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VERY HIGH CYTOTOXICITY OF BLEOMYCIN INTRODUCED INTO THE CYTOSOL OF CELLS IN CULTURE

B. PODDEVIN, S. ORLOWSKI,* J. BELEHRADEK JR and L. M. MIR†
Laboratoire de Biochimie–Enzymologie (U.R.A. 147 CNRS; U. 140 INSERM), Institut Gustave-Roussy, 94805 Villejuif, France

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Abstract—We observed previously *in vitro* that the cytotoxicity of bleomycin (BLM), an anticancer drug in current use, was greatly potentiated by exposing cultured cells to appropriately chosen electric pulses. We then showed *in vivo*, on tumor-bearing mice, that the same electric pulses also potentiated the antitumoral activity of BLM. In the present work, we demonstrate on DC-3F cells *in vitro*, that this potentiation is closely related to cell electropermeabilization and the consequent direct internalization of BLM molecules in the cytosol. The survival response curve (SRC) of the electropermeabilized (EP) cells exposed to BLM (plotted as logarithm of survival versus external drug concentration) shows a linear pattern usual for the SRCs of intact cells exposed to current cytotoxic drugs, though in the nanomolar range of concentrations. We have succeeded in determining the relation between BLM cytotoxicity on EP cells and the number of electroloaded BLM molecules per cell (that is the average number, per cell, of BLM molecules internalized into the cytosol). We conclude that (1) BLM molecules possess very intense cytotoxic activity which in non-EP cells is drastically limited by the intact plasma membrane; and (2) in these intact cells, the plasma membrane is responsible for the unusual upward concave curvature of the SRC resulting from exposure to BLM.

Bleomycin (BLM‡) is a hydrosoluble antibiotic which was first isolated by Umezawa *et al.* in 1966 [1], and exhibits cytotoxic activity against mammalian cells. BLM induces single- and double-strand DNA breaks. BLM cytotoxicity is closely related to the latter [2]. A very small amount of BLM associates with cells, presumably because of its hydrophilic nature [3]. Although a few reports have produced information about the association with cells of tritiated BLM in the micromolar concentration range [3–5], the method of BLM internalization is still not clear. Neither the amount of internalized BLM required to induce cytotoxicity nor the pathways used by BLM to enter the cells are known at present. In addition, there are various explanations for the upward concave curvature of the SRC of cells exposed to BLM, none of which are satisfactory [6–9].

Electropermeabilization of living cells is obtained by submitting the cells to very short intense electric pulses which modify their plasma membrane structure [10]. Non-permeant molecules, i.e. molecules which normally do not cross the intact plasma membrane, can cross the membrane of EP cells and either leave the cells, or if present in the external medium enter directly into the cytosol [11, 12]. Under suitable electrical conditions, the perturbations caused are reversible and transient. Plasma membrane integrity is recovered after a period of time which depends mainly on the temperature at which the cells are maintained [13]. After recovery, cells behave again like control cells which have not been submitted to electric pulses. Metabolite leaks from EP cells do not seem to affect cell viability. During the electropermeabilization procedure, cells become very sensitive to the external presence of non-permeant cytotoxic molecules such as pokeweed antiviral protein (a ribosome inactivating protein equivalent to a chain A-only ricin-like toxin) [14]. We found previously that electropermeabilization highly potentiates BLM cytotoxicity toward cell cultures *in vitro* [14]. Subsequently, we demonstrated that, *in vivo*, the same electric pulses increase the antitumor activity of BLM [15, 16].

In this report, we show that EP DC-3F cells exposed to BLM exhibit a conventional linear SRC. We also took advantage of a very sensitive BLM labeling method [17] to analyse BLM association with EP cells, even at very low concentrations. Comparison of the gain in cytotoxicity with the gain in association mediated by cell exposure to electric pulses revealed that the BLM molecule possesses very potent cytotoxic activity once it reaches the cytosol.

MATERIALS AND METHODS

Preparation of radiolabeled CoBLM. Lyophilized BLM (15 mg) (Roger Bellon, France) was dissolved in isoosmotic PBS at pH7, diluted with the same buffer to the desired concentration and stored at

* Present address: S.B.P.M.-D.B.C.M. and U.R.A. 1290 CNRS Cen Saclay, F-91191 Gif/Yvette, France.
† Corresponding author: Laboratoire de Biochimie–Enzymologie, Institut Gustave-Roussy, 39, rue Camille Desmoulins, F-94805 Villejuif Cedex, France. Tel. (33)1-45-59-47-92; FAX (33)1-46-78-41-20.

‡ Abbreviations: EP cells, electropermeabilized cells; NEP cells, non-electropermeabilized cells; BLM, Bleomycin; CoBLM, cobalt chelated form of BLM; LY, Lucifer Yellow; PBS, phosphate buffered saline; SRC, survival response curve; MEM, modified Eagle's medium.

−20°. $^{57}CoCl_2$ (440 Ci/mmol) (Amersham, U.K.) in a 50 mM HCl solution was diluted to the desired specific radioactivity in a 50 mM HCl solution of non-radioactive $^{59}CoCl_2$. The final pH of the labeled $CoCl_2$ solution was adjusted to 7 with 0.375 M Na_2CO_3. For the final CoBLM concentrations above 10 μM, BLM and $CoCl_2$ solutions were mixed in a molar ratio BLM/$CoCl_2$, 1/0.95 just before use and incubated for 1 hr at room temperature (24°). The other CoBLM solutions were obtained by dilution in PBS of a solution containing 10 μM BLM and 9.5 μM $CoCl_2$.

Electrical devices. Cells were electropermeabilized using a square wave pulse generator [14]. The output voltage from this generator is between 0 and 1000 V and can be maintained for periods of 5–150 μsec. This square wave signal can be delivered either *ad lib.* or at a preprogrammed frequency ranging from 0.1 to 10 Hz. The shape and parameters of the pulses actually delivered to cell suspensions were checked through an oscilloscope connected to the generator.

Cells and culture conditions. DC-3F cells, a spontaneously *in vitro* transformed Chinese hamster lung fibroblast line [18], were maintained as monolayers in 75 cm^2 tissue culture flasks (Becton Dickinson, Pont de Claix, France) in complete medium consisting of MEM supplemented with 8% fetal calf serum (Gibco, Cergy-Pontoise, France), 100 units/mL penicillin (Specia, Paris, France) and 100 μg/mL streptomycin (Sarbach, Suresnes, France). Cells were grown at 37° in a humid incubator with an air mixture containing 5% CO_2. Under these culture conditions, cell population doubling time was 12 hr as measured by cell counts at regular intervals using a model BZ Coulter Counter (Coultronics, France). Experiments were performed using cultures in the logarithmic phase of growth. Cells were seeded in T75 flasks at a density of 10^7 cells/flask, cultured for 24 hr and treated with CoBLM or BLM. Just before treatment, cells were trypsinized with 3 mL of a trypsin:EDTA solution prepared by dissolving 0.5 g/L of trypsin (Sigma Chemical Co., St Louis, MO, U.S.A.) and 0.5 g/L of EDTA (Merck Darmstadt, F.R.G.) in 137 mM NaCl; 5 mM KCl; 24 mM $NaHCO_3$; 50 mM glucose and 0.002% Phenol red. The average diameter of trypsinized cells was measured under a light microscope by comparison with the graduations of a micrometer, using a camera lucida. From this diameter, cell volume was calculated, assuming that under the above conditions cells were spherical.

Electric shock procedure. After trypsinization of exponentially growing cells and inactivation of trypsin by the complete medium, cells were washed three times in serum-free S-MEM (Gibco) supplemented with 0.5 mM Ca^{2+}. Cells were then resuspended in this medium at a density of 2.2×10^7 cells/mL and ice-cooled. Aliquots of 0.09 mL of the monodispersed cell suspension were mixed with 0.01 mL of BLM, CoBLM or solutions of LY at 10 times the final concentration. Fifty microlitres of the resultant mixture were deposited immediately between two flat electrodes 2 mm apart and exposed to the electric treatment (eight pulses of 0.1 msec and 1500 V/cm, or 1200 V/cm as specified, at a frequency of 1 Hz). After the shock, the cell suspension was incubated for 5 min at 24° in a plastic well (Nunc, Denmark). NEP cells were treated similarly except for the electric field exposure. In the absence of BLM or CoBLM, the electrical conditions used in these experiments led to a low cell mortality of less than 10%. About 3% of the electropulsed cells remained unpermeabilized at 1500 V/cm and 12% at 1200 V/cm [11].

Cytotoxicity studies. At the end of the post-shock incubation, cells were diluted about 200,000 times and seeded in triplicate in complete culture medium (500 cells/cell culture dish, 60 mm in diameter) for colony inhibition assay. Colonies of at least 50 cells were counted after 5 days. Results were expressed as percentages of the number of colonies obtained with control cells treated in the absence of drug. Absolute cloning efficiency of the NEP controls was usually about 70%.

Determination of LY uptake. Cells exposed to LY (M_r = 450) (Sigma) underwent the same post-shock incubations and were then washed four times by centrifugation at 20° in PBS, resuspended in 0.5 mL of PBS and sonicated (3 × 15 sec at 100 W; Vibra Cell model 500, Sonics & Materials, U.S.A.). The fluorescence of the sonicated cells was read at 25° with a SFM 25 spectrofluorimeter (Kontron, Switzerland) at excitation and emission wavelengths of 423 and 555 nm, respectively. The readings were compared with those obtained under the same conditions from the same amount of non-shocked, washed and sonicated cells to which known quantities of LY had been added. The average intracellular LY concentration was calculated from the average volume of the cells and from the percentage of cells actually permeabilized in the electropulsed population.

Quantification of CoBLM association with DC-3F cells. At the end of the post-shock incubation with the drug, the cells were diluted in 2 mL of complete medium and washed four times by dilution in 15 mL of complete medium, and centrifugation at room temperature for 10 min at 1000 rpm. At the end of each centrifugation, a 2 mL fraction of supernatant was collected for radioactivity measurement and 12.5 mL were discarded. The pellet was kept in 0.5 mL of supernatant, resuspended and diluted in 15 mL of complete medium before the next centrifugation.

After the fourth centrifugation, the radioactivity of the last pellet resuspended in 0.5 mL of supernatant (designated $[P_4 + S_4(0.5)]$) and the radioactivity of the 2 mL fraction of the corresponding supernatant (designated $S_4(2)$) were measured on a MR 252 Automatic Gamma Counting System (Kontron). The exact specific activity (designated A) of the labeled CoBLM preparation used for the experiment was checked on the same γ counter. Cells were then pipetted, diluted 1000-fold in Isoton (Coultronics, France) and counted on a model BZ Coulter Counter: the number of cells in the pellet P_4 was N_4.

Thus it was possible to determine (1) the radioactivity, P_4, associated with the last cellular pellet:

$$P_4 = [P_4 + S_4(0.5)] - S_4(2)/4$$

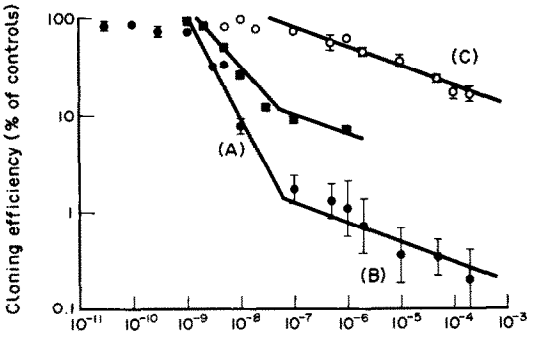

Fig. 1. BLM cytotoxicity on electropulsed and non-electropulsed cells. DC-3F cells were suspended in serum-free S-MEM supplemented with 0.5 mM Ca^{2+}, at a density of 2×10^7 cells/mL, and incubated in the presence of bleomycin for 5 min at room temperature. Cells were either exposed (closed circles) or not (open circles) to electric pulses (eight pulses of 0.1 msec and 1500 V/cm at a frequency of 1 Hz) at the beginning of the incubation with BLM. Then, cells were diluted 200,000 times and seeded in triplicate for colony inhibition assay. Closed squares correspond to the SRC for cells electropulsed under the same conditions except for the intensity of the electric field which, in this case, was 1200 V/cm. For A, B and C symbols, see text.

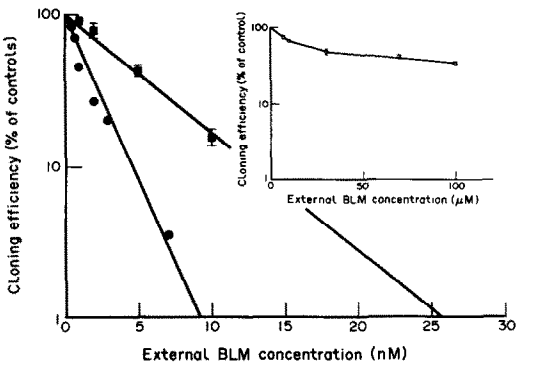

Fig. 2. SRC of EP DC-3F cells exposed to bleomycin. Closed circles and closed squares correspond to cells exposed to electric pulses at 1500 and 1200 V/cm, respectively. Inset shows the SRC of control non-electropulsed DC-3F cells exposed to bleomycin under the same incubation conditions as pulsed cells.

and (2) the average amount of molecules of CoBLM, n_4, associated with one cell of this pellet:

$$n_4 = (P_4 \times [\text{CoBLM}] \times 6.02 \times 10^{23})/(N_4 \times A).$$

As we had established previously that the cellular recovery of the washing steps was 93%, we were able to quantify, from the value of the radioactivity in the 2 mL fraction of supernatant after each centrifugation, $S_i(2)$ (cpm), the evolution of the CoBLM association with cells during the washing steps using the following formulas:

$$P_i = P_{i+1} + 7.5 \times S_{i+1}(2) - S_i(2)/4 \qquad i = 1,\ldots,4$$

$$n_i = (P_i \times 0.93^{(4-i)} \times [\text{CoBLM}] \times 6.02 \times 10^{23})/(N_4 \times A) \qquad i = 1,\ldots,4.$$

RESULTS

BLM cytotoxicity on EP cells

Both electropulsed and non-electropulsed cells were incubated for 5 min in the presence of BLM concentrations ranging from 1 mM to 0.01 nM. Electric pulses were delivered at the beginning of incubation with the drug. Cytotoxicity was measured by the relative cloning efficiency of the treated cells (Fig. 1). To permit comparison between BLM cytotoxicity on electropulsed and non-electropulsed cells, the logarithm of cell survival was plotted against the logarithm of the BLM concentration. For cells electropulsed at 1500 V/cm, the minimal concentration at which BLM cytotoxicity was detected was close to 1 nM, i.e. more than 100 times lower than the corresponding concentration for non-electropulsed cells (Fig. 1). For BLM concentrations above this minimal cytotoxic concentration, the plot of the survival of the electropulsed cells was biphasic and was fitted by two straight segments (A and B, Fig. 1), whereas the plot of non-shocked cell survival was monophasic (segment C, Fig. 1). The ordinate of the inflexion point between segments A and B corresponded to the survival of about 3% of the cells pulsed at 1500 V/cm which, under our experimental conditions, was identical to the previously reported proportions of NEP cells among cells exposed to the electric pulses [11]. In addition, the slopes of segments B and C seemed identical. Thus, segment B was the SRC of a 3% fraction of the electropulsed cell population which exhibited the same characteristics as the non-electropulsed population. Consequently (1) the biphasic survival pattern could be explained as follows: segment A corresponded to the BLM cytotoxicity to the entire electropulsed cell population (both EP and NEP) and segment B to the BLM cytotoxicity to those electropulsed cells which were not permeabilized; and (2) at 70 nM BLM, abscissa of the inflexion point, virtually all the EP cells were killed by BLM. Moreover, at this concentration, its cytotoxic effect on non-electropulsed cells was not detectable.

An equivalent biphasic pattern was obtained by treating cells at 1200 V/cm; in this case, the ordinate of the inflexion point corresponded to 12% of the treated cells, which again was identical to the previously reported fraction of NEP cells among the cells exposed to these electric pulses [11]. Nevertheless, higher external concentrations of BLM were needed at 1200 V/cm than at 1500 V/cm to obtain the same cytotoxic effect.

Using conventional coordinates (logarithm of survival as a function of the external BLM concentration), the plot of the SRC for the cells actually electropermeabilized was linear (Fig. 2) and did not exhibit the upward concave curvature usually observed with the SRC of NEP cells (inset to Fig. 2 and Refs 6 and 7). This plot shows that, for the cells electropermeabilized at 1500 V/cm, the external

BLM LD_{50} was as low as 1.3 nM, i.e. about 1000 times lower than the LD_{50} for NEP cells.

Relations between cytotoxicity potentiation and cell electropermeabilization

We performed several experiments with variations of the usual electropermeabilization protocol.

If cells are incubated at 37° instead of 24°, it is known that the electropermeabilized state is short-lived because membrane integrity is recovered faster [13]. Here, at 37°, BLM cytotoxicity to EP cells (characterized by the LD_{50}) decreased to three times less than at 24°. Cells are not permeabilized if pulsed at only 800 V/cm [11]. Here, at 800 V/cm, we did not observe any potentiation of BLM cytotoxicity (data not shown). Figure 2 shows that at 1200 V/cm, a suboptimal electric field intensity for DC-3F cell electropermeabilization [11], cytotoxicity potentiation was smaller than at 1500 V/cm, optimal for permeabilization. At 1200 V/cm the SRC slope was only 40% of the slope at 1500 V/cm.

Consequently, BLM cytotoxicity was closely correlated with cell permeabilization. Further experiments were designed to show a relation between cytotoxicity potentiation and the effective presence of BLM in the external medium during electric pulse treatment.

ATP leaks occurring during the permeabilized state could lead to hypothetical changes in cell metabolism and could therefore potentiate BLM cytotoxic efficiency. To avoid such leaks, ATP (2 mM) was added to cell suspensions before the simultaneous exposure of the cells to BLM and electric pulses: no changes in cytotoxicity potentiation were observed.

In a second assay, cells suspended in S-MEM medium were incubated for 1 hr at 37° in the presence of either 3 nM or 1 μM BLM, washed twice with S-MEM medium and then pulsed. In this assay, electric shocks occurred between 20 and 40 min after the last contact of the cells with BLM. No significant differences were detected between BLM cytotoxicity to electropulsed cells and cells incubated under the same conditions but not pulsed. This observation suggests that the electric pulses did not possess mechanisms able to potentiate the activity of the BLM molecules already associated with the cells.

In a third assay, cells were pulsed, incubated in the absence of BLM at 24° for different periods and only then exposed to the drug for 5 min. Cytotoxicity potentiation decreased as the interval between pulse delivery and drug exposure increased, and within 5 to 10 min cytotoxicity almost returned to within its normal values for intact cells. The kinetics of the decrease in BLM cytotoxicity potentiation were similar to the kinetics reported previously under the same experimental conditions for the decrease in LY uptake as the interval increased between pulse delivery and the addition of LY to the medium [14].

Thus the potentiation of BLM cytotoxicity was maximal when this drug was present in the external medium during electric pulse treatment, i.e. when membrane permeability was maximal.

Lucifer Yellow uptake

In an attempt to understand the difference between the levels of BLM cytotoxicity observed on cells electropermeabilized at 1500 and 1200 V/cm (Fig. 2), we measured LY uptake under the same experimental conditions, as regards electric pulse delivery and post-shock incubation, as those used for the cytotoxicity determination reported in the previous section. Table 1 shows that at 1200 V/cm and in the presence of 1 mM LY in the external medium, the amount of internalized LY per EP cell was about 40% of the amount internalized per cell at 1500 V/cm. As electroloading of exogenous non-permeant or slightly permeant molecules is non-specific and proportional to the external molecule concentration, BLM internalization at 1200 V/cm might be only 40% of its internalization at 1500 V/cm. The 40% internalization found with LY suggests that the difference between the levels of BLM cytotoxicity observed at 1200 and 1500 V/cm (Fig. 2) was due to a difference in BLM electroloading. If true, this would explain why at 1500 V/cm the external BLM concentration required to obtain a given level of toxicity was only 40% of the concentration required at 1200 V/cm. It, therefore, seems that both at 1500 and 1200 V/cm the toxicity level is determined by the number of electroloaded BLM molecules per EP cell. Consequently, the potentiation of BLM cytotoxicity is not only related to the presence of BLM in the external medium at the time of cell permeabilization but also to the amount of electroloaded BLM molecules.

Table 1. Quantification of LY uptake after cell electropermeabilization at 1200 and 1500 V/cm

	0 V/cm (Pinocytosis)	1200 V/cm	1500 V/cm
C_{ext}	0.22×10^{-6}	1.36×10^{-6}	3.41×10^{-6}
F_{per}	0	0.88	0.97
C_{int}	0.06×10^{-3} [0]	0.36×10^{-3} [39]	0.92×10^{-3} [100]

Electric treatment of DC-3F cells in the presence of 1 mM external LY, preparation of cell extracts (volume $V_{ext} = 0.5$ mL), measurements of LY fluorescence and LY concentration C_{ext} in these extracts, and determination of average cell volume V_{cel} were performed as described in Material and Methods. Each cell extract contained an average of $N = 1.9 \times 10^6$ cells. In this experiment, V_{cel}, calculated for samples of either non-pulsed cells or cells pulsed at 1200 or 1500 V/cm, was 0.94 pL. Pinocytosis (P) was measured by the concentration of LY associated with cells in the absence of electric pulses ($P = (C_{ext} \times V_{ext})/(N \times V_{cel}) = 0.06 \times 10^{-3}$ M). As LY is a non-permeant molecule that does not interact with the plasma membrane, its electroloaded concentration C_{int} in the fraction F_{per} of EP cells corresponds to the difference between total LY association and pinocytosis and is given by $C_{int} = (C_{ext} \times V_{ext} - N \times V_{cel} \times P)/(N \times V_{cel} \times F_{per})$. The percentage of electroloading in relation to electroloading at 1500 V/cm is given in square brackets.

CoBLM association with EP and NEP cells

To confirm and extend the results obtained by the indirect approach of LY uptake determination, we quantified the electroloading of labeled BLM at the

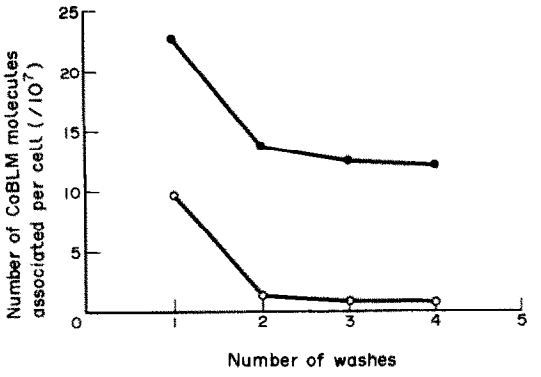

Fig. 3. Efficiency of washing procedure. Cells were incubated for 5 min in the presence of CoBLM and then washed four times by centrifugation at room temperature for 10 min at 1000 rpm. At the end of each centrifugation, the number of CoBLM molecules per EP cell (at 1500 V/cm; closed circles) and NEP cell (open circles) was determined as described in Material and Methods.

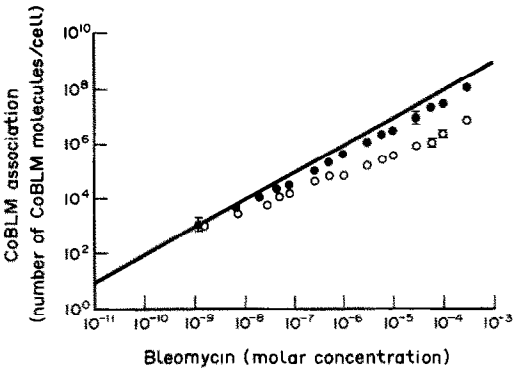

Fig. 4. Association of CoBLM with EP and NEP cells. DC-3F cells were suspended in serum-free S-MEM supplemented with 0.5 mM Ca^{2+}, at a density of 2×10^7 cells/mL, and incubated in the presence of bleomycin for 5 min at room temperature. Cells were either exposed (closed circles) or not (open circles) to electric pulses (eight pulses of 0.1 msec and 1500 V/cm at a frequency of 1 Hz) at the beginning of the incubation with BLM. Then, cells were washed four times and the number of CoBLM molecules associated per cell was determined. The continuous straight line corresponds to the association which would have been measured if the cell-associated CoBLM concentration had been identical to the external concentration, considering that the average cell volume was 1.4 pL in these experiments.

external concentrations at which BLM cytotoxicity potentiation was observed. These experiments were only performed at 1500 V/cm, optimal electric field intensity for electropermeabilization of DC-3F cells.

The conditions for these association experiments were identical to those for the cytotoxicity experiments except that (1) BLM was replaced by [^{57}Co]BLM in the incubation media and (2) at the end of the 5 min incubation, cells were washed four times, and the associated radioactivity was counted. The number of molecules associated per cell was calculated from the radioactivity bound to the cellular pellets resulting from the washing procedure (see Materials and Methods). In this calculation, we did not take into account the NEP fraction among the electropulsed cell population because this fraction (close to 3% of the electropulsed cell population) was not large enough to alter the association results significantly. Figure 3 shows a typical example of the step-by-step analysis of the washing procedure. At each step in this procedure, the same amount of radioactivity per cell was washed away from both EP and NEP cells. This means that the difference between CoBLM association between these two cell types, as detected in the last pellet, already existed in the first pellet and at each subsequent step of the washing procedure. After the third wash, no significant decrease in the associated radioactivity per cell was observed, indicating that the remaining radioactivity was tightly bound to the cells. We, therefore, decided to use the radioactivity measured in the fourth pellet to evaluate CoBLM association with cells.

The average number of CoBLM molecules associated with one EP or NEP cell was determined after incubation in the presence of CoBLM for a large range of concentrations (Fig. 4). Results were plotted using double logarithmic coordinates as in the cytotoxicity experiments illustrated in Fig. 1. Figure 4 shows that (1) at every concentration tested, more CoBLM always associated with EP than with NEP cells; (2) the ratio of BLM association with EP to NEP cells was not constant as it increased from 2 at low concentrations of 1–200 nM to 15 at high concentrations of 30 μM–1 mM; and (3) if the "concentration of CoBLM associated with the cells" is defined as the ratio of the average number of CoBLM molecules associated per cell to the average volume of DC-3F cells (1.4 pL), there was no overconcentration of BLM in the cells, even the EP cells.

In Figure 5, the association ratio, i.e. the ratio of the cell-associated CoBLM concentration to the external CoBLM concentration, was plotted against the external CoBLM concentration. The curve thus obtained showed that at low concentrations the association ratio decreased as the external CoBLM concentration increased but, for concentrations above 1 μM, a plateau was reached at about 38% for EP cells and about 3% for NEP cells. For each concentration tested, we calculated the difference between the respective association ratios of the two cell types and plotted it as a function of the external CoBLM concentration (inset to Fig. 5). Throughout the concentration range tested this difference was constant: about 35% of the external CoBLM concentration. Thus CoBLM association with EP cells could be considered as the sum of two components: one was similar to the CoBLM association observed with NEP cells and the other, present in EP cells only, was proportional to the external concentration throughout the concentration range explored and, consequently, was non-saturable. This non-saturable component present only in EP cells should be considered as the electroloaded BLM and corresponded to an

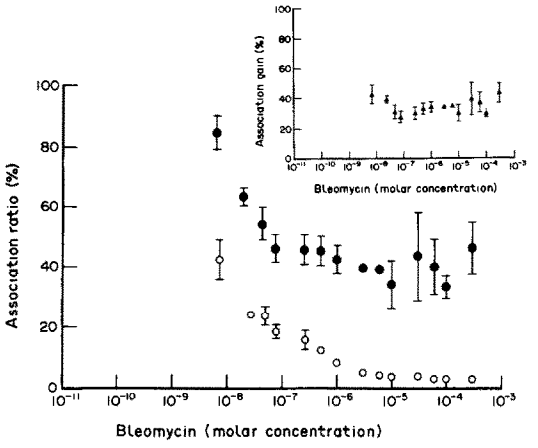

Fig. 5. Association ratio (defined as the ratio of the DC-3F cell-associated CoBLM concentration to the external CoBLM concentration) for EP and NEP cells. The association ratio is reported as a percentage of the external CoBLM concentration, 100% corresponding to the situation in which the cell-associated CoBLM concentration would be identical to the external concentration (straight line of Fig. 4). Closed circles, EP cells (at 1500 V/cm); open circles, NEP cells. Inset shows the difference between the association ratios of the two cell types when exposed to the same external CoBLM concentrations under the same conditions.

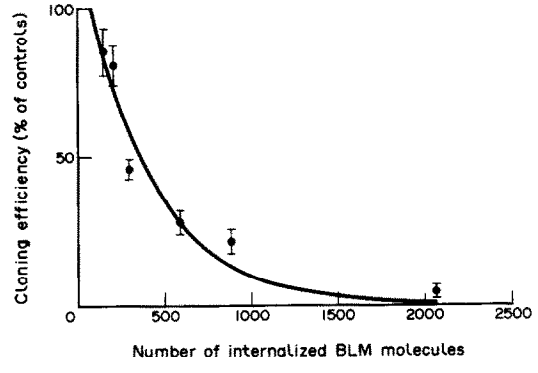

Fig. 6. Correlation between the number of internalized BLM molecules per cell and BLM cytotoxicity. Abscissae (linear scale), calculated from the difference between the association of CoBLM with EP (at 1500 V/cm) and NEP cells (see inset of Fig. 5 and text), correspond to the number of electroloaded BLM molecules at the external BLM concentrations leading to the cytotoxicities reported in ordinates (linear scale too). It is worth noting that the fitting curve suggests that a minimal amount of internalized BLM (in the region of 100 molecules) is necessary to induce cytotoxicity.

association of about 300 molecules per cell at 1 nM external CoBLM, and about 3×10^8 at 1 mM CoBLM.

The relationship between the number of CoBLM molecules associated per cell through this non-saturable component and BLM cytotoxicity to EP cells is shown in Fig. 6. The LD_{50} of BLM for these cells corresponded to an average number of 400 electroloaded molecules/cell. Virtually all the EP cells were killed when an average number of a few thousand molecules per cell was electroloaded into the cytosol.

DISCUSSION

The cytotoxicity of BLM depends greatly on its ability to induce double-strand breaks in DNA [2]. RNA may also be a relevant cellular target for BLM [19]. Even if BLM can perturb plasma membranes [20, 21], this effect has not been correlated to cytotoxicity and it can be assumed that therapeutically relevant targets of BLM are, therefore, intracellular. BLM cytotoxicity can be modulated by simultaneous or consecutive exposure of cells to BLM and to various agents interacting with the plasma membrane, including polyene antibiotics [22], ethanol [23] and local anesthetics [24], which all increase cell sensitivity to BLM. This suggests that the plasma membrane could act as a barrier which impedes BLM in reaching its intracellular target.

Under suitable conditions, electric pulses reversibly and transiently permeabilize the plasma membrane of cells in culture without affecting their viability [10]. Unlike chemical treatments, cell permeabilization by electrical treatment involves no addition and no withdrawal of chemical constituents at the plasma membrane level and recovery of cell integrity occurs spontaneously: the plasma membrane reorganizes itself and its initial structure is restored after a period whose length depends mainly on the temperature at which the cells are maintained [13]. EP cells can load exogenous, non-permeant molecules which cannot cross the plasma membrane in the absence of electropermeabilization but can enter EP cells and then remain internalized in the cytosol after cell recovery from the electropermeabilized state [11]. The permeation structures involved in the crossing of the plasma membrane of EP cells are not yet clear but electric pulses should not change the permeability of the nuclear membrane. For a given external concentration of a non- or slightly permeant compound, electroloading results in a homogeneous uptake of the exogenous molecules into the EP cells, as shown by our previous results using molecules as different as LY [11] and antibodies [25]. Electroloading is believed to be non-specific [26] and the molecular weight of exogenous compounds seems to be the main parameter governing their internalization into cells electropermeabilized under the same conditions: electroloading efficiency decreases as the molecular weight of the exogenous molecules increases. In the case of the DC-3F cells, which were electropermeabilized at 1500 V/cm according to the same protocol, we found previously that the internalized concentration was 100% of the external concentration for LY ($M_r = 450$) [11], 20% for oligonucleotide fragments ($M_r = 3000$) [12], 10% for pokeweed antiviral protein ($M_r = 30{,}000$) [11] and about 0.1% for antibodies ($M_r = 150{,}000$ [25]. These observations are in good agreement with other published results showing that the electroloading efficiency of iodine propidium ($M_r = 660$) was much

greater than that of macromolecules such as 70,000 dalton dextrans [27]. Given the non-specific nature of electroloading, we can assume that BLM and CoBLM electroloading should be equivalent.

In the case of BLM ($M_r = 1500$), we showed earlier that electric pulses greatly potentiate BLM cytotoxicity [14]. Here, all the data reported in Results concerning the relationship between cytotoxicity potentiation and cell permeabilization show that this potentiation is, in fact, due exclusively to the uptake of electroloaded molecules, and not to some unidentified mechanism induced by the electric field increasing the cytotoxic activity of BLM molecules already present inside the cells before the delivery of electric pulses. From our previous observations [11] concerning the distribution inside the cells of electroloaded LY (a highly fluorescent, non-permeant molecule), it may be assumed that once in the cytosol, BLM molecules can easily reach the nucleosol (through the ultrastructurally defined, aqueous pores of the nuclear membrane) in order to bind and break DNA. The determination of a correlation between level of BLM electroloading and BLM cytotoxicity on EP cells should provide quantitative information about BLM efficiency after its internalization into cell cytosol.

To quantify BLM electroloading, we needed labeled BLM with a specific radioactivity high enough to allow the detection of its presence even after exposure of the cells to BLM concentrations in the the nanomolar range, at which BLM already displays cytotoxicity to EP cells. Therefore, we recently developed and described a method of preparing [^{57}Co]BLM suitable for use at these low concentrations [17]. [^{57}Co]BLM appeared to be the best labeled compound for quantitative investigation of non-specific BLM electroloading because of the close resemblance of their molecular weight and chemical structure, of the high specific radioactivity of the chelate, and of the very high stability of the CoBLM coordination complex. Indeed, after the association of Co(II) to BLM metal is oxidized spontaneously and the complex Co(III)BLM is almost undissociable [28]. However, some of the biological properties of CoBLM such as its cytotoxicity and the extent of its association with NEP cells differ from those of BLM [4, 5, 29]. CoBLM is not cytotoxic and this absence of cytotoxicity has been shown to be due to its complete inability to cause DNA breaks [29]. The specific properties of CoBLM association with NEP cells are under investigation (manuscript in preparation). As explained earlier [11, 14], the choice of 5 min as the period of cell incubation with BLM or CoBLM was based on the LY uptake revealed by fluorescence microscopy. This period corresponds to the minimum time necessary for cell resealing at room temperature (24°) after electric pulse delivery. Here, cells were brought into contact with BLM or CoBLM, under the same conditions, for BLM cytotoxicity studies or for measurement of CoBLM association with cells, respectively.

CoBLM association was evaluated from the count of the radioactivity bound to cellular pellets obtained at the end of the washing procedure. The step-by-step checking of the washing procedure showed that the increase in association observed for EP cells after the last wash was already detectable after the first wash and remained constant throughout the washing procedure, even for amounts of electroloaded molecules as large as 10^8 molecules/cell. We can, therefore, assume that (1) the membrane perturbations induced by the electrical treatment do not significantly modify the efficiency of cell wash; (2) no loss of electroloaded CoBLM occurs during the washing procedure which lasts about 80 min; and (3), consequently, electroloaded molecules cannot easily cross the plasma membrane after it recovers its integrity. Thus, as expected, electroloaded CoBLM molecules behave as if they were irreversibly trapped inside the cells. This argues against BLM crossing plasma membrane by simple diffusion.

Comparison of CoBLM association with EP and NEP cells suggests that the association which occurs with NEP cells also occurs with EP cells. This is in good agreement with the current notion of electropermeabilization as a process which induces reversible perturbation of a limited area of the cell surface [30]. For EP cells, total association is the sum of the "NEP component" and the non-saturable component which corresponds to the electroloading, i.e. to the direct internalization into the cytosol (35% of the external CoBLM concentration, under our experimental conditions). Details on the "NEP component" of the association, that is the complete study of BLM association to intact, NEP DC-3F cells, will be presented and discussed elsewhere (manuscript in preparation). This study suggests that only very small amounts of BLM can reach the cytosol of intact cells, even at high external BLM concentrations. Under these conditions, it is easy to understand why electropermeabilization, which allows the uptake into the cytosol, even at very low external concentrations, of substantial amounts of BLM (see below), so greatly increases BLM cytotoxicity.

As discussed before, BLM and CoBLM electroloading are probably equivalent, and the measurement obtained by the use of [^{57}Co]BLM can be used to establish relations between the number of BLM molecules present in the cytosol and the cytotoxicity they cause (Fig. 6). Indeed, BLM cytotoxicity was detected within the nanomolar concentration range on EP cells. The LD_{50} on these cells was about 1.3 nM, i.e. 1000 to 10,000 times lower than the LD_{50} on NEP cells. Virtually all the EP cells were killed at the very low BLM concentrations of between 10 and 100 nM with which no significant cytotoxicity was detected on NEP cells. Thus, at these concentrations, the BLM-associated fraction present on both NEP and EP cells ("the NEP component") did not display significant cytotoxicity.

The SRC for EP cells exposed to nanomolar BLM concentrations exhibits a conventional mono-exponential pattern (Fig. 2). This shows that when the plasma membrane does not limit internalization of BLM by the cells, the drug does not display the unusual SRC. Consequently, our results indicate that for NEP cells, the crossing of the plasma membrane is the element responsible for the unusual

upward concave curvature of the BLM SRC. It should be remembered that liblomycin, a BLM derivative possessing a highly lipophilic residue that replaces one of the usual amine residues of natural BLM, exhibits a monophasic SRC at concentrations in the micromolar range [31]. This suggests that the lipophilic residue allows bleomycin to cross the plasma membrane but probably reduces its cytotoxic activity considerably, once it is in the cytosol. In this connection, we demonstrated here that BLM is intrinsically a very powerful cytotoxic compound, since the LD_{50} for EP cells corresponded to an average of only 400 molecules/cell after their internalization into the cytosol (Fig. 6). This shows that BLM is one of the compounds with the greatest known intrinsic toxicity, second only to substances conveying genetic information like viral DNAs or RNAs and to the ribosome-inactivating proteins like ricin, gelonin or modeccin, which are vegetal or microbial toxins that kill one cell as soon as a single molecule is present in the cytosol. This is probably due to the particular mode of action of BLM by which it can damage DNA, a crucial cell target, catalytically and irreversibly. The high intrinsic cytotoxicity of BLM contrasts with the pattern of the SRC of intact cells exposed to the drug and supports our observations that in NEP cells, simple diffusion of BLM cannot occur through the plasma membrane in either an inward or outward direction. Consequently, for these cells, a mechanism not related to passive transport by simple diffusion must be responsible for BLM internalization and cytotoxicity (manuscript in preparation).

In the present investigation, we described the main features of BLM cytotoxicity potentiation by cell electropermeabilization and demonstrated that this potentiation is closely related to cell permeabilization and to the direct internalization of BLM into the cytosol. Our results support the notions that (1) once bleomycin reaches the cytosol, it is endowed with especially intense cytotoxic activity; (2) when the plasma membrane is bypassed, the SRC of cells exposed to BLM is a conventional monophasic plot which is in agreement with the cytotoxicity models based on the radiologist's target theory [32]; (3), probably, in intact cells no simple diffusion of BLM occurs through the plasma membrane; and (4) the plasma membrane drastically limits BLM cytotoxicity and is no doubt responsible for the unusual upward concave curvature of the SRC of intact cells exposed to BLM. A grasp of the mechanisms involved in BLM association with the plasma membrane and in BLM crossing of the membrane would certainly be the key to an understanding of the cytotoxic activity of BLM at the cellular level.

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