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Internalisation of the Bleomycin Molecules Responsible for Bleomycin Toxicity: A Receptor-mediated Endocytosis Mechanism

Géraldine Pron,† Nawel Mahrour,* Stéphane Orlowski,‡ Omar Tounekti,*§ Bruno Poddevin,*‖ Jean Belehradek Jr* and Lluis M. Mir*¶*

*Laboratoire de Physicochimie et Pharmacologie des Macromolecules Biologiques, UMR 1772 CNRS, Institut Gustave Roussy, 94805 Villejuif, France; and ‡SBPM/DBCM, CEA and URA 2096 CNRS, Centre d'Etudes de Saclay, 91191 Gif sur Yvette, France

ABSTRACT. Bleomycin (BLM) does not diffuse through the plasma membrane but nevertheless displays cytotoxic activity due to DNA break generation. The aim of the study was to describe the mechanism of BLM internalisation. We previously provided evidence for the existence of BLM-binding sites at the surface of DC-3F Chinese hamster fibroblasts, as well as of their involvement in BLM cytotoxicity on DC-3F cells and related BLM-resistant sublines. Here we report that A253 human cells and their BLM-resistant subline C-10E also possessed a membrane protein of *ca.* 250 kDa specifically binding BLM. Part of this C-10E cell resistance could be explained by a decrease in the number of BLM-binding sites exposed at the cell surface with respect to A253 cells. The comparison between A253 and DC-3F cells exposing a similar number of BLM-binding sites revealed that the faster the fluid phase endocytosis, the greater the cell sensitivity to BLM. Moreover, the experimental modification of endocytotic vesicle size showed that BLM cytotoxicity was directly correlated with the flux of plasma membrane area engulfed during endocytosis rather than with the fluid phase volume incorporated. Thus, BLM would be internalised by a receptor-mediated endocytosis mechanism which would first require BLM binding to its membrane receptor and then the transfer of the complex into intracellular endocytotic vesicles, followed by BLM entry into the cytosol, probably from a nonacidic compartment. BIOCHEM PHARMACOL **57**;1: 45–56, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. bleomycin; endocytosis; electropermeabilisation; receptor-mediated endocytosis; drug uptake; drug-binding proteins

Bleomycins are a family of water-soluble glycopeptidic antibiotics that were first isolated by Umezawa in 1966 [1]. BLM** cytotoxicity on mammalian cells depends on its ability to induce single- and double-strand DNA breaks. BLM is used in several standard cancer chemotherapies, including treatments of head-and-neck carcinomas and malignant lymphomas. *In vitro* electropermeabilisation of cells in the presence of BLM revealed the very high intrinsic cytotoxicity of the drug [2–5]. Indeed, as few as 500 BLM molecules introduced into the cytosol of a cell are sufficient to kill this cell [3]. Under optimal conditions, electropermeabilised cells are much more sensitive to BLM as compared with nonelectropermeabilised cells [2, 3]. BLM cytotoxicity on nonelectropermeabilised cells thus appears greatly limited by the fact that BLM crosses the plasma membrane very poorly. Indeed, we have shown that BLM is unable to enter the cells by passive diffusion through their plasma membrane [3]. Binding experiments on cultured cells under various conditions showed that the amount of BLM associated to the cells is always low and that BLM is never overconcentrated with respect to the external incubation medium [6, 7]. Moreover, equilibrium and kinetic measurements of BLM association to cells indicated a saturable binding of the drug, with an apparent affinity determined as 4.5 [8], 20 [6], or 32 μM [7]. However, the detailed mechanisms of BLM association with cells and of BLM internalisation have not yet been described.

Recently, we showed that DC-3F cells, a Chinese hamster pulmonary fibroblast cell line, possess a membrane protein of about 250 kDa capable of specifically binding BLM [9]. To study the involvement of this binding site in BLM cytotoxicity, we developed BLM-resistant cells from DC-3F cells and observed a relative decrease in the number of membrane BLM-binding sites, which could explain the

† Present address: ALCIMED Biotechnologies, 75017 Paris, France.
§ Present address: Institut National des Sciences Appliquées et de la Technologie, BP 676, 1080 Tunis Cedex, Tunisia.
‖ Present address: GENSET Inc., 75011 Paris, France.
¶ Corresponding author: Dr. Lluis M. Mir, Laboratoire de Physicochimie et Pharmacologie des Macromolécules Biologiques, UMR 1772 CNRS, Institut Gustave-Roussy, 39 rue Camille Desmoulins, 94805 Villejuif Cédex, France. Tel. 33 1 4211 4792; FAX 33 1 4211 5276; E-mail: luismir@igr.fr.

** *Abbreviations*: BLM, bleomycin; Co-BLM, cobalt-bleomycin; ^{57}Co-BLM, ^{57}cobalt-bleomycin; IC_{50}, bleomycin concentration reducing cell survival by 50%; LY, Lucifer Yellow; MEM, minimum essential medium; and SRC, survival response curve.

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resistance of certain mutant cells [10]. We then wondered whether such BLM receptors could be detected at the surface of other cells.

Here, we report studies performed on head-and-neck human carcinoma cells, namely the A253 cell line, which is very sensitive to BLM, and its BLM-resistant subline C-10E [11, 12]. Lazo and co-workers determined a 40-fold resistance for C-10E cells with respect to the parental sensitive A253 cells. They described an increased BLM metabolisation in the C-10E cells, possibly due to an increased BLM hydrolase activity, and a decreased BLM association to C-10E cells. Both changes could explain C-10E cell resistance [12]. We also analysed here the difference in sensitivity to BLM of the A253 and DC-3F cells in terms of the plasma membrane permeability barrier. The present study led us to establish correlations between cell sensitivity to BLM and both the amount of the 250 kDa membrane BLM-binding protein and the constitutive endocytotic activity of the cells.

MATERIALS AND METHODS

Chemicals

BLM was purchased from the Laboratoires Roger Bellon. It was stored at 1 mg/mL in 0.9% NaCl at −20°. LY dipotassium salt (Sigma Chemical Co.) was solubilised up to 50 mM with PBS without Ca^{2+} and Mg^{2+} (GIBCO BRL) and kept at 4°. All other reagents were obtained from Sigma Chemical Co.

Cells and Cell Culture

DC-3F cells, a Chinese hamster spontaneously transformed fibroblast [13], were maintained as monolayers in tissue culture flasks (Falcon) in a culture medium ("complete MEM") consisting of MEM supplemented with 8% foetal bovine serum (Eurobio), 100 units/mL of penicillin (Sarbach) and 125 μg/mL of streptomycin (Sarbach). 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 0.02% solution (GIBCO BRL).

A253 cells, a human head-and-neck carcinoma cell line [11], were cultivated as monolayers in McCoy 5A medium (GIBCO BRL) supplemented with 10% foetal bovine serum and antibiotics. A253 cells and their BLM-resistant subline C-10E were kindly provided by Professor John S. Lazo, Pittsburgh, PA. C-10E cells were obtained by mutagenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine followed by selection in the presence of BLM and culture in stepwise increasing BLM concentrations in the growth medium [12]. They were routinely maintained in the presence of 50 nM of BLM. The A253 population doubling time was approximately 24 hr, whereas that of C-10E was 36 hr.

BLM Cytotoxicity on Intact Cells

SRCs of cells exposed to BLM were obtained as follows: exponentially growing cells in monolayers were incubated for 3 hr at 37° in their usual culture medium containing various concentrations of BLM, then washed once, trypsinised, counted, and plated in triplicate (500 cells/dish for the DC-3F cells and 2000 cells/dish for the human cells) for the determination of the survival fraction by the measure of their relative cloning efficiency [10]. The absolute cloning efficiency of control DC-3F cells was about 70% after 5 days, whereas that of control A253 or C-10E cells ranged between 10 and 15% after 10 days of growth. Because of the lack of homogeneity of the human colonies, viable colonies were hand-counted under a light microscope.

Since 150 mM KCl appeared to be toxic for cells beyond 1 hr of incubation, we compared BLM cytotoxicity in KCl with that in MEM either for 1-hr incubations at various BLM concentrations or for 15-min incubations at 20 μM. BLM cytotoxicity in the complete MEM was similar to that observed in MEM without serum.

For the comparison between BLM cytotoxicity at 4° and at 37°, DC-3F cells were incubated with 20 μM BLM for various times and then treated as described above. For the 4° experiments, cells were cooled on ice for 15 min before incubation with BLM. To study the effect of weak amines on BLM cytotoxicity, DC-3F cells were preincubated for 1 hr at 37° with either 20 mM NH_4Cl or 100 μM chloroquine. Then, BLM was added to the medium for 3 hr and the cells were treated as above. In experiments involving medium acidification, conditions were similar to those used in diphtheria toxin uptake studies [14]. Briefly, cells were incubated with 20 μM BLM at 4° for 5 min. Then, medium acidification was obtained by addition of 50 mM HCl in the amounts necessary to reach pH 4.0. Cells were kept at this pH for 5 to 10 min, washed, and plated for colony efficiency determinations.

BLM Cytotoxicity on Electropermeabilised Cells

The BLM cytotoxicity on electropermeabilised cells was determined according to Poddevin *et al.* [3]. Briefly, 10^6 cells suspended in S-MEM (GIBCO BRL) supplemented with 0.5 mM $CaCl_2$ were mixed with various concentrations of BLM at room temperature and immediately subjected to electric pulses (8 square wave pulses of 1250 V/cm and 100 μs delivered at a frequency of 1 Hz by a PS-15 electropulsator from Jouan). Five minutes later, the cells were extensively diluted and the cytotoxicity was determined by the measurement of the relative cloning efficiency as above.

Association of Radiolabelled BLM with Cell Membranes

Radiolabelled BLM was prepared according to Poddevin *et al.* [15] by mixing BLM with ^{57}cobalt chloride (Amersham)

in a 3:1 molar ratio. Under these conditions, all the cobalt-57 is chelated by the BLM. The absence of free cobalt-57 was checked by gel filtration through P10 columns (Pharmacia). For the determination of the association of radiolabelled BLM with cell membranes [9], monolayers of cells in 60 mm-diameter Petri dishes (Falcon) were rinsed with Ca^{2+} and Mg^{2+} containing cold PBS at pH 7, and incubated at 4° for 15 min. PBS was then removed and cells were incubated at 4° for 15 min with 800 μL of ^{57}Co-BLM diluted in PBS to the suitable concentration. The cells were then washed 8 times with cold PBS. The radioactivity of the final washing medium was always counted to ensure the washing efficacy. To prepare crude membrane extracts, cells were incubated in the presence of 1 mL of lysis solution (200 mM Tris–HCl; 1 mM EDTA; 1 mM DL-dithiothreitol; 0.4 mM of phenylmethylsulfonyl fluoride; 7 μM pepstatin; 4 μM leupeptin) for 5 min at 4° and collected with a rubber policeman. The Petri dishes were rinsed with a second mL of lysis solution. After a 25-min incubation at 4°, the cells were homogenised using a Potter Dounce B (30 strokes). A first centrifugation (500 *g*, 15 min, 4°) left unbroken cells and nuclei in the pellet. Then the supernatant was centrifuged for one hour at 4° at 20,000 *g*. The final pellet, i.e. the cellular membranes, and the supernatant, i.e. the cytosolic fraction, were counted separately on an MR 252 Gamma Counter (Kontron).

The protein content of each pellet was determined by the method of Bradford using the Bio-Rad Protein Assay with immunoglobulin G as a standard (Bio-Rad S.A.). In each experiment, one of the dishes was incubated in BLM-free PBS, washed 8 times like the others, and then trypsinised in order to determine the number of cells per dish. Knowing the specific activity of the labelled ^{57}Co-BLM (maximum 17 Ci/mmole), we were then able to transform the radioactivity of each membrane fraction into the corresponding number of BLM molecules associated with the membranes of one cell. Experimental data were fitted using least square linear regression.

Electrophoretic Characterisation of BLM-binding Membrane Proteins

Crude membrane extracts were prepared from A253 and C-10E cells as described above, except that the ^{57}Co-BLM incubation step was omitted. Known amounts of membrane proteins, ranging from 50 to 150 μg, were incubated with 3 μM ^{57}Co-BLM for 1 hr at room temperature. For these experiments, we reversed the sequence of the two steps in the previous experiments, i.e. incubation with radiolabelled ^{57}Co-BLM and preparation of membrane extracts, in order to reduce the amount of radioactivity handled to a minimum. Solubilisation of membrane fractions was achieved by incubating the samples with 1% SDS for 1 hr at room temperature. Proteins were separated on a 4.5% polyacrylamide gel containing 0.1% SDS as previously described [9], and autoradiographies were performed using MP-Hyperfilms (Amersham).

Incorporation of Co-BLM into Entire Cells

Monolayers of DC-3F cells in 60-mm Petri dishes were preincubated in complete MEM or in 150 mM KCl for 15 min at 37° or at 4°. ^{57}Co-BLM was then added to a final concentration of 20 μM, with or without a 100-fold excess of nonradioactive Co-BLM, for 15 min at 37° or at 4°. Cells were then washed 8 times at 4° with the medium used during their preincubation (i.e. either complete MEM or 150 mM KCl), trypsinised, counted, and centrifuged. The amount of radioactivity associated with the cellular pellet in the absence of unlabelled BLM defined the amount of total BLM incorporation, and that associated with the cellular pellet in the presence of an excess of unlabelled BLM defined the amount of nonspecific BLM incorporation. The difference between these two values gave the specific incorporation of BLM.

Determination of Lucifer Yellow Incorporation into Cells

Cells in 6-well plates (Falcon) were incubated for various times with 1 mL of culture medium containing either 1 mM LY for incubations at 37° or 3 mM LY for incubations at 4°. LY uptake was also determined in DC-3F cells preincubated in 150 mM KCl for 15 min and then incubated in the presence of 1 mM LY in 150 mM KCl for 15 min at 37°. After the incubation with LY, cells were washed 3 times with PBS, trypsinised, and centrifuged. With parallel experiments, we determined that about 40% of the initial pool of cells was lost at the end of this procedure, so we corrected the initial number of cells by this factor. The cellular pellets (2 to 3×10^6 cells in 100 μL) were lysed with 100 μL of 10% SDS for 15 min at room temperature, then 22 μL of DNAse I at 1 mg/mL in 1 mM $MgCl_2$ (Sigma Chemical Co.) was added for an additional 15-min incubation at 37°. One hundred μL of 10% SDS was again added to achieve complete solubilisation and the final volume was adjusted to 1 mL with PBS. The incorporated fluorescence was measured using a 23/B Spectrofluorometer (Kontron), set at 423nm for excitation and 555nm for emission. The base line was obtained with LY-free lysates. The fluorescence of cells that were incubated with LY for less than 5 sec matched the base line, which confirmed the washing efficacy. The standard curves were obtained by adding specified concentrations of LY to LY-free cell lysates.

Fluorescence Microscopy

For fluorescence microscopy observations, DC-3F cell monolayers were incubated with 1 mM LY either in complete MEM or in 150 mM KCl for 15 min at 37°. They were washed at 4°, trypsinised and centrifuged as above, resuspended in 100 μL of PBS, kept on ice, and observed at

room temperature within the 5 min following their removal from the ice in order to avoid cell alterations due to light irradiation. Control cells were obtained by electropermeabilising DC-3F cells in the presence of 1 mM LY, under the conditions described above. Routine and preliminary experiments were performed using a Photomicroscope III (Zeiss). For confocal microscopy, LY was excited with the 488 nm line from an argon ion laser and detected through a 530/30 nm bandpass filter with the ACAS 570 Meridian (Okemos).

RESULTS

BLM Cytotoxicity on either Intact or Electropermeabilised Human Cells

The SRCs of the two human cell lines, A253 and C-10E, and of the Chinese hamster cells DC-3F reported for comparison, are shown in Fig. 1A. They are linear beyond the minimal inhibitory BLM concentration when plotted in double logarithmic coordinates (logarithm of BLM concentration versus logarithm of cell survival). This is linked to the fact that in the standard representation (logarithm of drug concentration versus cell survival) the SRC of cultured cells exposed to BLM usually displays a continuous upward concave curvature [3]. A253 cells were very sensitive to BLM, since the BLM concentration inducing 50% lethality (IC_{50}) was 40–50 nM under our experimental conditions (3 hr exposure to the drug). C-10E cells were about 6-fold resistant to BLM, with an IC_{50} of 250–300 nM. This resistance index is lower than the 40-fold resistance previously determined by Lazo and coworkers for the same cells by a growth inhibition test with a 4-day exposure to BLM [12]. The IC_{50} of DC-3F cells was 2 μM, as previously reported [10]. The minimal inhibitory BLM concentrations were about 20 nM for A253 cells, 80 nM for C-10E cells, and 400 nM for DC-3F cells. As previously shown [3, 10], the slopes of the SRCs, linearised in double logarithmic plots, permit a direct comparison of BLM net influxes into different cell lines. For the three SRCs, least square regressions gave the following slope values: -0.98 ± 0.10 for A253 cells, -0.60 ± 0.05 for C-10E cells, and -0.46 ± 0.06 for DC-3F cells.

When electropermeabilised in the presence of BLM, the two human cell lines exhibited virtually the same minimal inhibitory BLM concentration (about 1 nM) and the same slope (-1.15 ± 0.15) in a double logarithmic plot (Fig. 1B). Thus, after electropermeabilisation, C-10E cells appeared to be as sensitive as their parental A253 cells, both cell lines having an IC_{50} of about 2 nM. Surprisingly, DC-3F cells were also as sensitive as the two human cell lines, with an IC_{50} of 1.5 nM. Therefore, the differences in BLM sensitivities among these three cell lines seem to be due to differences in BLM internalisation, i.e. to differences in the BLM crossing of the plasma membrane.

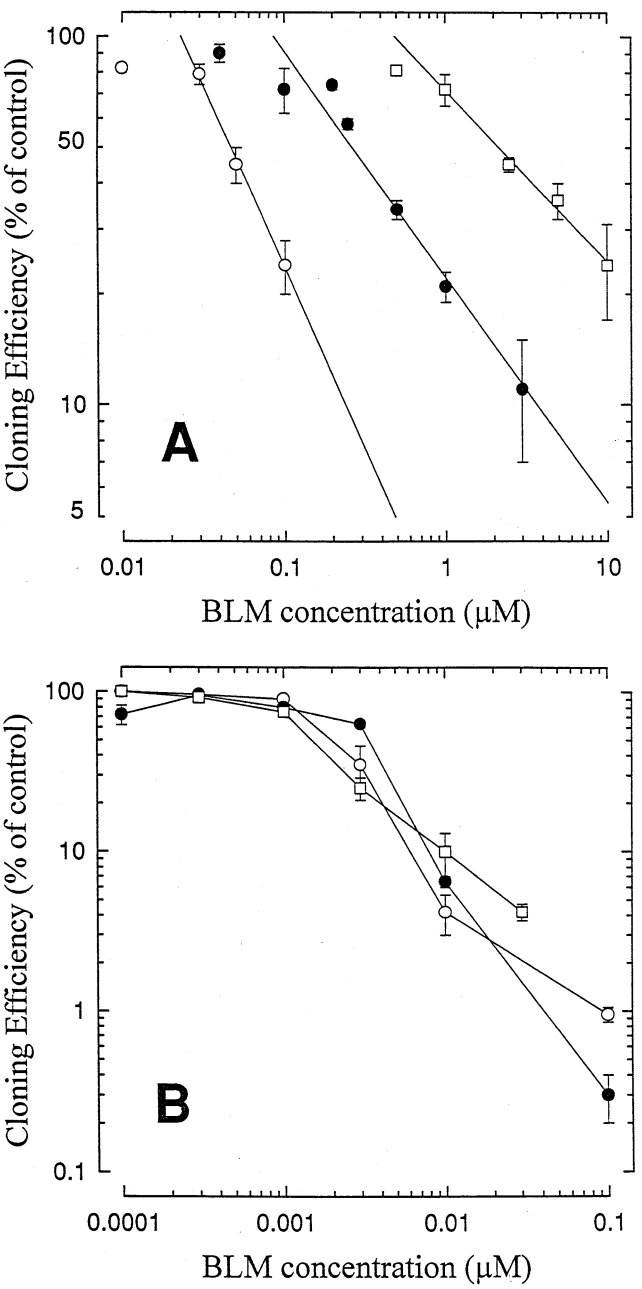

FIG. 1. (A) SRCs of intact cells incubated with BLM for 3 hr at 37°. Means ± SD of three independent sets of triplicates are represented. Hollow circles, A253 cells; filled circles, C-10E cells; hollow squares, DC-3F cells. Straight lines are regressions of the experimental points for survival levels <80%. (B) SRCs of cells electropermeabilised in the presence of BLM as described in Materials and Methods. Means ± SD of at least 2 independent sets of triplicates are represented. Hollow circles, A253 cells; filled circles, C-10E cells; hollow squares, DC-3F cells.

Association of Radiolabelled BLM with the Plasma Membranes of Human Cells

We used BLM radiolabelled with ^{57}Co [15] because the complex is known to be very stable, because it is noncytotoxic, thereby allowing measurements of BLM association with living cells at any time and any external concentra-

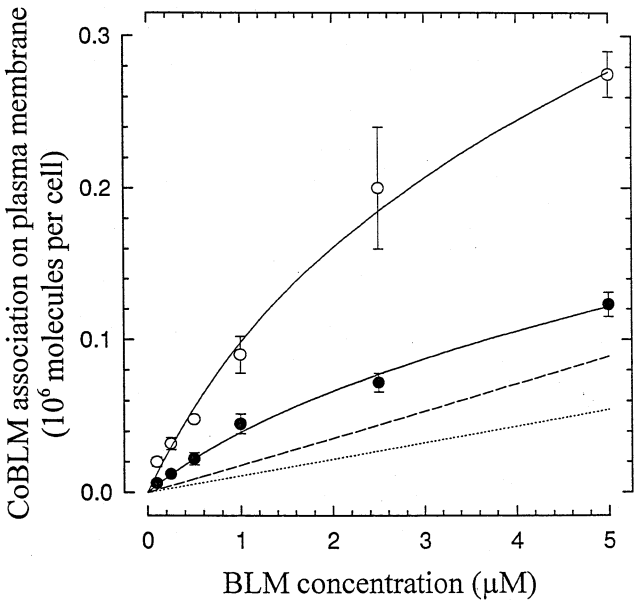

FIG. 2. Association of radiolabelled ^{57}Co-BLM with the plasma membranes of A253 cells (hollow circles) and C-10E cells (filled circles). Cells were incubated with ^{57}Co-BLM for 15 min at 4° and washed. Crude membrane extracts were prepared as described in Materials and Methods. Means ± SD of three independent experiments (each point in triplicate) are represented. Dashed and dotted lines represent the nonspecific component of the association with the plasma membranes prepared from the A253 cells and the C-10E cells, respectively. For each cell line, curves represent the sum of this linear nonspecific component and of a hyperbolic saturable component, corresponding to the presence of 280,000 and 100,000 BLM-binding sites per cell for the A253 cells and the C-10E cells, respectively, and to a half-saturating BLM concentration of 2.5 μM for both cell lines.

tion, and because Co-BLM and unchelated BLM compete for ^{57}Co-BLM binding on the living cell surface.*

Since the cells were exposed to Co-BLM at 4°, in order to largely block membrane traffic, and then washed extensively before membrane preparation, only the plasma membranes were expected to be labelled. For both human cell lines, we observed an association pattern typical of the superimposition of a specific Co-BLM binding component and a nonspecific component (Fig. 2). The fits of the specific Co-BLM binding components gave for A253 cells: 300,000 ± 40,000 binding sites per cell with a half-saturating BLM concentration of 2.7 ± 0.7 μM; and for C-10E cells: 97,000 ± 14,000 binding sites with a half-saturating BLM concentration of 2.3 ± 0.7 μM (Fig. 2 and data not shown). Thus, at each labelled BLM concentration, the membranes of C-10E cells specifically associated about three times less Co-BLM than did the membranes of A253 cells. We have previously reported that the specific component of Co-BLM association with plasma membranes of DC-3F cells was characterised by 260,000 ± 70,000 binding sites per cell and a half-saturating BLM concentration of 3.7 ± 1.2 μM [10]. Thus, the membranes of DC-3F cells specifically associated roughly as much labelled BLM as did the membranes of A253 cells.

* B. Poddevin, G. LeBourhis, S. Orlowski and L.M. Mir, manuscript in preparation.

Electrophoretic Characterisation of BLM-binding Membrane Proteins from Human Cells

Using a semi-denaturing gel electrophoresis procedure [9], we succeeded in labelling a BLM-binding protein of about 250 kDa in both human cell membrane extracts (Fig. 3). An excess of nonradioactive Co-BLM was able to decrease the labelling of this band, indicating the specificity of Co-BLM binding (data not shown; see [9] for similar results on the DC-3F cells). The comparison of lanes 2 and 7 shows that the radioactive band intensity was roughly five times lower in the C-10E cell extract than in the A253 cell extract. This difference is of the same order of magnitude as the decrease in the number of BLM-binding sites observed above.

Lucifer Yellow Incorporation into A253 and DC-3F Cells

The relationship between BLM association to cell membranes and BLM cytotoxicity prompted us to investigate

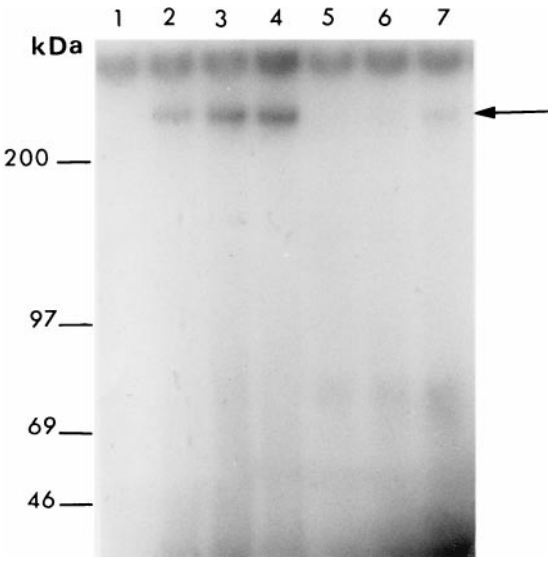

FIG. 3. Detection of a BLM-binding protein among the membrane proteins of the human cells. Crude membrane extracts were isolated, incubated with ^{57}Co-BLM for 1 hr, solubilised with 1% SDS, and separated on a semi-denaturing polyacrylamide gel as described in Materials and Methods. Lanes 2, 3, and 4: membrane proteins from A253 cells (50, 100, and 150 μg, respectively); lanes 5, 6, and 7: membrane proteins from C-10E cells (50, 100, and 150 μg, respectively). Co-BLM alone was loaded as control in lane 1. It was located in the migration front (not visible on the autoradiography presented here). Some residual radioactivity, due to the presence of Co-BLM alone, is seen at the bottom of the wells. The arrow indicates the position of the radioactive band detected around 250 kDa. The position of the molecular mass markers was revealed by Coomassie blue staining and reported on the figure.

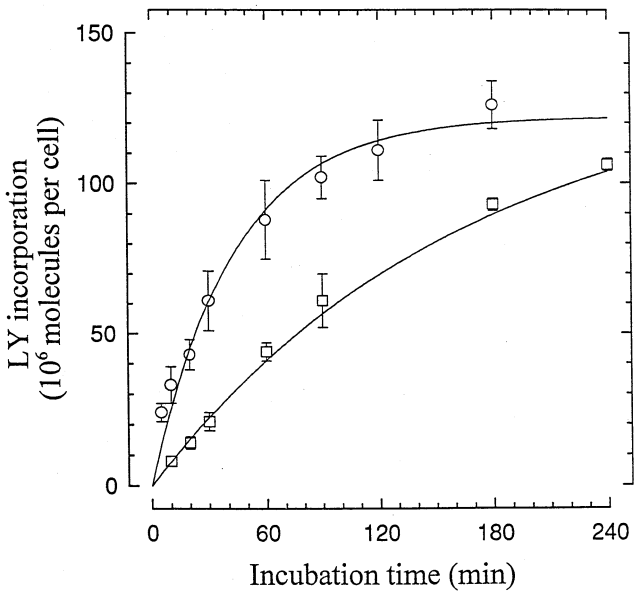

FIG. 4. Time-dependence of LY uptake in A253 cells (circles) and DC-3F cells (squares). Cells were incubated with 1mM of LY at 37° in their own culture medium, washed, trypsinised, lysed with SDS, and the amount of LY incorporated per cell was determined by fluorescence (see Materials and Methods). Experiments with A253 cells were repeated twice and those with DC-3F cells were repeated three times.

the physiological state of the treated cells from the point of view of their endocytotic activity. LY is a usual probe for fluid phase endocytosis [16]. LY incorporation into both DC-3F and A253 cells reached a plateau for prolonged incubation times (Fig. 4). This plateau value, which corresponds to a balance between LY influx (endocytosis) and LY efflux (exocytosis), was similar in the two cell lines. In contrast, the initial slope of the LY incorporation curve, which reflects the endocytotic influx rate of the cells, was three times higher for A253 than for DC-3F cells. The C10-E cells also presented an initial slope higher than that of the DC-3F cells (data not shown).

Effects of Temperature, NH_4Cl, or Chloroquine on BLM Cytotoxicity

Further experiments designed to confirm the involvement of endocytosis in BLM cytotoxicity were performed using the DC-3F cells. Indeed, their sensitivity to BLM can be much more precisely determined than that of the A253 and C10-E cells because of the higher absolute cloning efficiency of the DC-3F cells (see Materials and Methods).

It is well known that temperature changes alter the endocytotic activity of cells [17]. Thus, we tested the influence of temperature on BLM cytotoxicity. We chose a BLM concentration as high as 20 μM in order to allow the determination of BLM lethal effects after short incubation times of the cells in the presence of BLM. When DC-3F cells were incubated at 37° in the presence of BLM for increasing times, cell survival first rapidly decreased for short incubations and then decreased more progressively for longer incubations. In contrast, when the cells were incubated at 4°, cell survival remained nearly constant for incubation times ranging between 15 and 90 min (data not shown).

Chloroquine and ammonium chloride are known to inhibit the acidification of intracellular vesicles such as lysosomes and endosomes, and consequently to perturb the regular pathway of receptor-mediated endocytosis [18]. Control experiments performed in the absence of BLM showed that the incubation of DC-3F cells with 20 mM NH_4Cl or 100 μM chloroquine had no effect on their survival. The SRCs determined for DC-3F cells in the presence of either of the two weak amines showed that chloroquine had a slight protective effect for the low concentrations of BLM (<5 μM), whereas NH_4Cl increased BLM cytotoxicity only for higher BLM concentrations (>5 μM) (Fig. 5). Interestingly, in both cases, a

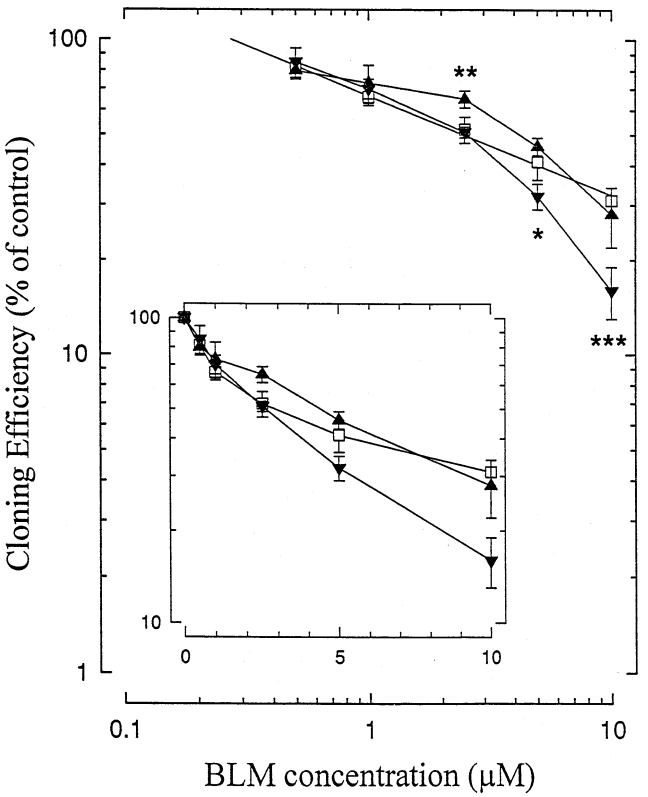

FIG. 5. Influence of NH_4Cl and chloroquine on BLM cytotoxicity. Inset: same curves in semilogarithmic coordinates. DC-3F cells were preincubated for 1 hr with either 20 mM of NH_4Cl (up triangles) or 100 μM of chloroquine (down triangles) or neither of these (hollow squares), before the addition of BLM for 3 hr at 37°. Cloning efficiencies were normalised with respect to the absolute cloning efficiency of cells incubated with the same concentration of the amines in the absence of BLM. Means ± SD of two independent sets of triplicates are represented. Statistical significances between the experimental points are indicated, according to Student's *t*-test, as: * for $P < 0.05$, ** for $P < 0.01$, and * for $P < 0.001$.**

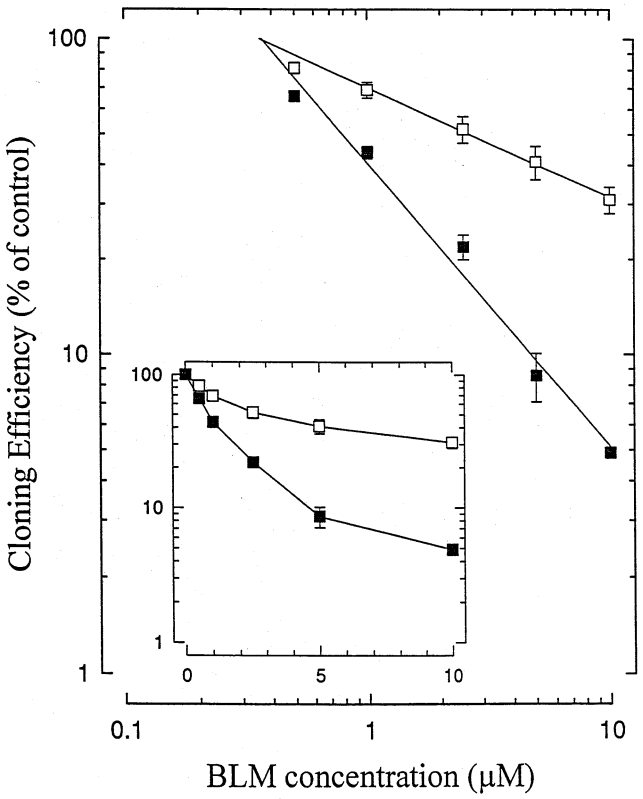

FIG. 6. SRCs of DC-3F cells exposed to various concentrations of BLM for 1 hr at 37° in complete MEM (hollow squares) or in 150 mM of KCl (filled squares). Inset: same curves in semilogarithmic coordinates. Points are means ± SD of 2 independent sets of triplicates.

double logarithmic plot for the SRC lost the linearity characterising the control SRC of the cells exposed to BLM. This corresponds to the fact that, in the usual semilogarithmic coordinates, the SRCs in the presence of a weak amine display an upward concave curvature much less marked than the control SRC (inset of Fig. 5).

Effects of High External K^+ Concentrations on BLM Cytotoxicity and on BLM-binding Sites

Potassium ions had already been reported to stimulate endocytosis [19, 20]. BLM cytotoxicity on DC-3F cells incubated for 1 hr at 37° appeared to be greater in 150 mM KCl than in complete MEM, with an IC_{50} decreasing from 2.5 to 0.8 μM. The double logarithmic representations of the SRCs are both linear, with a slope in 150 mM KCl (-0.89 ± 0.07) much higher than that found in the complete MEM (-0.34 ± 0.03), whereas the minimal cytotoxic BLM concentration, 300 nM, was identical for the two conditions (Fig. 6). In the usual semilogarithmic coordinates, the SRC in 150 mM KCl displayed an upward concave curvature, as in complete MEM (inset of Fig. 6).

The association of Co-BLM with the membranes of DC-3F cells incubated either in complete MEM or in 150 mM KCl (at 4°) showed that cells presented the same number of BLM-binding sites at their cell surface in these two different media, with identical half-saturating BLM concentrations (data not shown).

Effect of High External K^+ Concentration on Endocytotic Activity of Cells

In order to understand the effects of potassium ions on BLM cytotoxicity, we observed, using confocal fluorescence microscopy, living DC-3F cells incubated in the presence of LY. As shown in Fig. 7, LY fluorescence distribution was very different depending on whether cells were incubated (at 37°) with LY in complete MEM or in 150 mM KCl. In MEM, the fluorescence was concentrated in large intracellular vesicles, the number of which varied from 10 to 20 per cell, with an average radius of approximately 1 μm (Fig. 7A). After incubation in 150 mM KCl, LY fluorescence revealed a few large vesicles as well as the presence of a large number of small intracellular dots. (Fig. 7B). Similar fluorescence distributions were observed in the case of the

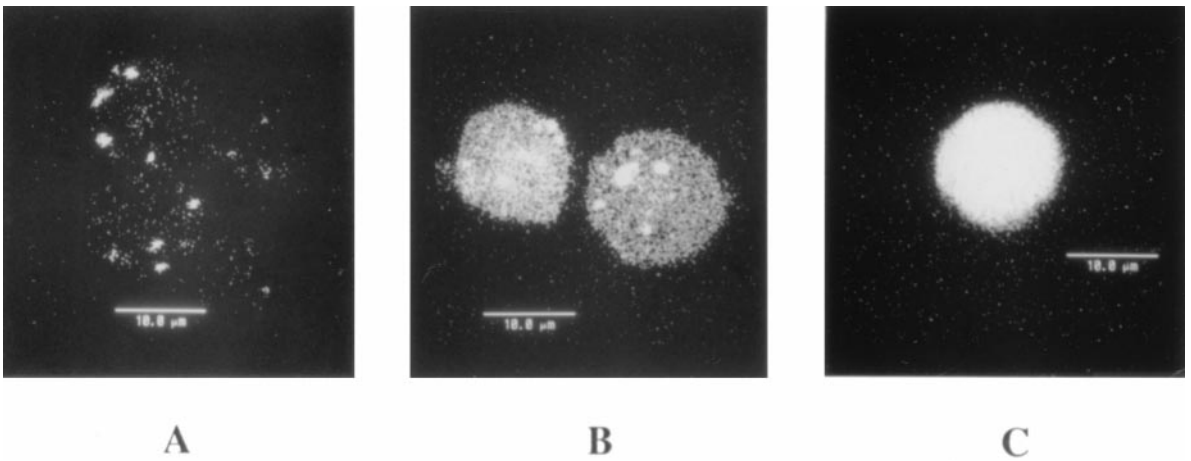

FIG. 7. Confocal microscopy pictures of LY fluorescence distribution in DC-3F cells incubated for 15 min with 1 mM of LY either in complete MEM (A) or in 150 mM KCl (B) or electropermeabilised in the presence of 1 mM of LY in complete MEM (C). The sections correspond to fields above or below the cell nucleus.

TABLE 1. Effects of various incubation conditions on LY uptake, Co-BLM incorporation, and BLM cytotoxicity in DC-3F cells

Incubation medium	LY incorporation (10^6 molecules/cell)	Nonspecific Co-BLM association (10^6 molecules/cell)	Specific Co-BLM association (10^6 molecules/cell)	BLM cytotoxicity (relative survival of BLM-treated cells)
Complete MEM/4°	2.5 ± 0.8	0.045 ± 0.015	0.115 ± 0.055	79% ± 12%
Complete MEM/37°	8.1 ± 0.6	0.120 ± 0.025	0.530 ± 0.065	58% ± 5%
KC 150 mM/37°	10.1 ± 3.2	0.150 ± 0.075	7.2 ± 1.0	13% ± 1%

The external concentrations were 20 μM for both Co-BLM and BLM, and 1 mM for LY. Co-BLM and LY incorporation are expressed as the number of molecules incorporated per cell for a 15-min incubation. BLM cytotoxicity is reported as the relative cloning efficiency of cells exposed to BLM for 15 min. Values are means ± SD of at least three independent determinations.

human cells (data not shown). The punctiform nature of the distribution in cells incubated in 150 mM KCl was confirmed by comparing it with the image of LY incorporated into cells electropermeabilised in the usual medium. Indeed, we observed in this case a diffuse and continuous fluorescence (Fig. 7C) as expected, since LY has a direct access from the external medium to the cytoplasm when cells are electropermeabilised [21]. This homogeneous distribution of LY fluorescence was unambiguously different from that detected in KCl-incubated cells, and we concluded that after incubation of cells in 150 mM of KCl, LY fluorescence was mainly localised in many very small intracellular vesicles. Because the smaller the vesicles, the greater the surface-to-volume ratio, these observations indicate that high external K^+ concentrations increase the total membrane area internalised much more dramatically than the total medium volume incorporated. Thus, variations in the external KCl concentration could make it possible to distinguish between membrane-bound receptor-mediated endocytosis and fluid phase endocytosis, which is only linked to the volume internalised into the cell.

Relationship between Endocytosis, BLM Association, and BLM Cytotoxicity under Various Incubation Conditions

For the analysis of these relationships, we considered separately 1) the specific component of ^{57}Co-BLM association with DC-3F cells (i.e. the radioactivity that can be displaced by an excess of nonradioactive BLM) and 2) the nonspecific component (unaltered by an excess of nonradioactive BLM). We tried to correlate the data concerning each of these components with either the characteristics of the fluid phase endocytotic activity of the cells (evaluated by LY incorporation) and the BLM cytotoxic effect on these cells. When the temperature of the incubation medium was increased from 4° to 37°, both the specific and nonspecific components of the Co-BLM association, as well as the LY uptake, increased by a similar factor of about 3–4 (Table 1). On the contrary, BLM cytotoxicity moderately but significantly increased (Table 1). When we compared the cytotoxicity and cell association of BLM at 37° in either 150 mM of KCl or complete MEM, BLM cytotoxicity was largely enhanced in KCl, as shown in Fig. 6 and in Table 1, and concomitantly, the amount of specifically incorporated Co-BLM was also greatly increased (more than 12 times). Conversely, the nonspecific association of Co-BLM and the LY uptake were not significantly different between the two media (Table 1).

DISCUSSION

Characterisation of the Human Cell Lines A253 and C-10E

After electropermeabilisation, i.e. under conditions for which BLM cytosolic entry is not restricted, the resistant C-10E cells are as sensitive to BLM as their parental line A253 (Fig. 1B). Thus, the main resistance mechanism of C-10E cells is likely linked to a decrease in the plasma membrane crossing. We had previously shown that the slopes of the SRCs linearised in double logarithmic plots allowed a direct comparison of BLM net influxes into different cell lines [3, 10]. The observation that the slope of such cytotoxicity plots of BLM on intact C-10E cells is lower than that of their parental A253 cells (Fig. 1A) confirms that BLM influx is reduced in the resistant subline.

This result seems to exclude any internal resistance mechanism in the C-10E cells, such as an increase in DNA repair or in intracellular BLM metabolisation as described for other BLM-resistant cell lines derived from A253 cells [22]. In particular, we can rule out the presence of BLM hydrolase in the cytosol, where it would have degraded the BLM molecules internalised after cell electropermeabilisation, resulting in a decrease in BLM cytotoxicity. Since Morris *et al.* previously reported an increase in BLM metabolisation, possibly due to BLM hydrolase, in cell homogenates from C-10E cells [12], our results indicate that BLM hydrolase might be confined to internal vesicles.

We have shown here that both human A253 and C-10E cells possess a membrane protein that is able to specifically bind BLM (Figs. 2 and 3), with a half-saturating BLM concentration and an approximate molecular mass similar to those of the protein found in DC-3F Chinese hamster cells [9, 10]. We have also found that C-10E cell membranes possess three to five times fewer BLM-binding sites than those of their parental cells (Fig. 2). This is in good agreement with previous observations by Morris *et al.*

showing that C-10E entire cells associated three times less BLM A2 than did the A253 cells [12]. Thus, the whole of our data imply that the resistance of C-10E cells, related to the decreased BLM influx in these cells, seemed to be due to a decreased number of membrane BLM-binding sites. Therefore, these sites would be instrumental in the internalisation of the BLM molecules responsible for BLM cytotoxicity, as in the case of the DC-3F cells and their related BLM-resistant mutant cells [10].

Comparison between Human and Hamster Sensitive Cells

The membrane BLM-binding sites appear to be a common protein in cells from various origins, reinforcing our conclusion that they play a major role in determining the sensitivity of cells to BLM [10]. However, we found that A253 cells are about 40-fold more sensitive to BLM than DC-3F cells at the IC_{50} level (Fig. 1A). The observation that both cell lines, when electropermeabilised, are similarly sensitive to BLM (Fig. 1B) suggests a higher BLM influx into intact A253 cells than into intact DC-3F cells. This is in agreement with the fact that the slope of the linearised SRC for A253 cells is higher than that for DC-3F cells. However, these two cell lines have similar numbers of BLM-binding sites on their surface and similar half-saturating BLM concentrations for these sites. Therefore, the higher BLM influx into A253 cells cannot be accounted for by an increase in their ability to bind BLM at their surface.

BLM influx into cells is also obviously dependent on the speed at which every BLM molecule is internalised. Since it had already been reported that the BLM internalisation pathway could involve intracellular vesicles from the endocytotic apparatus [23], we used LY as a fluid phase endocytosis marker and found that the initial rate of LY uptake per cell was about three times higher for human than for DC-3F cells (Fig. 4). We concluded that the fluid phase endocytotic rate of A253 cells is three times faster than that of DC-3F cells. Consequently, we investigated the relationship between BLM cytotoxicity and fluid phase endocytosis by varying experimental conditions known to modify the general endocytotic machinery of the cells.

Effects of Temperature, Weak Bases, and Acidification on BLM Cytotoxicity

BLM cytotoxicity was almost independent of the incubation time with cells at 4°, although BLM cytotoxicity increased continuously with the incubation time at 37°. Experiments performed with LY confirmed that endocytosis was greatly reduced at 4° when compared with measurements at 37°. This suggests that endocytosis has to be functional to obtain BLM internalisation and the cytotoxic effects of BLM. At 4°, BLM would be less toxic because BLM internalisation would be blocked during the incubation period at this temperature, concomitantly with the endocytosis inhibition.

Chloroquine and ammonium chloride were able to alter BLM cytotoxicity on DC-3F cells, in particular with a disappearance of the upward concave curvature typical of SRC for cells exposed to BLM (Fig. 5). This confirms that intracellular endocytotic compartments would seem to be involved in the internalisation pathway of cytotoxic BLM molecules. When considering the survival levels of DC-3F cells exposed to BLM at either <5 μM or >5 μM, we observed that the two weak amines had unexpected opposite effects. Lazo *et al.* had already reported the sensitising effect of NH_4Cl on BLM cytotoxicity [23]. Dean *et al.* showed that the major effect of chloroquine inside the cells could be the inhibition of intervesicular fusions, some of which are essential to constitutive endocytosis [18]. Thus, there is no contradiction between the slight protective effect of chloroquine and the more pronounced opposite action of NH_4Cl that relies on the alkalinisation of the acidic vesicles.

We transitorily acidified the external medium (pH 4.0) for 5–10 minutes after cell incubation in the presence of BLM to determine if an acidification like that found in some internal vesicles could be responsible for BLM translocation across the membrane, as in the case of diphteria toxin [14]. The experiment was performed at 4° to block the endocytosis and to ensure that any increase in cytotoxicity was due to an increase in the direct transfer of plasma membrane-bound BLM across the plasma membrane. BLM cytotoxicity was not affected by this medium acidification (data not shown). Therefore, BLM translocation into cytosol probably involves nonacidic intracellular compartments.

Effects of High External K^+ Concentrations Allowing to Distinguish between Fluid Phase Endocytosis and Membrane-dependent Internalisation

Nonspecific and specific Co-BLM cellular associations were increased from 4 to 37° by a factor of about 3 and 4 respectively, concomitantly with an increased BLM cytotoxicity, whereas LY incorporation was increased by a factor of about 3 (for 15-min incubations, see Table 1). In contrast to these moderate effects of temperature that do not allow one to detect differential effects on nonspecific and specific Co-BLM cellular associations, the presence in the incubation medium of high K^+ concentrations does make it possible to distinguish the cytotoxic efficacies of the two types of associated BLM. On the one hand, both specific incorporation of Co-BLM and BLM cytotoxicity were greatly enhanced by incubation in 150 mM K^+ as compared with incubation in the usual culture medium. On the other hand, neither the nonspecific incorporation of Co-BLM nor the LY uptake were modified by exposure of DC-3F cells to 150 mM of K^+. Moreover, the number of BLM molecules nonspecifically associated to each DC-3F cell and the number of LY molecules incorporated are roughly proportional to their extracellular concentrations in the incubation medium (Table 1). Thus, the effects of experimental variations of temperature as well as of K^+

concentration strongly suggest that nonspecific BLM incorporation into the cells corresponds to the fluid phase endocytosis of BLM.

BLM cytotoxicity appears to be correlated with the specific Co-BLM cellular association due to the BLM-binding sites on plasma membrane. The double logarithmic representations of the SRC of DC-3F cells exposed to BLM in the presence or absence of K^+ ions show a marked difference between their slopes (Fig. 6), suggesting that the increase in BLM cytotoxicity caused by K^+ ions might be due to an increase in BLM influx into the cells. However, this increased BLM influx cannot be accounted for by an increase in BLM binding at the cell surface in 150 mM K^+ (at 4°), since no difference either in the number of binding sites or in their affinities for BLM was detected under these conditions. Moreover, the increased BLM influx cannot be accounted for by an increase in the size of the volumes internalised by fluid phase endocytosis. Therefore, we studied the effect of K^+ ions on endocytosis by observing the LY fluorescence distribution inside DC-3F cells using conventional as well as confocal fluorescence microscopy. It appeared that, in the usual culture medium, the endocytotic vesicles were significantly larger in diameter and fewer in number than in 150 mM K^+ (Fig. 7). Since the total fluid phase endocytosed volume seems roughly the same in the two cases (Table 1), the greater the number of vesicles after cell incubation in the K^+ medium, the larger the total surface of membrane internalised. According to our microscopic observations, it seems that there are roughly 1000 times more intracellular vesicles in cells incubated in 150 mM of K^+ than in MEM. Since the total volume of these vesicles remains constant, the average diameter of each vesicle is roughly ten times smaller than the vesicle diameter observed in MEM. Therefore, the membrane area of each vesicle is 100 times smaller in 150 mM of K^+ but the total vesicle membrane area is 10 times larger in 150 mM of K^+ than in MEM. If we assume that the nonspecific incorporation of BLM is directly proportional to the total endocytosed volume and that the specific incorporation of BLM is directly proportional to the total endocytosed surface of plasma membrane, it is now understandable how 150 mM K^+ can increase roughly 10-fold the specific uptake of BLM, and hence BLM cytotoxicity, while keeping virtually constant the nonspecific incorporation of BLM (Table 1). This provides strong experimental evidence for the hypothesis that the BLM molecules responsible for the lethal effects of this drug on cells are internalised by means of plasma membrane engulfment during the endocytotic activity of the cell following BLM binding on specific proteins, which can be considered as "receptors". This defines the BLM uptake pathway relevant for its cytotoxicity as a mechanism of receptor-mediated endocytosis.

Proposed Mechanism of BLM Internalisation

In conclusion, we propose that the BLM molecules responsible for cytotoxicity first bind to the BLM-binding sites at the cell surface, and that further internalisation occurs when these complexes are endowed in intracellular vesicles, taking advantage of the constitutive endocytosis of the cells that leads to a permanent flux of membrane from cell surface into the cell and vice versa.

Receptor-bound, potentially cytotoxic BLM molecules have to escape the endocytotic pathway before the recycling of the vesicle at the cell surface or its fusion with lysosomes, where they would be degraded. The mechanism by which the BLM molecule transported by its binding protein will be translocated from an intracellular vesicle into the cytosol is still unknown. Our experiments only indicate that this step probably involves a nonacidic compartment. The mechanism of internalisation of the BLM molecules responsible for the cytotoxicity of this drug shares some features with those of other toxins, in particular ricin, for which internalisation by receptor-mediated endocytosis is well documented [24–26]. The B chain in the entire ricin binds to any galactose residue exposed at the cell surface, i.e. about 1 to 2×10^7 binding sites per cell [26], whereas the catalytic A chain of ricin binds to the mannose-specific receptor present at the cell surface (1.5 to 2.0×10^5 sites per cell) of certain cells such as the macrophages and the Kupffer cells of the liver. The ricin molecules internalised through this specific receptor are the most cytotoxic [26]. Since BLM can specifically bind a specific membrane protein, the BLM uptake mechanism appears to resemble the second ricin uptake pathway. The actual nature of the proteinic BLM receptor and its exact physiological role remain to be identified.

It should be emphasised that since nonspecific BLM incorporation, which corresponds to fluid phase endocytosis, occurs independently of specific internalisation and does not contribute to BLM cytotoxicity, BLM molecules entering cells in the fluid engulfed during fluid phase endocytosis should remain confined to the intracellular vesicles and never reach the cytosol. This accounts for the large increase in BLM cytotoxicity on electropermeabilised cells compared to intact cells, whereas BLM association to the cells is only moderately increased by cell electropermeabilisation [3]. Indeed, most of the BLM molecules incorporated into electropermeabilised cells directly enter the cytosol where they are then able to exert their cytotoxic effects, whereas only a small fraction of BLM molecules associated to intact nonpermeabilised cells, i.e. the "specific" fraction, bound to the membrane BLM receptors, will be translocated into the cytosol and become cytotoxic. Therefore, cell electropermeabilisation [27], which allows the massive introduction of BLM directly into the cytosol of isolated cultured cells [3] as well as of cells in tissues *in vivo* [28], seems to be a fruitful method to potentiate BLM cytotoxicity, and its application for a new human anticancer therapy, electrochemotherapy, seems promising [29–32].

Our assumption that the mechanism of BLM internalisation is a receptor-mediated endocytosis helps elucidate results like those of Matkhanov *et al.*, who showed that

BLM cytotoxicity can be increased by coupling the drug to molecules possessing membrane receptors like insulin or calcitonin [33]. More generally, the BLM internalisation mechanism, which depends on the number of BLM receptors at the cell surface and on the endocytotic activity of the cells, can explain the differences in BLM cytotoxicity among various cell types. It can also explain both the limited BLM antitumor effects and the absence of the usual side effects observed in clinics with most of the other anticancer drugs. In particular, it could be also responsible for the particular sensitivity of normal lung and cutaneous tissues, thought to be until now the result of reduced BLM hydrolase activity [34].

In conclusion, we have demonstrated that BLM internalisation in intact cells occurs via a receptor-mediated endocytosis mechanism. Therefore, natural BLM internalisation clearly appears to be a slow and limited process, and enhanced BLM cytotoxicity can be achieved by increasing BLM influx into the cells, as in the case of the electrochemotherapy. Interestingly, the BLM internalisation mechanism accounts for BLM's unusual biological characteristics that make it so different from the other anticancer drugs *in vitro* as well as *in vivo*.

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