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INVOLVEMENT OF MEMBRANE BLEOMYCIN-BINDING SITES IN BLEOMYCIN CYTOTOXICITY

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Abstract—The authors have recently shown the existence of bleomycin (BLM)-binding sites at the surface of DC-3F cells. In order to study the involvement of these sites in the sensitivity of the cells to bleomycin several BLM-resistant cell lines from DC-3F cells were analysed. These mutants were obtained by electrotransfection of the Sh *ble* gene (D/BlmI cells) or the Sh *ble*-βGal fusion gene (D/BlmII cells) and/or by continuous culture in the presence of BLM (D/BlmIR and D/Blm40 cells). The resistance levels of the D/BlmII and D/Blm40 cells were 50- and 22-fold, respectively, determined at the EC_{50} level. The D/BlmI cells were only 2-fold resistant, whereas D/BlmIR cells were so resistant that almost no cytotoxicity was detected up to 200 μM BLM external concentration. Electropermeabilization was used in an attempt to bypass the plasma membrane of the cells and permit the distinction between internal resistance and membrane resistance. The former was observed when the products of the transfected genes were present. With respect to membrane resistance, differences were detected in the number of BLM-binding sites in several mutant cell lines, which could account for the differences in cell sensitivity to BLM. This suggests that the BLM-binding sites found at the cell surface may play a crucial role in BLM internalization and consequently in its cytotoxicity.

Key words: bleomycin; cytotoxicity; resistance; BRP; binding sites; electropermeabilization

Bleomycin (BLM)‡ is a watersoluble antibiotic first isolated by Umezawa *et al.* in 1966 [1]. It exhibits cytotoxic activity against mammalian cells by inducing single- and double-strand DNA breaks [2]. It is used clinically in the treatment of several types of solid tumours, including squamous cell carcinoma and malignant lymphoma. Studies performed on DC-3F cells in culture showed that BLM possesses very high intrinsic cytotoxicity: 500 molecules of BLM introduced in the cytosol of an electropermeabilized cell are sufficient to kill it [3]. The plasma membrane has been shown to be responsible for the unusual biphasic SRC of cells exposed to BLM [3]. A very small amount of BLM associates with the cells, presumably because of its hydrophilic nature [4]. Although many authors have stressed the role of the plasma membrane in limiting BLM uptake, little is known about the comprehensive mechanism of BLM internalization. However, when the plasma membrane is transiently and reversibly permeabilized, by using short and intense electric pulses for example [5], BLM cytotoxicity is greatly increased [3, 6]. *In vivo* application of this finding resulted in a new antitumour treatment [7–9].

This group recently demonstrated the existence of specific BLM binding sites on the plasma membrane of DC-3F cells [10]. In attempting to characterize these BLM-binding sites special interest was taken in another previously described BLM-binding protein, the BRP. This 14 kDa protein is encoded by the BLM-resistance Sh *ble* gene, cloned from a tallysomycin-producing actinomycete [11]. The BRP binds BLM almost irreversibly in a 1:1 ratio and prevents it from cleaving DNA [12]. BLM-resistance of cells harboring a Sh *ble* gene could be accounted for by a sequestering effect of the BRP [12].

In this paper the isolation and characterization of BLM-resistant cells obtained from DC-3F cells by electrotransfection of the Sh *ble* gene or the Sh *ble*-βGal fusion gene, and/or by culture in the continuous presence of BLM, are described. Internal, cytosolic resistance and resistance due to membrane alterations have been discriminated by comparing the sensitivity to BLM of mutant cells which were subjected to electropermeabilization with those of cells which were not. Moreover, the number of BLM-binding sites at the surface of all mutant cells, and their affinity for BLM, have been determined.

MATERIALS AND METHODS

Cells and cell culture. DC-3F cells, a spontaneously transformed Chinese hamster fibroblast, were maintained as monolayers in tissue culture flasks

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‡ Abbreviations: BLM, bleomycin; BRP, bleomycin-resistance protein; DTT, DL-dithiothreitol; EC_{50}, bleomycin concentration necessary to reduce cell survival by 50%; MIC, minimal cytotoxic concentration; PMSF, phenylmethylsulphonide fluoride; SRC, survival response curve.

(Falcon, Becton Dickinson, Le Pont de Claix, France) in culture medium ("complete MEM") consisting of MEM supplemented with 8% foetal calf serum (Eurobio, Les Ulis, France), 100 units/mL penicillin (Specia, Paris, France) and 125 μg/mL streptomycin (Sarbach, Suresnes, France). They were grown at 37° in a humid incubator with an air mixture containing 5% CO_2. Under these culture conditions exponentially growing DC-3F cells had a population doubling time of 10 hr. Cells were harvested with a suitable amount of trypsin 0.05%:EDTA solution (Gibco, Cergy-Pontoise, France). DC-3F cells were regularly thawed from an initial batch to avoid variations in the number of BLM binding sites due to cell ageing [10].

DC-3F cells were also cultured in the presence of increasing concentrations of BLM in the culture medium. After 4 months, the surviving cells were cloned and one of these clones (designated D/Blm40) was studied. This resistant clone was routinely cultured in the presence of 40 μg/mL BLM (27 μM).

Chemicals and plasmids. Bleomycin (Laboratoires Roger Bellon, France) was solubilized in 0.9% NaCl at a concentration of 1 mg/mL. Radiolabelled BLM was prepared (as described by Poddevin *et al.* [13]) by mixing bleomycin with ^{57}Co chloride (Amersham, U.K.) in a 3:1 molar ratio. The pH of the mixture was adjusted to 7 with sodium bicarbonate and the Co–BLM complex (below referred to as CoBLM) allowed to stabilize for 1 hr at room temperature before use. The maximum specific activity of our radiolabelled bleomycin was 17 Ci/mmol of BLM.

E. coli containing pUT 523 (3.9 kb) and pUT 531 (6.8 kb) plasmids were provided by Société Cayla (Toulouse, France). pUT 523 plasmid contained the Sh *ble* gene under the control of the SV40 enhancer and promotor. pUT 531 plasmid contained the Sh *ble*-βGal fusion gene under the control of the same enhancer/promotor. Plasmid DNA was prepared according to usual procedures including two cesium gradients [14]. Purified BRP was also kindly provided by Société Cayla. All other chemicals were obtained from Sigma (La Verpilliére, France).

BLM cytotoxicity on intact cells. Cells were plated in drug-free medium in 25 cm^2 tissue culture flasks so as to be almost confluent 2 days later. BLM was then added to the medium at various concentrations, and the exponentially growing cells incubated for 3 hr at 37°. Controls were incubated with 0.9% NaCl rather than BLM. Cells were then washed, trypsinized and plated in triplicate in 60 mm diameter Petri dishes at a density of 500 cells/dish. After 5 days colonies were fixed and counted and the relative cloning efficiency determined.

BLM cytotoxicity on electropermeabilized cells. Cells were dissociated by trypsinization and centrifuged in complete MEM. They were then washed twice by centrifugation in S-MEM (Gibco) supplemented with 0.5 mM $CaCl_2$. The final pellet was resuspended at a concentration of 25×10^6 cells/mL and cooled on ice for 10 min. Ten microlitres of 5-fold concentrated BLM were mixed with 40 μL of the cell solution and the mixture was immediately subjected to electropermeabilization under the optimal conditions previously described (eight electric pulses of 100 μsec and 1300 V/cm delivered at a frequency of 1 Hz by a PS-15 electropulsator; Jouan, Nantes, France) [6]. After pulse delivery cells were kept at room temperature for 5 min and then immediately diluted ~200,000 times in complete MEM. They were plated for relative cloning efficiency determination as described above.

Obtention of mutants by electrotransfection. DC-3F cells were prepared for electropermeabilization as described above. They were adjusted to a final concentration of 22×10^6 cells/mL. Forty-five microlitres of this solution (~10^6 cells) were mixed with 5 μL of pUT 523 or pUT 531 DNA at a concentration of 2.5 mg/mL. The mixture was kept on ice for 10 min and then subjected to electropermeabilization using the same electric parameters as those reported above for BLM cytotoxicity on electropermeabilized cells. After pulse delivery cells were kept on ice for 10 min and then at 37° for a further 10 min. After dilution in complete MEM cells were plated in three 24-well plates (Falcon) at a density of 10,000 cells/well, as well as in two 60 mm diameter Petri dishes (about 130,000 cells per dish), and cultured at 37°. BLM-resistant cells were selected by two consecutive 48 hr long exposures to 15 μM BLM at day 1 and 3 after electrotransfection. Cells were once again cultured in complete medium without BLM (day 5). BLM-resistant clones were already detectable at day 7.

For the observation of the karyotype the chromosomes were stained using the G-banding technique [15]. For the X-Gal staining assay cells were grown on cover slips placed on the bottom of Petri dishes. They were stained as described by Bonnerot *et al.* [16]. Briefly, cell monolayers were fixed with 1% formaldehyde and 0.2% glutaraldehyde at room temperature, washed twice with PBS and incubated with the staining buffer [4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 0.4 mg/mL X-Gal, 2 mM $MgCl_2$] for 3 hr at 42°. The cover slips were then removed, washed with water and dried before being mounted in permount. Photographs were taken with a Photomicroscope II (Zeiss).

Association of radiolabelled BLM with cell membranes. Monolayers of cells in 60 mm diameter Petri dishes were rinsed with cold PBS, pH 7, and maintained on ice for 10 min. The PBS was then removed and the cells incubated at 4° for 15 min with 800 μL CoBLM diluted to suitable concentrations in PBS. The cells were then washed eight times with cold PBS. The last washing was counted each time to ensure washing efficacy. Crude membrane extracts were then prepared as follows: cells were incubated with 1 mL of lysis solution (200 mM Tris–HCl, 1 mM EDTA, 1 mM DTT, 0.4 mM PMSF, 7 μM pepstatin, 4 μM leupeptin) for 5 min at 4°. They were collected with a rubber policeman and the plates were rinsed with 1 mL of lysis solution. After 25 min incubation at 4° cells were homogenized using a Dounce B (30 strokes). A first centrifugation (500 *g*, 15 min, 4°) separated unbroken cells and nuclei, and the supernatant was centrifuged for 1 hr at 4° at 20,000 *g*. The final pellet consisting of the cellular membranes and the supernatant (i.e. the cytosolic fraction) were counted separately on a MR 252 Gamma Counter (Beckman).

The protein content of each fraction was determined by the method of Bradford using the Bio-Rad Protein Assay (Bio-Rad, Ivry sur Seine, France). In each experiment one of the dishes was incubated in BLM-free PBS, washed eight times like the others and then trypsinized in order to determine the number of cells per dish. By knowing the specific activity of the labelled BLM it was then possible to transform the radioactivity of each membrane fraction in the corresponding number of BLM molecules associated with the membranes of one cell, as described previously [3].

Visualization of BLM-binding proteins. Crude membranes and cytosolic extracts were prepared as above, except that incubation with CoBLM was omitted. Membrane proteins (125 μg), cytosolic proteins (300 μg) and purified BRP (25 μg) were incubated with 3 μM CoBLM for 1 hr at room temperature, solubilized with 1–2% SDS for 1 hr at room temperature without heating and separated on polyacrylamide gels containing 0.1% SDS in Tris (6.25 mM), glycine (192 mM) and EDTA (1.25 mM), according to this group's previous report [10]. Immunoblots were performed as described by Zeheb *et al.* [17], using an antibody directed against the BRP kindly provided by Société Cayla.

RESULTS

Obtention of mutants by electrotransfection

The electrotransfection of pUT 523 plasmid resulted in the growth of 10 colonies after the initial 4 day selection in the presence of 15 μM BLM. Each colony appeared independently in separate wells of 24-well plates and was therefore considered as a clone. After a second 4 day selection in the presence of 15 μM BLM only one clone grew regularly and survived. This clone was separated into two subpopulations: D/BlmI cells maintained in drug-free medium and D/BlmIR cells maintained in the continuous presence of 10 μM BLM. A karyological analysis of the mutant cells revealed no major changes during the selection of these mutants cells. In particular, neither homologous staining regions nor double minute chromosomes were detected, even in the case of the D/BlmIR cells which expressed high amounts of the transfected Sh *ble* gene (see below).

The electropermeabilization of DC-3F cells in the presence of pUT 531 DNA, followed by a 4 day selection by exposure of the cells to 15 μM BLM, led to the emergence of one large colony and three small ones in one of the Petri dishes. In order to

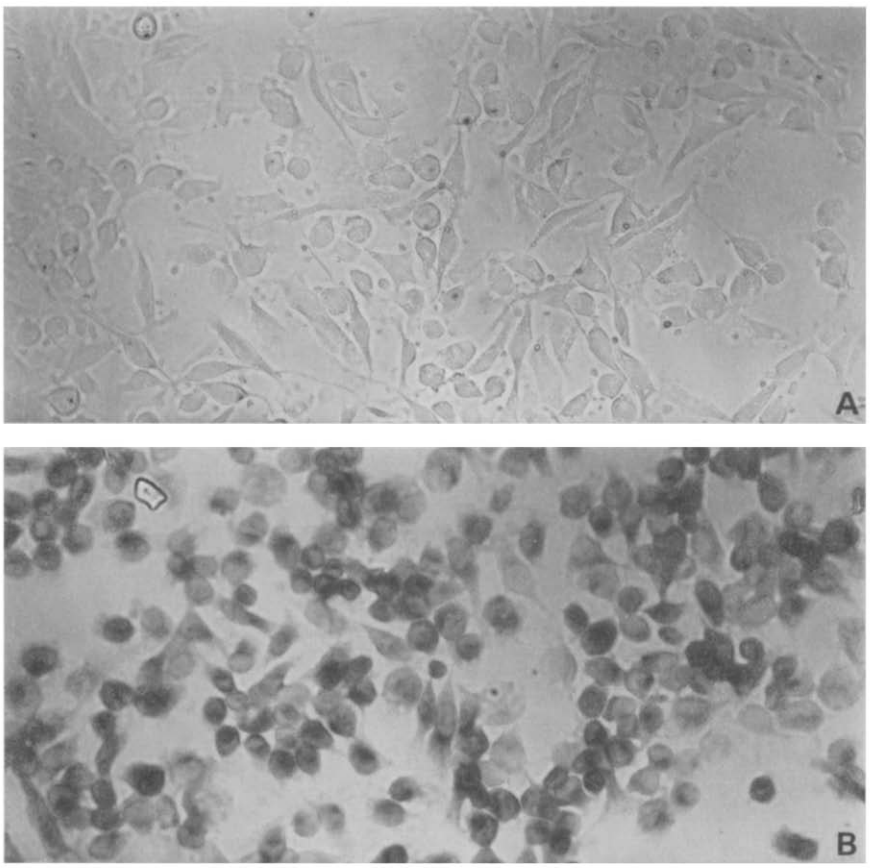

Fig. 1. X-Gal staining assay of D/BlmII cells (B) with DC-3F cells as negative control (A) (magnification 500×).

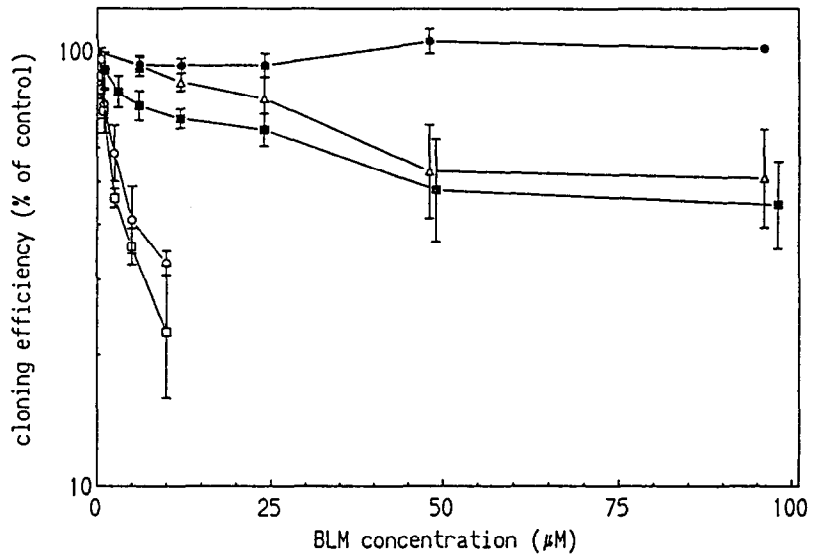

Fig. 2. BLM cytotoxicity on intact DC-3F cells and their related BLM-resistant cell lines. Cells were exposed to BLM for 3 hr at 37°, washed and then plated for relative cloning efficiency determination as described in Materials and Methods. Each point is the average of at least two independent determinations. □, DC-3F cells; ■, D/Blm40 cells; ○, D/BlmI cells; ●, D/BlmIR cells; △, D/BlmII cells.

obtain a clone derived from a unique cell the four colonies were trypsinized and replated under cloning conditions (0.7 cells/well) in a 96-well plate. Five days later five distinct clones were obtained, only one of which grew after a second 4 day selection in the presence of 15 μM BLM. This clone was also divided into two parts, one cultured in drug-free medium and called D/BlmII, the other cultured in the continuous presence of 10 μM BLM and called D/BlmIIR. The staining assay using X-Gal showed that all the D/BlmII cells were blue, proving that the Sh *ble*-βGal fusion gene had been incorporated and was expressed in the cytoplasm of these cells (Fig. 1).

All experiments presented below were performed on cell populations prepared after ~30 passages after transfection.

BLM cytotoxicity on intact cells

After a 3 hr exposure to various concentrations of BLM the D/BlmI and D/Blm40 cells, like the parental DC-3F cells, exhibited the classic biphasic SRC of intact cells exposed to BLM. The SRC of the D/BlmII cells showed that almost no cytotoxicity was detected below 10 μM BLM (Fig. 2). BLM concentration corresponding to 50% cloning efficiency (EC_{50}) (2 μM for the parental DC-3F cells) was 100 μM for the D/BlmII cells, giving a resistance level of 50-fold (Table 1). The D/BlmIIR cells, cultivated in the presence of 10 μM BLM for more than 20 passages, responded exactly like the D/BlmII cells (data not shown). The D/Blm40 cells showed a 22-fold resistance level with an EC_{50} of 45 μM. Surprisingly, the D/BlmI cells showed only a slight resistance of 2-fold with an EC_{50} of 3.5 μM, whereas the D/BlmIR cells appeared to be extremely resistant. Indeed, at 200 μM, almost the maximum concentration possible in this kind of experiment, the percentage survival for D/BlmIR cells was still 80% (data not shown). Consequently, the resistance level of these cells could not be determined.

In order to try to determine the D/BlmIR cell

Table 1. Characteristics of BLM-resistant mutant cells

Cells	Obtention	Global resistance	Internal resistance	Membrane resistance
DC-3F	–	1	1	–
D/BlmI	BRP	2	3	No
D/BlmII	BRP-βgal	50	30	Yes
D/Blm40	+BLM	22	2	Yes
D/BlmIR	BRP; + BLM	?	4000	?

Global resistance is the ratio of the EC_{50} of intact, resistant cells to the EC_{50} of intact DC-3F cells. Internal resistance is the ratio of the EC_{50} of electropermeabilized, resistant cells to the EC_{50} of electropermeabilized DC-3F cells. Membrane resistance results from the comparison between global and internal resistances.

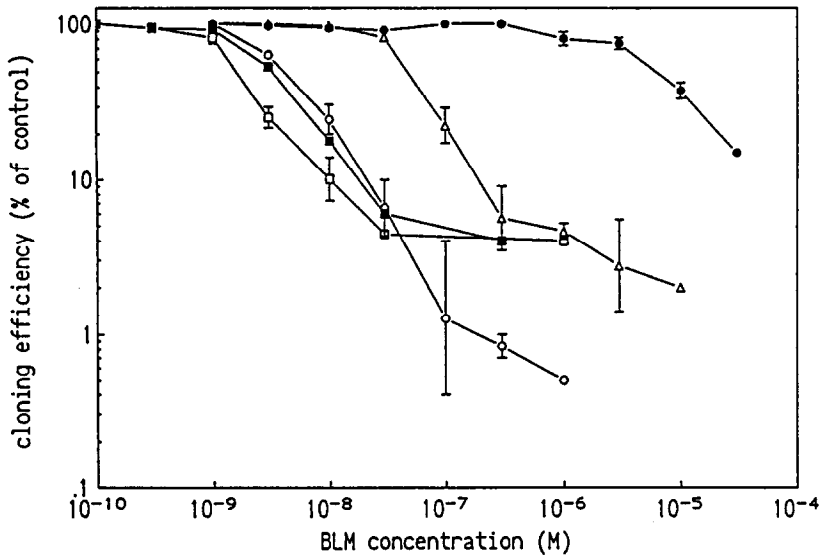

Fig. 3. BLM cytotoxicity upon electropermeabilized DC-3F cells and their related BLM-resistant cell lines. Cells were subjected to electric pulses in the presence of BLM, then extensively diluted and plated for relative cloning efficiency determination, as described in Materials and Methods. Each point is the average of at least two independent determinations. □, DC-3F cells; ■, D/Blm40; ○, D/BlmI cells; ●, D/BlmIR cells, △, D/BlmII cells.

resistance level more accurately the D/BlmIR and DC-3F cells were exposed to BLM for 24 hr instead of 3 hr before being plated for a relative cloning efficiency determination. The D/BlmIR cell SRC has a shoulder, since no cytotoxicity was detected below 50 μM, whereas that of DC-3F cells was still concave (data not shown). Even in this case, the EC_{50} for D/BlmIR cells was not reached, because at 250 μM 67% cell survival for the D/BlmIR cells had been obtained. After seven passages without BLM in the culture medium D/BlmIR maintained the same resistance levels (result not shown).

BLM cytotoxicity on electropermeabilized cells

When plotted using double logarithmic coordinates (log of electropermeabilized cell survival vs log of external BLM concentration) all tested cell lines exhibited a biphasic linear response, except for the D/BlmIR cells (Fig. 3). For this last strain the BLM concentration which could be prepared was technically limiting and the portion of the SRC with the lowest slope could not be observed. The first decay in all the graphs which describes cytotoxicity in actually electropermeabilized cells [3] always had similar slopes.

For DC-3F cells the MIC was 0.7 nM and the EC_{50} 1.6 nM. D/Blm40 cells were nearly as sensitive as the parental DC-3F cells when electropermeabilized. D/BlmI cells were once again only slightly resistant, ~3-fold (Table 1), with an EC_{50} of 4.5 nM. For D/BlmII and D/BlmIIR cells (data not shown) the MIC was 20 nM and the EC_{50} 47 nM, giving a resistance level of ~30-fold. D/BlmIR cells proved to be extremely resistant when electropermeabilized, with an MIC of 2.5 μM and an EC_{50} of 6.3 μM. Their resistance level, precisely determined at the EC_{50} level, could therefore be estimated to be 4000-fold. The same resistance level was obtained with D/BlmIR cells cultured in the absence of BLM for seven passages (data not shown).

Association of radiolabelled BLM with cell membranes

The association of CoBLM with the plasma membranes of the different cell lines is shown in Fig. 4. All the curves were best fitted by the sum of a linear non-saturable component (C) and a hyperbolic saturable component, defined by the parameters N and $K_{1/2}$. According to previous results [10], these parameters are assumed to be the number of plasma membrane BLM-binding sites per cell (N) and the half-saturating concentration of these sites ($K_{1/2}$). The values of C, N and $K_{1/2}$ are given in Table 2. No major variations of the half-saturating concentrations were detected among the different cell lines. In contrast, the number of membrane BLM-binding sites varied over a wide range, from 400,000 for D/BlmI cells to 20,000 for D/BlmIR cells. The non-specific component C was higher for the D/Blm40 cells and accounted for the high association level of CoBLM to the plasma membrane of these cells (Fig. 4). This increase might be due to the morphological differences of the D/Blm40 cells, which extend over the surface of the culture dish and present sharp extensions (data not shown).

Visualization of the BLM-binding proteins

Membrane proteins. For each cell line a membrane protein of ~250 kDa which was able to bind BLM was detected (Fig. 5A), in agreement with this group's previous results on DC-3F cells [10]. The intensity of the radioactive band correlated qualitatively with the number of membrane BLM-

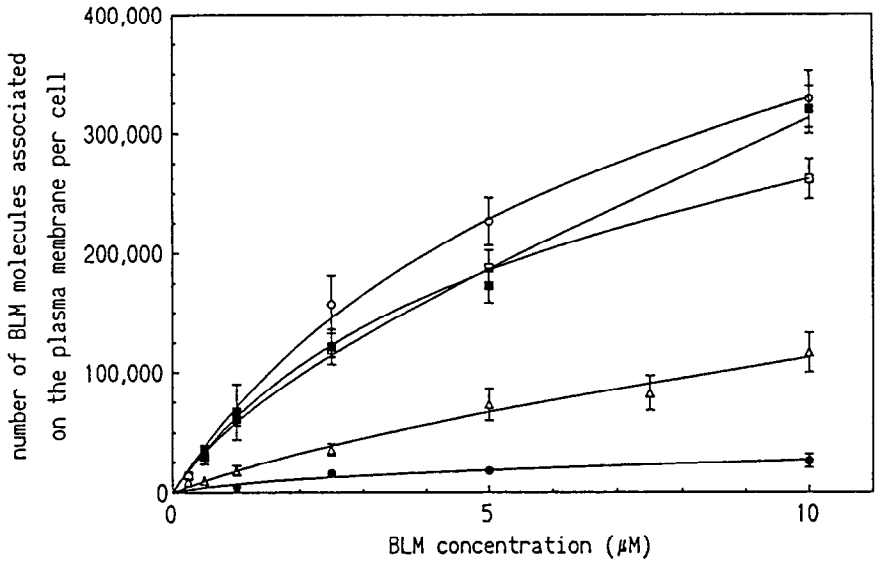

Fig. 4. Association of CoBLM on the plasma membranes of DC-3F cells and its related BLM-resistant cell lines. Cells in monolayers were incubated with CoBLM for 15 min at 4° and membranes were then isolated as described in Materials and Methods. Each point is the mean of at least three determinations. □, DC-3F cells; ■, D/Blm40 cells; ○, D/BlmI cells; ●, D/BlmIR cells; △, D/BlmII cells.

binding sites per cell determined by the association studies above (see Table 2).

Cytosolic proteins. In the cytosolic fractions (Fig. 5B) a protein of 14 kDa in the cytosol of the D/BlmIR cells, almost certainly the BRP, was visualized. No other cytosolic BLM-binding protein was detected by this method either in the D/BlmIR cells or in the other cells. However, by using a standard immunoblot technique with an antibody directed against the BRP, a protein of 150 kDa in the cytosol of the D/BlmII cells, which was assumed to be the BRP-βGal fusion protein (data not shown), could be labelled.

DISCUSSION

Many BLM-resistant cell lines have previously been isolated from mouse cells [18, 19], rat cells [20], hamster cells [21, 22] and human cells [23–26]. The resistance levels were often low, ranging from 3- to 20-fold, and only in three cases were the resulting resistance levels higher [19, 24, 25]. Several resistance mechanisms have been proposed: decrease in BLM accumulation within the cells [23–25]; elevated DNA repair activity [23, 24]; and increased inactivation of BLM [20, 22], resulting particularly from an increase in BLM hydrolase activity [25–27]. The hypothesis of membrane alteration has been postulated by several authors [18, 20, 21] but the implied mechanisms have never been analysed. Similarly, when a decrease in BLM accumulation has been shown [23–25] the mechanism responsible for this reduced uptake has not been elucidated.

By using DC-3F cells, this group has recently identified a membrane protein of 250 kDa able to bind BLM [10]. The authors wondered whether differences in the number of these binding sites, or in their affinities for BLM, could alter cell sensitivity

Table 2. Parameters of the association curves shown in Fig. 3, determined by the use of least square regression

Cell line	Number of binding sites per cell (N)	Half-saturating concentration $K_{1/2}$ (μM)	Non-saturable component (BLM molecules/min/cell) (C)
DC-3F	260,000 ± 70,000	3.7 ± 1.2	7200
D/BlmI	400,000 ± 30,000	5.2 ± 0.8	7200
D/BlmII	60,000 ± 16,000	4.6 ± 2.9	7200
D/Blm40	100,000 ± 15,000	1.9 ± 0.6	23,000
D/BlmIR	20,000 ± 3000	4.1 ± 1.6	1300

Number of membrane BLM-binding sites per cell (N), half-saturating concentration of these sites ($K_{1/2}$) and non-saturable component (C). Values are means ± SEM. The correlation coefficients r^2 were always greater than 0.97. For D/BlmIR and D/Blm40 cells supplementary points up to 25 μM BLM (not shown) were used to obtain the above parameters.

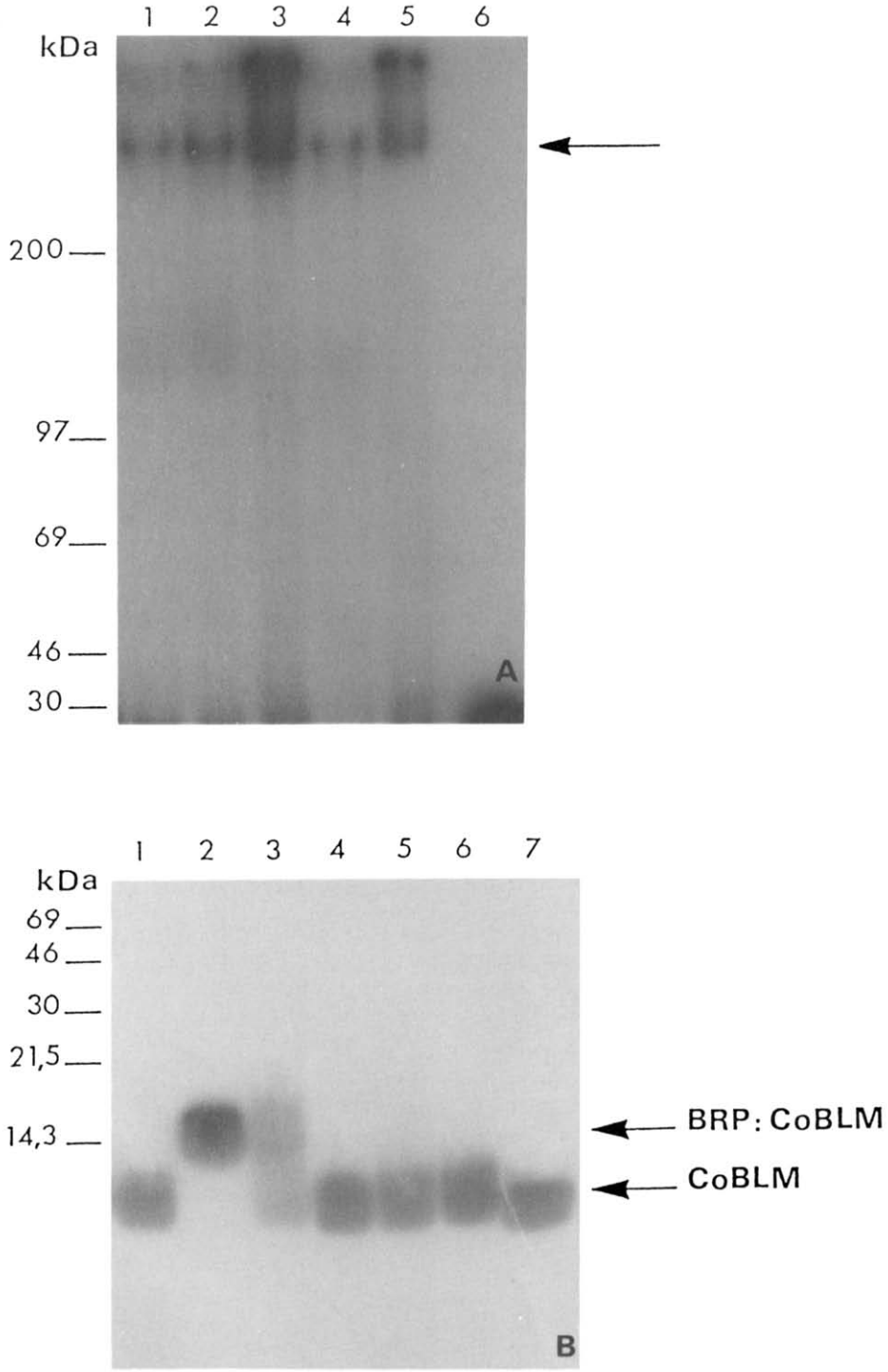

Fig. 5. Visualization of BLM-binding proteins in membrane extracts (A) or in cytosolic fractions (B). For each cell line, 125 μg of membrane proteins or 300 μg of cytosolic proteins pre-incubated with 3 μM CoBLM were loaded per well. (A) Lane 1, D/BlmII cells; lane 2, D/BlmIR cells; lane 3, D/BlmI cells; lane 4, D/Blm40 cells; lane 5, DC-3F cells; CoBLM alone was loaded as control in lane 6. (B) Lane 1, D/BlmII cells; lane 2, 25 μg purified BRP; lane 3, D/BlmIR cells; lane 4, D/BlmI cells; lane 5, D/Blm40 cells; lane 6, DC-3F cells; CoBLM alone was loaded as control in lane 7. Free CoBLM is located at the migration front. Retardation of CoBLM in lanes 2 and 3 is due to the presence of the BRP, a BLM-binding protein of 14 kDa.

to BLM. In order to clarify the role of this protein in BLM cytotoxicity BLM-resistant cells were prepared. Their global resistance was determined by measuring BLM cytotoxicity on intact cells in monolayers, and their internal resistance determined by measuring BLM cytotoxicity on electropermeabilized cells. Whatever the mechanism of resistance due to membrane-related limitations of drug influx might be, it was bypassed after cell electropermeabilization. Consequently the level of internal resistance could be evaluated directly. Thus, the resistance level strictly related to alterations of BLM influx across the plasma membrane could be derived from the difference between the global and internal resistance levels (Table 1).

In Fig. 2, BLM cytotoxicity upon electropermeabilized cells is plotted using a log–log scale, because in such coordinates, as shown previously [3], the SRCs of cells exposed to BLM became linear plots and their slopes described BLM influx within cells. Indeed, the SRCs of cells electropermeabilized either under optimal conditions (maximal influx) under sub-optimal conditions (intermediate influx), or non-electropermeabilized (basal, i.e. very low influx) were compared. The result, as evidenced in Fig. 1 of Ref. [3], was that the slope increased with the influx. Consequently, a log–log representation of the non-electropermeabilized cell SRCs allowed comparison of BLM influxes among the present mutant cells.

The first type of BLM-resistant cells were obtained by electrotransfection of the Sh *ble* gene (D/BlmI cells) or the Sh *ble*-βGal fusion gene (D/BlmII cells). The efficiency of the electrotransfection was 10^{-6}, which seemed very low, but could be explained by the very strong selection pressure imposed on the cells after electrotransfection. As the cytotoxicity curve of BLM had a plateau, a 4 day exposure to 15 μM BLM was chosen to ensure any false mutant that could have appeared with lower BLM concentration was eliminated. Subsequently, the result of the electrotransfection by the staining assay of the protein BRP-βGal (using X-Gal in D/BlmII cells), or by the detection of proteins BRP and BRP-βGal in the cytoplasms of D/BlmIR and D/BlmII cells, respectively (using gel electrophoresis), were checked. Since D/BlmIR cells are directly derived from D/BlmI cells by a long-term culture of these cells in the presence of 10 μM BLM, it could then be assumed that the D/BlmI cells had also incorporated the Sh *ble* gene.

The D/BlmI cells actually showed a slight 2-fold resistance whether electropermeabilized or not. The slope of their SRC in a log–log plot (-0.36 ± 0.02) is quite similar to that of DC-3F cells (-0.40 ± 0.06) (data not shown), which suggests a similar BLM influx in these cells. Moreover, no major differences in the specific association of BLM with cell membranes with respect to the parental DC-3F cells at an equivalent passage were detected, which proves that these cells had not undergone any membrane alteration and that the BRP was responsible for their resistance.

D/BlmII cells have a global resistance level of 50-fold with respect to their parental cells. After electropermeabilization they are 30-fold resistant, which suggests that the predominant, but not unique, mechanism of resistance is an internal one which can be related to the presence of the BRP-βGal protein detected in their cytoplasm. This internal BLM-trapping protein is most probably cytoplasmic because of its size (150 kDa), whereas the BRP has been shown to have a nuclear localization [19]. The BRP-βGal protein could trap every BLM molecule which slowly enters the cytoplasm of non-electropermeabilized cells until all the proteins are saturated, which would result in raising the BLM cytotoxicity threshold. This phenomenon would then account for part of the resistance of intact D/BlmII cells, depicted by the shoulder of the SRC. However, the difference between the global and internal resistance levels suggests that some membrane resistance occurred in these intact cells. Their SRC slope in a log–log scale (-0.125 ± 0.001) shows that the BLM influx was reduced within these cells (data not shown). This decrease in BLM uptake could be accounted for by the decrease in the number of BLM-binding sites detected, which could give rise to a decrease in BLM binding and further internalization.

According to already published data [3], the electropermeabilization of DC-3F cells in the presence of 0.7 nM BLM, the MIC on the DC-3F cells, results in the internalization of an average of 200 BLM molecules/cell, sufficient to kill the first cell. At 20 nM BLM, the MIC on the D/BlmII cells, 6000 molecules of BLM are internalized/cell. Therefore, in D/BlmII cells, among these 6000 BLM molecules, only 200 molecules remain efficient and are responsible for the beginning of the cytotoxic effect. The other 5800 molecules should be inactivated before reaching DNA, most probably because they would be trapped by the BRP-βGal protein. Since the BRP binds BLM almost irreversibly in a 1:1 molar ratio [12], it can be deduced that on average D/BlmII cells may possess at least 5800 BRP-βGal proteins in their cytoplasm.

The second type of BLM-resistant cells were obtained by continuous culture in the presence of BLM in the culture medium. D/Blm40 cells, isolated after 4 months of culture in the presence of increasing BLM concentrations in the culture medium, are 22-fold resistant to BLM. They are almost as sensitive as the DC-3F cells when electropermeabilized, which suggests that their resistance is linked to a reduced BLM influx. Indeed, their SRC slope in a log–log plot (-0.101 ± 0.002) (data not shown) is significantly lower than that of their parental sensitive cells, which indicates a decrease in BLM influx in these cells. As with D/BlmII cells, this decrease in BLM influx correlates with the decrease in the number of BLM-binding sites detected. It is worth noticing that D/Blm40 and D/BlmII cells have similar numbers of membrane BLM-binding sites and similar SRC slopes under non-electropermeabilization conditions. This reinforces the assumption that the decrease in the number of BLM-binding sites is directly responsible for the low slope of these two BLM-resistant cell SRC as compared with those of DC-3F and D/BlmI cells, and that it governs the resistance observed.

D/BlmIR cells, which derive from D/BlmI cells

by culture in the continuous presence of 10 μM BLM, present an unusually high global resistance level, and the EC_{50} level could not be determined. Their resistance level under electropermeabilization conditions, which could be determined precisely, is 4000-fold, proving that these cells have a very high internal resistance, presumably due to the high amount of BRP which was detected in their cytosolic fraction. As for D/BlmII cells, the average number of BRPs expressed per cell was calculated to be 600,000 molecules/cell. Because the entire SRC of these intact cells could not be determined it was impossible to predict whether membrane resistance accounts for part of the global resistance. Yet, a great decrease in the number of BLM-binding sites at the surface of these cells was observed. By reference to D/BlmII and D/Blm40 cells, it can be hypothesized that membrane resistance also occurs in D/BlmIR cells, possibly to a greater extent due to the very small number of binding sites. Thus, the very high resistance of D/BlmIR cells could be due to a large expression of the BRP combined with a dramatic decrease in their BLM-binding ability at the cell surface, resulting in a large decrease in BLM internalization. This particular procedure of mutant obtention selected the clone of D/BlmI cells, probably because they had a very high transitory expression of BRP just after electrotransfection. These cells could have lost their high expression during subsequent passages without BLM (D/BlmI cells), whereas they would have retained it when BLM pressure was maintained in the culture medium (D/BlmIR cells). Subsequently, continuous exposure to BLM may have induced the decrease in the number of membrane BLM-binding sites in the D/BlmIR cells by a mechanism similar to the D/Blm40 cell adaptation to stepwise increases in BLM concentration in the culture medium. A combination of internal and membrane resistance, similar to that presently described for BLM, has already been reported for some human cell lines resistant to methotrexate. In this case, cells concomitantly presented an elevated level of intracellular dihydrofolate reductase and defective methotrexate transport due to a decrease in its maximal rate V_{max} [28, 29].

In conclusion, from these results it appears that, besides the mechanisms of internal resistance, the number of BLM-binding sites at the cell surface plays an important role in cell sensitivity to the drug. The relationship between BLM sensitivity and the number of membrane BLM-binding sites is currently being investigated for cells with various origins to determine whether BLM association with these sites might be a key step in BLM internalization in the cells and in its cytotoxicity. This could provide new insights into understanding why BLM is an antitumour drug slightly more toxic with respect to tumour cells than normal cells. Moreover, other appropriate and effective uses of this drug can be anticipated, as, for example, in the case of the new antitumour treatment, electrochemotherapy [7], based on the *in vivo* electropermeabilization of tumour cells [8].

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