Fig. 1) was almost completely inhibited by 1000- and 100-fold (data not shown) concentrations of non-cytotoxic BJP (○ in Fig. 1), cytotoxic non-labelled BJP or BSA, suggesting that there is no specific receptor for cytotoxic BJP. Figure 4(b) shows that essentially the same results were obtained at 10-fold concentrations of these competitors, although a higher level of scatter was observed with this data.

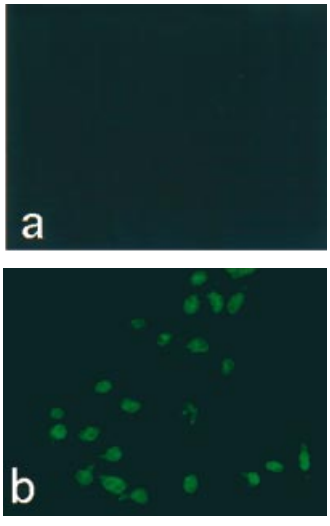

Figure 3. Immunofluorescence staining of intracellular Bence–Jones proteins (BJPs). After incubation of LLC-PK$_1$ cells with 1·0 μM of non-cytotoxic (a) or cytotoxic (b) BJP, the cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-human κ-chain goat immunoglobulin G (IgG), essentially as described.[19]

Cytochemical detection of biotin-labelled DNA fragmentation

It is known that TUNEL-positive staining is indicative of DNA fragmentation, which is found not only in histologically defined apoptotic cells but also in morphologically intact cells that are destined to go through cell death.[16] As shown in Fig. 5(a),

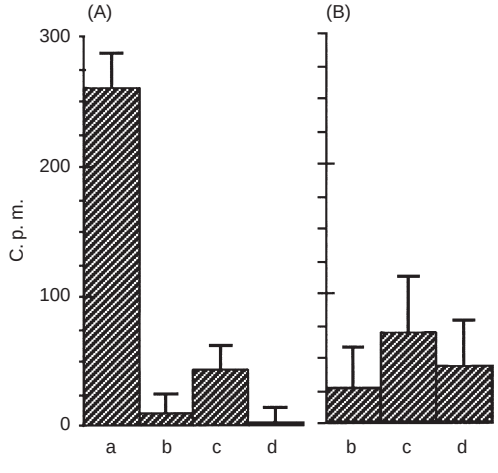

Figure 4. Binding of radiolabelled cytotoxic BJP to LLC-PK$_1$ cells in the absence (a) and presence of 1000-fold (A) and 10-fold (B) concentrations of non-cytotoxic BJP (b), BSA (c) and cytotoxic non-labelled BJP (d). c.p.m., counts per minute.

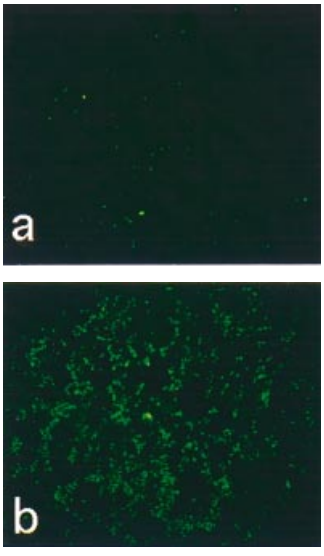

Figure 5. Cytochemical detection of DNA fragmentation. After incubation of LLC-PK_1 cells with 1·0 μM of non-cytotoxic (a) or cytotoxic (b) Bence–Jones proteins (BJPs), the cells were subjected to terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labelling (TUNEL) staining, as described previously.[15]

TUNEL staining was negative for cells treated with non-cytotoxic BJPs. However, TUNEL-positive staining was detected as green fluorescence on the nuclei of LLC-PK_1 cells that had been treated with cytotoxic BJPs (Fig. 5b). When biotinylated dUTP was omitted from the reaction process, no green fluorescence was detected (data not shown).

DNA ladder observed by electrophoresis

To further substantiate the above cytochemical results, nuclear DNA was cleaved into oligonucleosome fragments and analysed further, as described below. After treatment of LLC-PK_1 cells with cytotoxic BJP, cells were lysed and the DNA fragments present in the lysate were examined as described in the Materials and methods. Figure 6 shows the presence of fragmented DNA as a ladder-like prominent smear (lanes 2–4). These DNA ladders were not detected in LLC-PK_1 cells treated with BSA or non-cytotoxic BJP (lanes 1 and 5).

Relationship between cytotoxicity and catalytic activities

It was shown previously that the majority of BJPs had weak amidase activity[1,3,6] while ≈ 20% of BJPs examined had DNA-hydrolysing activity.[10] We therefore studied whether or not the nuclear localization of BJP is related to either enzymic activity. Figure 7 shows that BJPs with relatively high amidase activity (abscissa) were cytocidal (c–f). The most cytotoxic BJP (Fig. 1, ■) had the highest amidase activity (Fig. 7, ■), and was secreted by a 63-year-old male (patient c) who died of renal failure 4 years after the first diagnosis (average daily BJP excretion, 0·8 g). On the other hand, three patients (e–g) who secreted BJPs with relatively high amidase but no DNase activities exhibited mild symptoms, although their BJPs were moderately cytocidal *in vitro* (Fig. 1, ●). Three out of four BJPs with DNA-nicking activity (ordinate) were cytotoxic (a, c and d). However, one BJP with a relatively high DNase activity (b) was neither taken up by the cells nor cytotoxic. These results suggest that the amidase activity is more related to cytotoxicity than the DNase activity. It is possible, however, that BJPs have a number of other, as yet unknown, activities and more data are required to confirm this.

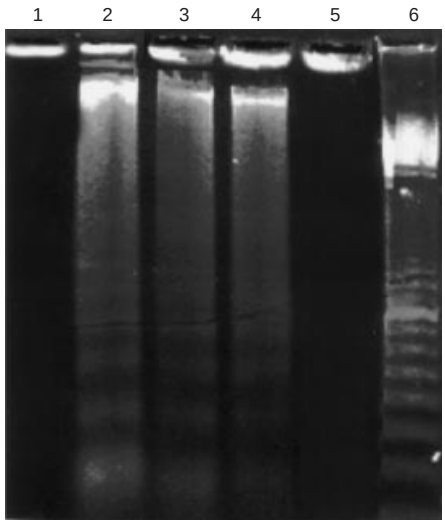

Figure 6. DNA fragmentation in LL-CPK_1 cells after incubation with Bence–Jones proteins (BJPs). LL-CPK_1 cells were incubated with BJPs and the DNA fragments present in the lysate were detected by the method of Hockenbery *et al.*[17] Lane 1, 2 μM of bovine serum albumin (BSA); lanes 2–4, 2, 1 and 0·25 μM of cytotoxic BJP, respectively; lane 5, 2 μM of non-cytotoxic BJP; lane 6, 100-bp DNA ladder marker (Amersham Pharmacia Biotech, Tokyo, Japan).

DISCUSSION

Among the various symptoms associated with multiple myeloma, clinically significant renal impairment has been

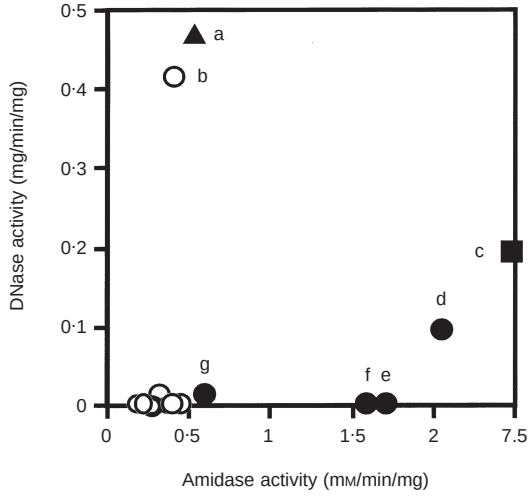

Figure 7. Relationship between amidase, DNase and *in vitro* cytotoxicity of Bence–Jones proteins (BJPs). Cell viability was determined as described in the text: ≈ 25% (■), 50% (●), 75% (▲) and 100% (○) viable after 1 hr of incubation with 1 μM of BJP. The symbols correspond to those given in Fig. 1.

reported to occur in 40–60% of patients and to be the second most common cause of death after infection.[20] Although the aetiology of renal failure is multifactorial, we highlighted the possibility that the catalytic activity of BJPs may contribute, to some extent, to the clinical process.[8] The present results show that a subset of BJPs entered LLC-PK_1 cells, gained access to the nucleus and induced DNA fragmentation. These results lend new support to the above hypothesis. However, the catalytic potential of individual BJPs differs greatly, not only in the degree of enzymic activities but also in the substrate specificity[3,8,10] and thus the extent that an individual BJP contributes to the aetiology may be very diverse, ranging from practically null to significant levels. If a BJP is taken up by the cell and has contact with protein(s) or nucleic acid(s) that are necessary for the maintenance of cell function, slow cleavage of a single peptide or nucleotide bond may be sufficient to lead to the gradual loss of function, resulting in cell death.

Madaio and co-workers[11,12,21] showed that some lupus anti-DNA autoantibodies and their Fab′ fragments entered the cell and gained access to the nucleus in a time-dependent manner. This suggests that the antigen-binding region is mainly responsible for both cell entry and nuclear localization. The present results obtained with BJPs are in general agreement with these previous findings obtained for intact anti-DNA autoantibodies. In the past few years, enormous progress has been made in understanding nuclear protein import.[22,23] Nuclear proteins synthesized on free ribosomes in the cytoplasm are translocated efficiently and precisely to the nucleus through the nuclear pore complex present in the nuclear envelope. The pore complex can accommodate the active transport of large molecules. This process is conferred by several nuclear localization signals. Among them, two signals were relatively well characterized: a single basic type consisting of a short stretch of highly basic amino acid residues, such as PKKKRKV; and a bipartite basic type consisting of two stretches of basic amino acid residues separated by a spacer of random amino acid residues, such as KRPAAIKKAG-QAKKKK. It is possible that some BJPs have a similar sequence in their CDRs. Foster *et al.*[24] showed that the nuclear localization-like motifs were present in CDR3 of nuclear localizing anti-DNA lupus antibodies.

Recently, Yanase *et al.*[21] reported that cellular entry of nuclear localizing anti-DNA antibodies is mediated by cell-surface binding to brush border myosin (myosin 1). The initial binding to this receptor provides the subsequent sorting to enter living cells. Imported autoantibodies interact with DNase 1, which was thought to be primarily responsible for apoptosis, and result in the attenuation of apoptosis. These results obtained with the intact anti-DNA antibodies and their Fab′ fragments were not in agreement, in several areas, with the present study for BJPs. For example, cytotoxic BJPs bound to non-specific cell-surface receptors (Fig. 4) and stimulated cell death rather than attenuation of apoptosis (Fig. 1). Furthermore, recent findings suggest that DNases other than DNase 1 are responsible for apoptosis, such as Ca^{2+}/Mg^{2+}-dependent endonuclease,[25] DNase γ[26] and caspase-activated DNase.[27] Further elucidation of these conflicting results between anti-DNA antibodies and BJPs will lead to a better understanding of the pathogenesis of multiple myeloma, as well as autoimmune diseases.

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