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Protection of Mammalian Cells from the Toxicity of Bleomycin by Expression of a Bleomycin-Binding Protein Gene from *Streptomyces verticillus*[1]

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A gene, *blmA*, encodes a bleomycin (Bm)-binding protein, designated BLMA, from Bm-producing *Streptomyces verticillus* and confers resistance to Bm in *Streptomyces* and *Escherichia coli* cells. In the present study, by transfection of the gene into COS-1 cells with a plasmid designated pEF-BOS/*blmA*, which contains a strong promoter from the human polypeptide chain elongation factor 1α, we transiently overproduced BLMA at a high level of approximately 4% of the whole cell protein. Although NIH/3T3 cells transfected with pEF-BOS/*blmA*, designated NIH/3T3-BR cells, stably expressed BLMA, its expression level was about 0.1% of the total protein. Using an anti-BLMA monoclonal antibody reported previously [Sugiyama *et al.* (1995) *FEBS Lett.* 362, 80-84], we revealed that BLMA is localized in the nucleus of pEF-BOS/*blmA*-transfected COS-1 and NIH/3T3-BR cells. Semi-permeabilized nuclear transport experiments showed that BLMA penetrates the nuclear envelope by energy- and transporter-independent passive diffusion, suggesting that the karyophilic nature of BLMA may be due to the acidic nature of the protein. NIH/3T3-BR cells were 130-fold more resistant to Bm than the host cells. NIH/3T3 cells exhibited a swollen nuclear envelope and a malformed spindle body and overexpressed at least 4 kinds of stress proteins including calreticulin and mitochondrial matrix protein P1 when exposed to 25 μg/ml of Bm, whereas NIH/3T3-BR cells grew without morphological alteration and expressed no stress proteins under the same conditions. Furthermore, reverse transcription-polymerase chain reaction and Northern blot analysis showed that the expression of interleukin-6, an inflammatory cytokine, is activated by addition of Bm in NIH/3T3 cells, but not in the NIH/3T3-BR cells. These results suggest that BLMA contributes to protection of mammalian cells from the inflammatory effect of Bm.

Key words: bleomycin-binding protein, bleomycin-resistance, heat shock proteins, nuclear localization, *Streptomyces verticillus*.

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Bleomycin (Bm) and its related antibiotics cause nucleotide sequence-specific DNA cleavage and inhibit the growth of both bacterial and mammalian cells (*1*). Since the Bm family of antibiotics inhibit effectively the growth of malignant cells, they are frequently used to treat human malignancies. The DNA-cleaving process seems to be mediated *via* a Fe^{2+} chelate of Bm that is capable of generating a reduced form of oxygen in proximity to susceptible site(s) in the DNA (*2*).

The antibiotic-producing microorganisms must be protected from the lethal effect of their own product. We have cloned and sequenced two independent genes, designated *blmA* and *blmB*, encoding Bm-resistance determinants from Bm-producing *Streptomyces verticillus* ATCC15003 (*3*). The genes *blmA* and *blmB* have been shown to encode a Bm-binding protein and Bm *N*-acetyltransferase, respectively (*3, 4*). We have physico-chemically characterized the *blmA* gene product, designated BLMA (*5*).

Streptoalloteichus hindustanus, which belongs to the family Actinoplanaceae and should be obviously distinct from *Streptomyces*, produces tallysomycin, which is also a member of the Bm family of antibiotics. The Sh*ble* gene product from *Streptoalloteichus hindstanus*, which confers resistance to tallysomycin, was also a binding protein with affinity for Bm (*6*). We have generated a monoclonal antibody against BLMA and made an immunological comparison between BLMA and the Sh*ble* proteins, showing that the Sh*ble* protein does not crossreact with the monoclonal antibody against BLMA (*5*). Furthermore, a polyclonal antibody raised against Sh*ble* protein in rabbit did not crossreact with BLMA (*5*). Thus, these Bm-binding proteins are functionally the same, but differ immunologically.

The Sh*ble* gene has been expressed in mammalian cells

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Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; Bm, bleomycin; CS, calf serum; ELISA, enzyme-linked immunosorbent assay; IC_{50}, concentration of antibiotic causing 50% inhibition of cell growth; IL, interleukin; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.

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and shown to confer Bm-resistance on the transfected cells (*7, 8*). However, the biological responses of the transfected cells to Bm have not been investigated.

In the present study, we expressed *blmA* in two kinds of mammalian cells and found that BLMA localizes within the nucleus. We suggest a mechanism for the nuclear transport of the protein and investigated some biological responses caused by exposing mammalian cells transfected with or without *blmA* to Bm.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-1 and NIH/3T3 cells were kindly provided by Mr. N. Nakamura, Mochida Pharmaceutical, Tokyo and Dr. E. Morita, Hiroshima University School of Medicine, respectively. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (CS) (Gibco, USA) and 10 mM HEPES in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Vector—An expression vector for mammalian genes, designated pEF-BOS (*9*), which contains a strong promoter from the human polypeptide chain elongation factor 1α and the SV40 replication origin, was kindly provided by Dr. S. Nagata, Osaka Bioscience Institute, Osaka.

Construction of Plasmid to Express BLMA in Mammalian Cells—To amplify the *blmA* structural gene (369 bp) having a *Xba*I site at both the 5'- and 3'-adjacent reagions, a sense 5'-GGGATCTAGAATGGTGAAATTCT-TGGGTGC-3' and an antisense 5'-GGAATCTAGATCAC-TCCCCCGCGGTGAAGT-3' oligonucleotide PCR primer were chemically synthesized. To ensure the expression of *blmA* in mammalian cells, we designed a sense-primer having an ATG codon instead of the GTG start codon contained originally in *blmA*. A plasmid p181EB1 (*10*) was used as a template DNA for amplification of the *blmA* structural gene by PCR. The amplified DNA fragment was inserted into the *Xba*I-digested pEF-BOS to generate pEF-BOS/*blmA*.

Transfection—pEF-BOS/*blmA* was transfected into the mammalian cells by the lipofection method using lipofectamine (Gibco, USA) according to the supplier's instruction manual.

Western Blotting—Cultured host cells or pEF-BOS/*blmA*-transfected cells were washed twice with PBS. Each cell mass was suspended in 100 μl of homogenizing buffer [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 2 mM 2-mercaptoethanol], then sonicated (Bioruptor, Cosmo Bio, Tokyo). After centrifugation of the cell-extracts for 20 min at 4°C at 10,000 $\times g$, the resulting supernatant was subjected to Tricine/SDS-PAGE (*11*), which is employed for the high resolution at low acrylamide concentration (10%) of small proteins such as BLMA. The proteins were electrophoretically transblotted at 150 mA for 1 h onto a nitrocellulose membrane (Hybond-C super, Amersham, England) and immunologically detected by using an anti-BLMA mouse monoclonal antibody (0.5 μg/ml), as described previously (*5*).

Enzyme Linked Immunosorbent Assay—Cell-extracts from host and pEF-BOS/*blmA*-transfected cells, prepared as described above, were diluted to 2.5 μg protein/ml with PBS and used for enzyme-linked immunosorbent assay (ELISA). ELISA was done as described previously (*5*).

Immunocytochemical Localization of BLMA—Host and pEF-BOS/*blmA*-transfected cells, grown on plastic coverslips (Celldesk, Sumitomo, Tokyo) in Bm-free medium, were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature. After fixation for 10 min, the cells were washed twice with PBS and permeabilized with PBS containing 0.2% Triton X-100 for 2 min at room temperature. After additional washing four times with PBS, the cells were used for immunostaining using 1 μg/ml of anti-BLMA mouse monoclonal antibody. Detection was performed with an Elite ABC kit (Vectastain, USA) according to the supplier's instructions.

Semi-Permeabilization Nuclear Transport Assay—COS-1 cells (2×10^5 cells), cultured on a glass coverslip in DMEM supplemented with 10% CS for 24 h, were harvested and used in the semi-permeabilization nuclear transport assay in the transport mixture consisting of 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM dithiothreitol, and 1 mM EGTA, as described previously (*12*). After the transport assay, cells were permeabilized with PBS containing 0.2% Triton X-100, followed by staining of BLMA with an Elite ABC kit using an anti-BLMA mouse monoclonal antibody.

Drug-Resistance Assay—To determine the susceptibility of cells to various antibiotics, cell survival was assayed based on the absorbance at 450 nm, as described previously (*13*). NIH/3T3 cells and pEF-BOS/*blmA*-transfected NIH/3T3 cells (2×10^3 cells) were grown in 0.6 cm^2 in 96-well plates in DMEM supplemented with 10% CS. After incubation for 12 h, the medium was replaced with the same medium supplemented with various drugs. After further incubation for 5 d, the surviving cells were counted using a Cell Counting Kit (Dojindo Laboratories, Kumamoto) with an automatic plate analyzer (Toyo Sokki, Kanagawa).

Two-Dimensional PAGE Analysis—NIH/3T3 cells and pEF-BOS/*blmA*-transfected NIH/3T3 cells, grown in DMEM containing 10% CS supplemented with 25 μg/ml of Bm for 72 h, were harvested and washed twice with PBS. The washed cells were suspended in homogenizing buffer [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 2 mM 2-mercaptoethanol] and sonicated, then centrifuged for 20 min at 4°C at 10,000 $\times g$. Protein concentrations in the supernatant were assayed by the Bradford method (*14*). A portion of proteins (450 μg) was subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as described previously (*15*), then stained with Coomassie Brilliant Blue R250.

Reverse Transcription-Polymerase Chain Reaction—NIH/3T3 cells and pEF-BOS/*blmA*-transfected NIH/3T3 cells, exposed for 48 h to 25 μg/ml of Bm (bleomycin A_2 sulfate) in DMEM supplemented with 10% CS, were trypsinized and harvested. mRNA from the harvested cells was isolated using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech., USA). A 2-μl (50 ng) portion of mRNA was used for reverse transcription-polymerase chain reaction (RT-PCR) using a RT-PCR high kit (TOYOBO, Osaka), according to the supplier's instructions. To amplify the interleukin-6 (IL-6) cDNA fragment of 229 bp, the following oligonucleotide primers were used: 5'-GACA-AAGCCAGAGTCCTTCAGAGAG-3' and 5'-CTAGGTTT-GCCGAGTAGATCTC-3' (Stratagene, USA). As an internal control, the set of primers to amplify a part of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (450

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bp), supplied in the same kit, was also utilized. The PCR conditions were as follows: for IL-6, 30 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C; for G3PDH, 24 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C. These DNA fragments, amplified by PCR, were subjected to 2% agarose gel electrophoresis.

Northern Blotting—NIH/3T3 cells and pEF-BOS/*blmA*-transfected NIH/3T3 cells, exposed for 48 h to 25 μg/ml of Bm in DMEM supplemented with 10% CS, were harvested and washed twice with PBS. Total RNA from each washed cell mass was prepared using Isogen (Nippon Gene, Tokyo) based on the method of Chomczynski (*16*). Ten micrograms of each RNA was electrophoresed in 1.2% agarose gel containing formaldehyde according to the standard procedures (*17*) and blotted onto a nylon membrane (Hybond N+) for Northern blot analysis. Northern blot analysis was performed using a Gene Images AlkPhos Direct labeling and detection kit (Amersham, England) following the supplier's instruction manual. IL-6 and G3PDH cDNA fragments amplified by RT-PCR (see above) were used as hybridization probes.

N-Terminal Amino Acid Sequence—The proteins separated and purified by 2D-PAGE were electrophoretically transferred onto ProBlott membranes (Applied Biosystems, USA) for determination of N-terminal amino acid sequences by Edman degradation using an autosequencer (Shimadzu, Kyoto).

RESULTS

Transient and Stable Expression of blmA—Since the GTG initiation codon originally contained in *blmA* was not recognized as a translational start signal in mammalian cells, we designed *blmA* PCR primers carrying an ATG codon instead of the GTG codon. To overproduce the gene product, BLMA, we generated a chimeric plasmid, designated pEF-BOS/*blmA*, which carries the modified *blmA* under the control of a strong promoter from the human polypeptide chain elongation factor 1α, which has a high transcriptional efficiency (*9*). After introduction of this plasmid into COS-1 cells, we analyzed the expression of BLMA in cell-extracts from the pEF-BOS/*blmA*-transfected cells grown for 72 h. Western blot analysis shows that BLMA was transiently overproduced (Fig. 1). To assay its expression level, the ELISA method using an anti-BLMA monoclonal antibody was employed. Figure 2B reveals that BLMA was produced much more in the pEF-BOS/*blmA*-transfected COS-1 cells cultured for 72 h than for 48 h after transfection. The BLMA produced was estimated to be approximately 4% of the whole cell proteins, based on the standard curve in Fig. 2A.

We tried to get a stable transformant of *blmA* using NIH/3T3 as a host cell. Such a transformant was obtained by culturing the NIH/3T3 cells transfected with pEF-BOS/*blmA* for 2 weeks in DMEM supplemented with 25 μg/ml of Bm (as bleomycin A_2 sulfate). Western blot analysis showed that the resulting Bm-resistant cells continuously produced BLMA and the production lasted for one month without Bm. Its expression level, estimated by ELISA, was approximately 0.1% of the whole cell proteins (data not shown). We designated the stable transformant of *blmA* as

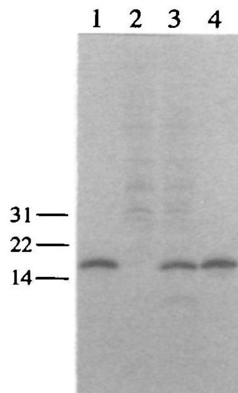

Fig. 1. **Western blot analysis of cell-extracts from COS-1 cells and pEF-BOS/*blmA*-transfected COS-1 cells.** Cells were harvested and sonicated, then 0.2-μg protein portions of each cell-extract were subjected to 10% Tricine/SDS-PAGE. The proteins were transblotted on a nitrocellulose membrane and stained with a Vectastain Elite ABC kit using an anti-BLMA monoclonal antibody. Lanes 1 and 4, purified BLMA (10 ng) obtained as described previously (*5*); lanes 2 and 3, cell-extracts from COS-1 cells and pEF-BOS/*blmA*-transfected COS-1 cells, respectively. Protein size in kDa is indicated.

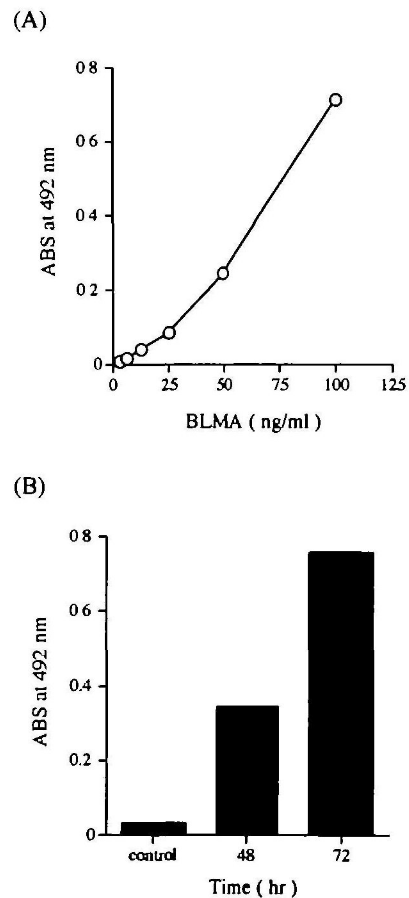

Fig. 2. **Expression level of BLMA in COS-1 cells.** BLMA, purified as described previously (*5*), was diluted to the given concentrations and assayed by ELISA using an anti-BLMA monoclonal antibody. (A) Standard curve for relationship between BLMA concentration and absorbance at 492 nm, (B) COS-1 cells incubated for the given times after transfection with pEF-BOS/*blmA*. The proteins in the cell-extract were assayed by ELISA. The cell-extract from COS-1 cells was used as a control.

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NIH/3T3-BR. Southern hybridization of the NIH/3T3-BR chromosomal DNA using *blmA* structural gene as a probe confirmed that only one copy of *blmA* was integrated into genomic DNA (data not shown).

Immunocytochemical Localization of BLMA—We determined the subcellular localization of BLMA in both pEF-BOS/*blmA*-transfected COS-1 and NIH/3T3-BR cells by immunostaining using an anti-BLMA monoclonal antibody. After permeabilization with Triton X-100, each cell was incubated sequentially with the anti-BLMA antibody, biotinylated secondary antibody, and horse-radish peroxidase-conjugated avidin-biotine complex. Figure 3 shows that the signal for BLMA was stronger in the nucleus of COS-1 cells than in that of NIH/3T3-BR cells. The signal strength may depend on the expression level of BLMA. No signals were detected in host COS-1 or NIH/3T3 cells.

Nuclear Transport of BLMA—Analysis by immunostaining of the subcellular localization of BLMA revealed that the protein localizes within the nucleus, even though BLMA has no nuclear localization sequence (*18*). To clarify how BLMA enters the nucleus, a semi-permeabilization nuclear transport assay was done as described previously (*12*). Digitonin-treated cells were incubated with or without BLMA in the transport mixture (see "EXPERIMENTAL PROCEDURE"), then immunostained using an anti-BLMA monoclonal antibody. As shown in Fig. 4A, a signal was observed in the cells incubated with BLMA, but not in those without BLMA (Fig. 4B). No signals were also seen in the cells treated with wheat germ agglutinin (Fig. 4C), which is an inhibitor of nuclear transport *via* interaction with nuclear pore complex (*19-21*), before incubation in the transport mixture containing BLMA. In our experiments, cytosolic fractions and ATP, which are necessary for the nuclear transport of proteins containing nuclear localization sequence of the SV40 large T antigen, as described previously (*12*), were not added to the transport mixture.

Drug Resistance—To evaluate the function of BLMA in mammalian cells, we examined the susceptibility of the

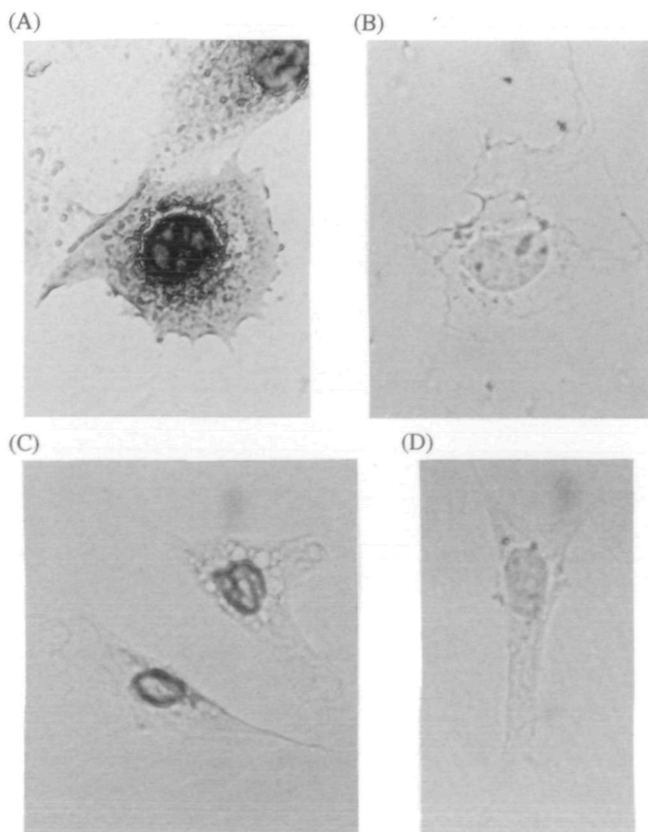

Fig. 3. **Immunocytochemical localization of BLMA.** pEF-BOS/*blmA*-transfected COS-1 and NIH/3T3-BR cells were fixed with paraformaldehyde and permeabilized with Triton X-100. After blocking with goat serum solution, cells were incubated with an anti-BLMA monoclonal antibody, then immunostained using a Vectastain Elite ABC kit. (A) COS-1 cells transfected with pEF-BOS/*blmA*, (B) COS-1 cells, (C) NIH/3T3-BR cells, (D) NIH/3T3 cells. All micrographs were photographed at ×400.

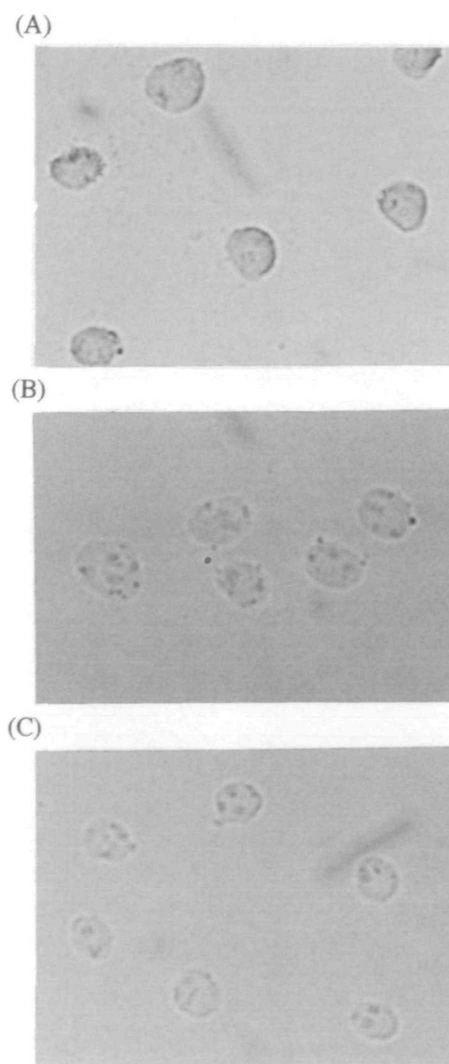

Fig. 4. **Nuclear transport of BLMA.** COS-1 cells grown on glass coverslips were treated with digitonin. The resulting permeabilized cells were incubated in the transport mixture with or without BLMA (20 μg), treated with Triton X-100, then stained with an Elite ABC kit using an anti-BLMA monoclonal antibody. The permeabilized cells, treated with wheat germ agglutinin (WGA) before incubation with the transport mixture in the presence of BLMA, were also stained by the same method. (A) Treated with BLMA, (B) without BLMA, (C) pretreated with WGA before incubation with BLMA. All micrographs were photographed at ×200.

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NIH/3T3-BR cells to several antibiotics. As shown in Fig. 5A, although the host NIH/3T3 cells were susceptible to Bm, the NIH/3T3-BR cells were resistant to Bm. IC_{50}s for Bm in the NIH/3T3 and NIH/3T3-BR cells were 0.49 and 62.5 μg/ml, respectively, indicating that Bm resistance of NIH/3T3-BR cells was about 130-fold higher than that of the host cells. We also determined IC_{50} of both cells to Bm analogues. The values in the host NIH/3T3 cells treated by liblomycin, peplomycin, and phleomycin were 6.25, 0.6, and 1.2 μg/ml, but those in NIH/3T3-BR cells were 50, 12.5, and 50 μg/ml, respectively. Figure 5B shows that the NIH/3T3-BR cells were susceptible to another DNA-cleaving agent, mitomycin C. In addition, the cell growth of NIH/3T3-BR cells was also inhibited by an aminoglycoside antibiotic, geneticin (data not shown). These results suggest that BLMA binds to the Bm family of antibiotics and exhibits the Bm-resistant phenotype in mammalian as well as in bacterial cells (*3*).

Cell Response to Bm—Biological responses of NIH/3T3-BR cells to Bm were examined. Host NIH/3T3 and NIH/3T3-BR cells were exposed to Bm (25 μg/ml) for 72 h, then the total protein from each cell-extract was analyzed by 2D-PAGE. Figure 6A shows that four protein spots in the electrophoretic profile of proteins from the NIH/3T3 cell-extracts were overproduced. The N-terminal amino acid sequences of the protein spots 1 and 4, marked in Fig. 6A, were DPAIYFKEQF and AKDVKFGADA, respectively. Those of the other two proteins (spots 2 and 3) could not be determined because their N-termini were blocked. Protein homology analysis using the FASTA homology program showed that the N-terminal amino acid sequences of protein spots 1 and 4 were identical with a part of calreticulin (Cal) and mitochondrial matrix protein P1 (HSP 60), respectively. Since the migration profiles of these two proteins on 2D-PAGE were consistent with the molecular mass and isoelectric point of the proteins, we concluded that the proteins were Cal and HSP60, respectively. Both of these proteins have been reported to be chaperonins which are induced by biophysical or biochemical stress (*22*). Such overexpression of the stress proteins was not observed in NIH/3T3-BR cells (Fig. 6B). In addition, no morphological alteration of the NIH/3T3-BR cells was observed when they were exposed to Bm, but the NIH/3T3 cells exhibited a swollen nuclear envelope and malformed spindle body (Fig. 7).

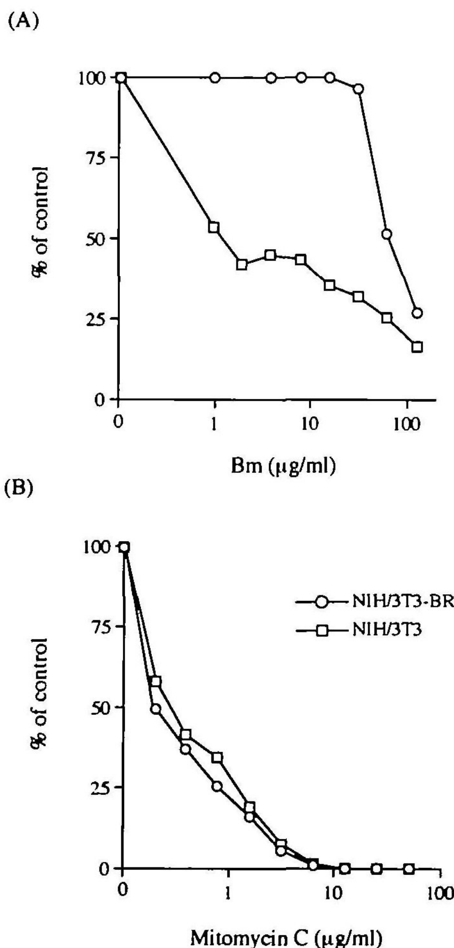

Fig. 5. **Dose-dependent survival curves of NIH/3T3 and NIH/3T3-BR cells exposed to Bm (A) or mitomycin C (B).** Cells were seeded in a 96-well plate and exposed to each drug at the given concentrations. After incubation for 5 d, the surviving cells were determined using a Cell Counting kit, as described in the experimental procedure.

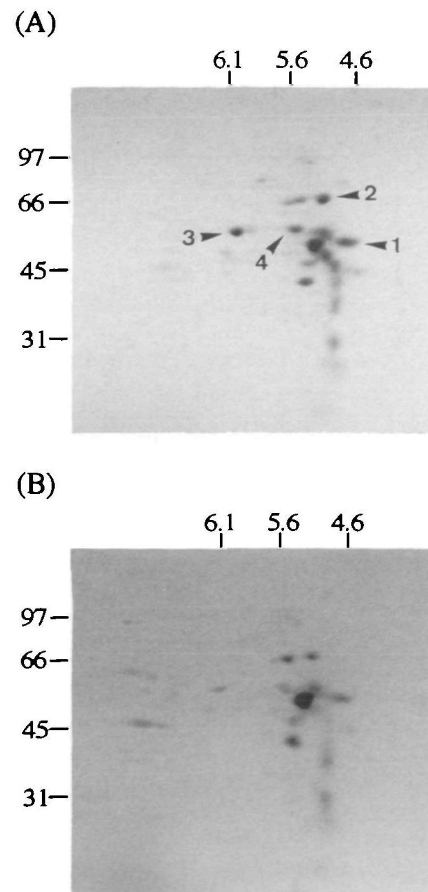

Fig. 6. **2D-PAGE analysis of the cell-extracts from NIH/3T3 (A) and NIH/3T3-BR (B) cells grown in the presence of Bm.** Cells were exposed to 25 μg/ml of Bm for 72 h, then harvested and sonicated. The resulting cell-extracts were subjected to 2D-PAGE. Bm-induced proteins are indicated by numbered arrowheads. Numbers to the left and top of the photographs indicate protein size in kDa and isoelectric point, respectively.

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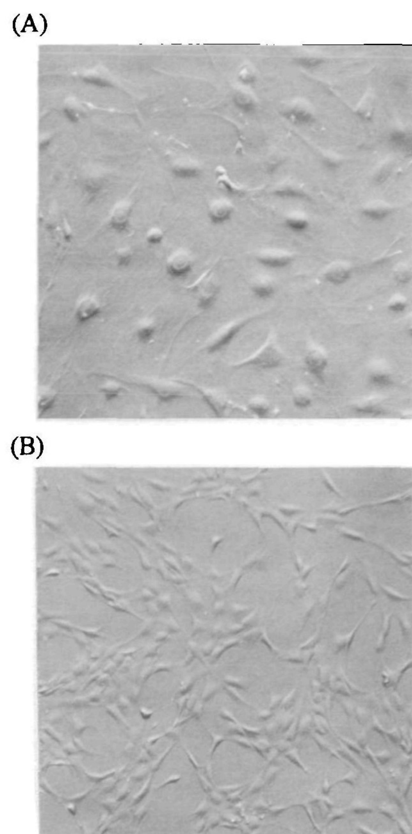

Fig. 7. **Morphology of NIH/3T3 (A) and NIH/3T3-BR (B) cells exposed to Bm.** Both types of cells were exposed to 25 μg/ml of Bm for 48 h, then photographed at $\times$40.

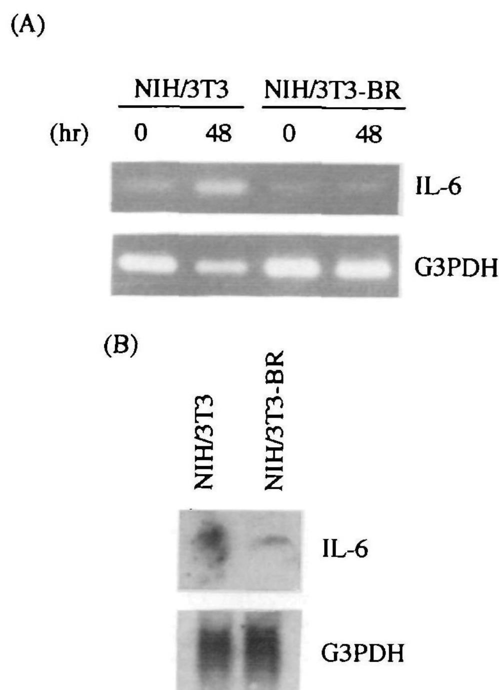

Fig. 8. **RT-PCR (A) and Northern blot analysis (B) of NIH/3T3 and NIH/3T3-BR cells exposed to Bm.** Each cell type (5×10^5 cells) was exposed to 25 μg/ml of Bm for 48 h, then harvested. The messenger and total RNAs isolated from each cell were used for RT-PCR and Northern blot analysis, respectively. The expression of G3PDH gene was measured as an internal control.

The expression of an inflammatory cytokine IL-6 in pulmonary endothelial cells is upregulated by Bm (*23*). We analyzed the expression level of IL-6 mRNA by means of RT-PCR and Northern blotting. Figure 8 shows that the expression of IL-6 mRNA increased in the wild-type NIH/3T3 cells, but not in the NIH/3T3-BR cells, when cells were exposed for 48 h to 25 μg/ml of Bm. On the RT-PCR, densitometric analysis using a densitograph (ATTO, ver. 4.0, Tokyo) showed that the IL-6 expression level in the wild-type NIH/3T3 cells was 12-fold higher than that in the NIH/3T3-BR cells.

DISCUSSION

A gene, *blmA*, which encodes a Bm-binding protein, confers resistance to Bm in *Streptomyces lividans* (*3*) and *Escherichia coli* (*3, 5, 10*). The present study showed that the bacterial gene is efficiently expressed and confers resistance to Bm in mammalian cells. In our preliminary experiments, however, the expression of the original *blmA* containing a GTG start codon was not successful. Therefore, we replaced GTG with ATG, which is generally observed as a start codon recognized in mammalian cells. Even though *blmA* has no consensus Kozak sequence (5′-ACCATGG-3′; the start codon is underlined and the 5′-terminal A can be replaced with G), which is preferable for the initiation of translation by eukaryotic ribosomes (*24*), we were successful in achieving the efficient expression of the ATG-substituted *blmA*. An explanation for this may be that *blmA* has an essential G for mammalian translation at position +4 (the ATG start codon is designated position +1 to +3), as described by Kozak (*24*).

The BLMA molecule, despite having no nuclear localization sequences, accumulates in the nucleus. The nuclear transport of the molecules occurs through the nuclear pore complex which spans the nuclear envelope (*25*). Cell microinjection studies have shown that the pore complex contains an aqueous channel that allows nonselective passive diffusion of molecules smaller than 30–40 kDa across the nuclear envelope (*26*). Most proteins which localize in the nucleus have nuclear localization sequences consisting of positively charged amino acids (*18*). Transport of most proteins through the pore complex requires ATP and cytosol fractions (*12*). Our semi-permeabilized cell transport study revealed that BLMA is imported without the addition of ATP and cytosol fractions into the nucleus, suggesting that, since BLMA is a small protein (13.2 kDa), it may penetrate into the nucleus by energy-independent passive diffusion. The accumulation of BLMA in the nucleus may be associated with the acidic nature of the protein. Because co-immunoprecipitants were not observed in the immunoprecipitation of nuclear extracts from the NIH/3T3 cells with BLMA using an anti-BLMA monoclonal antibody (our unpublished data), the acidic protein, BLMA, may interact with positively-charged components in the nucleus presumably by isoelectric force, thus preventing exclusion of the incorporated BLMA from the nucleus. The karyophilic nature of the protein may contribute to the protection of DNA from Bm's toxicity in the nucleus.

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The drug resistance profile of the NIH/3T3-BR cells was specific to the Bm family of antibiotics. This result indicates that *blmA* could be useful as a selective marker for the genetic manipulation in mammalian cells.

A dose of Bm (25 μg/ml) generates a swollen nuclear envelope, malformed spindle body, and overexpression of stress proteins such as Cal and HSP 60 in NIH/3T3 cells. This is the first report of Bm-induced stress proteins. Alterations in cell morphology and in the 2D-PAGE profile of the whole cell proteins, however, were undetectable in NIH/3T3-BR cells grown at the same concentration of Bm. Furthermore, RT-PCR and Northern blot analysis of the mRNA of imflammatory cytokine IL-6 showed no alternation of its expression in NIH/3T3-BR cells cultured in the presence of 25 μg/ml of Bm. It is well known that the continuous administration of the antitumor antibiotic Bm causes pulmonary fibrosis. In the development of the Bm-induced pulmonary fibrosis, the expression of cytokines, such as interleukins (*23, 27, 28*), transforming growth factor-β (*29-32*), and tumor necrosis factor-α (*33*), is reported to play an important role. The present study suggests that BLMA may prevent Bm-related pulmonary toxicity if *blmA* can be introduced into appropriate cells in lung tissue.

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