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IDENTIFICATION OF A PLASMA MEMBRANE PROTEIN THAT SPECIFICALLY BINDS BLEOMYCIN

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In this paper, the association of radiolabelled bleomycin on the plasma membrane of DC-3F cells is shown to be saturable, displaying classical features of a ligand-receptor binding. This association corresponds to the presence of 140,000 to 400,000 binding sites at the surface of the cells with a half-saturating concentration of 5μM. Moreover, using a gel electrophoresis procedure to separate membrane proteins incubated with radiolabelled bleomycin, we demonstrate the existence of a membrane protein of about 250kDa able to specifically bind bleomycin. This membrane bleomycin-binding protein could play a major part in the association of bleomycin with the cells and in its further internalization, a process that has not been elucidated yet.

Bleomycin (BLM) is a hydrosoluble antibiotic which was first isolated by Umezawa *et al* in 1966 (1), and exhibits cytotoxic activity against mammalian cells, by inducing single- and double-strand DNA breaks (2). It is clinically used in the treatment of many solid tumors, including squamous cell carcinomas and malignant lymphomas. Studies performed on DC-3F cells in culture showed that BLM possesses a very high intrinsic cytotoxicity : 500 molecules of BLM introduced in the cytosol of an electropermeabilized cell are sufficient to kill this cell (3). A very reduced crossing of the plasma membrane by BLM was shown to be responsible for the unusual biphasic survival response curve of cells exposed to BLM. In fact, a very small amount of BLM associates with cells, presumably because of its hydrophilic nature (4). Although many authors have stressed the limiting role of the plasma membrane in the uptake of BLM, little is known about the detailed mechanism of BLM internalization.

Recent studies in our laboratory suggested the existence of BLM specific binding sites on the cell surface. In this paper, we characterize the association of radiolabelled BLM on the plasma membranes of DC-3F cells. An adaptation of the gel retardation technique is described, which allowed us to visualize the putative BLM-binding site, *i.e.* a membrane protein able to tightly bind BLM.

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MATERIALS AND METHODS

Cells and chemicals : DC-3F cells were maintained as monolayers in MEM (Eurobio) supplemented with 8% foetal calf serum (Eurobio) and antibiotics. They were grown at 37°C in a humid incubator containing 5% CO_2. We used two different populations of DC-3F cells which had not the same number of *in vitro* passages : the "young" cells had about 50 passages and the "old" cells about 180 passages.

Radiolabelled BLM was prepared as described by Poddevin et al. (5) by mixing BLM (Laboratoires Roger Bellon, France) with [^{57}Co] Cl_2 (Amersham, France) in a 3:1 molar ratio. The pH of the mixture was adjusted to 7 with sodium bicarbonate and the [^{57}Co]BLM complex was allowed to stabilize for one hr at room temperature before use. For competition experiments, a 5-fold excess $CoCl_2$ was then added in order to chelate all the BLM. The maximum specific activity of our radiolabelled bleomycin was 17 Ci/mmole of BLM.

When not otherwise specified, reagents were obtained from Sigma Chemicals Co.

Association of radiolabelled BLM on cell membranes : Monolayers of cells in 60mm diameter Petri dishes were rinced with cold pH 7 Phosphate Buffered Saline (PBS), and maintained on ice for 10 min. PBS was then removed and cells were incubated at 4°C for 15 min with 800 μl [^{57}Co]BLM diluted to proper concentrations in PBS. Cells were then washed 8 times with cold PBS. The last washing was counted each time to ensure the washing efficiency. Crude membrane extracts were then prepared as following : cells were incubated with 1 ml of lysis solution (20mM Tris-HCl; 1mM EDTA; 1mM DTT; 0.4mM PMSF; 7μM pepstatin; 4μM leupeptin) for 5 min at 4°C, and then scraped using a rubber Policeman. The plates were rinsed with an other ml of lysis solution. After 25 min of incubation at 4°C, cells were homogenized using a Potter Dounce B (30 strokes). A first centrifugation (500g, 15 min, 4°C) separated unbroken cells and nuclei, and the supernatant was centrifuged one hr at 4°C at 20,000g. The final pellet, consisting of the cellular membranes, and the supernatant, *i.e.* the cytosolic fraction, were counted separately on a Gamma Counter MR 252 (Beckman).

The protein content of each fraction was determined by the method of Bradford (6) using the Bio-Rad Protein Assay . In each experiment, one of the dishes was incubated in BLM-free PBS, washed 8 times like the others and then trypsinized in order to determine the number of cells per dish. Knowing the specific activity of the labelled bleomycin, we were then able to transform the radioactivity of each membrane fraction in the corresponding number of bleomycin molecules associated with the membranes of one cell.

Gel electrophoresis procedure : Crude membrane extracts to be separated on the gels were prepared as above, except that the incubation with BLM was omitted. The final membrane pellet was resuspended in PBS at a protein concentration of 5mg/ml and stored at 4°C for one week maximum before use. The cytosolic fractions were concentrated up to 5mg/ml using an Automatic Speed-Vac (Savant). Twenty-five to 150 μg of either cytosolic or membrane proteins were incubated with 5 μl of 10μM [^{57}Co]BLM for one hr at room temperature. For competition experiments, 5 μl of non-radioactive CoBLM ranging from 20 to 500μM were added in the same time than [^{57}Co]BLM. Then, the extracts were solubilized in an equal volume of sample buffer (62.5mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.005% bromophenol blue) for one hr at room temperature. The samples were loaded on a 4.5% polyacrylamide gel (for a final volume of 10 ml, 1.5 ml of acrylamide:bisacrylamide 29:1 mixture (Bio-Rad), 2 ml Tris 250mM - glycine 1.92mM - EDTA 12.5mM (5X TGE), 0.1 ml SDS 10%, 0.1 ml ammonium persulfate 10%, 10 μl Temed and 6.3 ml distilled water). Samples were run at 30 mA for 3 to 4 hrs in TGE 0.5X supplemented with 0.1% SDS.

RESULTS

Association of [^{57}Co]BLM on cell membranes at 4°C

The association of [^{57}Co]BLM to DC-3F cell membranes is shown in Fig 1. An incubation time of 15 min was chosen in our experiments because at 4°C, a kinetic study of

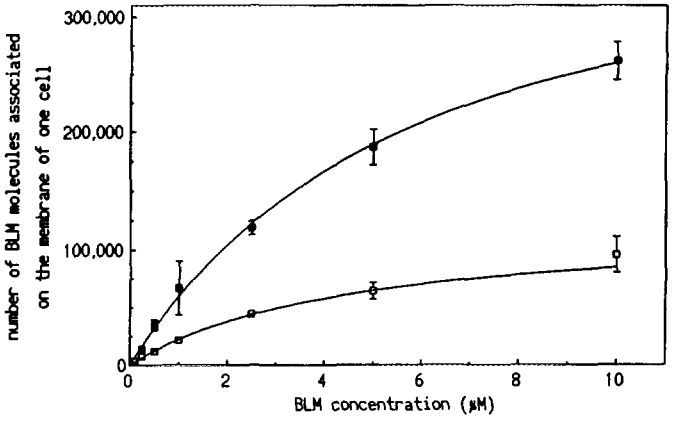

Figure 1 . Association of [^{57}Co]BLM on DC-3F cell plasma membranes.
Cells in monolayers were incubated with [^{57}Co]BLM for 15 min at 4°C and membranes were isolated as described in Materials and Methods. □ 180 passage-old DC-3F cells. ■ 50 passage-old DC-3F cells. Each point is the average of at least 3 independent experiments.

the association of [^{57}Co]BLM at 100nM, which is the lowest concentration we used, showed that the equilibrium was reached within 10 to 15 min (data not shown). The association profile appeared to be saturable, and it was best fitted to a rectangular hyperbola. The plateau value, which defines the maximum binding, appeared to depend on the number of *in vitro* passages of the cells. For 50 passage-old DC-3F cells ("young cells"), it was found to be 400,000 ± 20,000 BLM molecules bound per cell, whereas for 180 passage-old cells ("old cells"), only 140,000 ± 20,000 BLM molecules were bound per cell. The half-saturating concentration seemed to be nearly constant and equal to 5μM ± 1μM for both the "young" and the "old" cells. An excess of unlabelled BLM inhibited the association of [^{57}Co]BLM on membranes (data not shown).

Visualization of a membrane BLM-binding protein

Barranco et al. showed that freshly trypsinized CHO cells were transitorily resistant to BLM (7). We confirmed this result with DC-3F cells (manuscript in preparation). This observation argued in favor of a proteinic nature for the BLM-binding sites. In order to confirm this point, we incubated membrane proteins with radiolabelled BLM and separated them using polyacrylamide gel electrophoresis. The particular technique we used was a modification of the gel retardation assay, in which we simply added 0.1% SDS both in the gel and in the running buffer. The crude membrane extracts were solubilized with 1% SDS for 1 hr at room temperature in the absence of reducing agents. These were found to be the best conditions for the penetration of the samples in the gels and for the keeping of the binding of [^{57}Co]BLM.

On the autoradiographies of the resulting gels (Fig.2 and 3), it can clearly be seen that we succeeded in labelling one protein of about 250kDa. This molecular weight was only an approximation because protein separation was not performed under totally

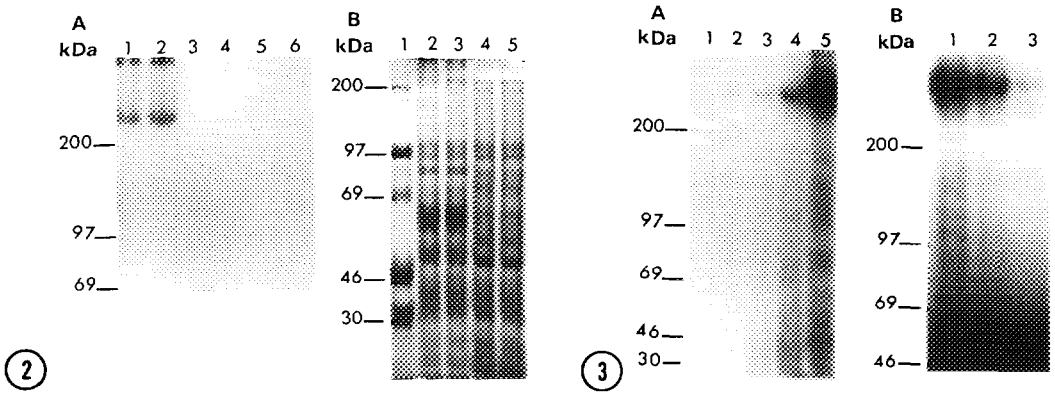

Figure 2 . Visualization of a membrane BLM-binding protein : (A) Crude cytosolic extracts (lanes 3 and 4, 60μg per well) or membrane extracts (lanes 1 and 2, 60μg per well) were prepared as described in Materials and Methods, then incubated with [^{57}Co]BLM, solubilized with 1% SDS and separated by gel electrophoresis. [^{57}Co]BLM alone was loaded as control in lane 6. (B) Coomassie Blue staining of a non-radioactive gel showing the cytosolic pattern (lanes 2 and 3) and the membrane protein pattern (lanes 4 and 5).

Figure 3 . Characterization of the BLM binding on the membrane BLM-binding protein : (A) Effect of the amount of membrane proteins loaded on the binding of BLM. (from lane 2 to 5: 25μg, 50μg, 100μg, 150μg). [^{57}Co]BLM alone was loaded as control in lane 1. (B) Competition between [^{57}Co]BLM and non-radioactive CoBLM. Ninety μg of membrane proteins was loaded in each well with 10μM [^{57}Co]BLM and non-radiolabelled CoBLM (lane 1: 0μM; lane 2: 50μM; lane 3: 500μM).

denaturing conditions. Indeed, the radiolabelled band was lost when the samples were heated in the presence of ß-mercaptoethanol prior to their loading into the gel (data not shown). However, the binding of BLM to this protein was certainly quite strong, because it was not lost even in the presence of 1% SDS. No band was detected in the cytosolic fraction (Fig 2.A), proving that the BLM-binding protein was exclusively a membrane protein. The Coomassie Blue staining of either cytosolic or membrane proteins is given in Fig 2.B. No major band seemed to correspond to the radiolabelled protein. A load of 25μg of membrane proteins was sufficient to detect the radiolabelled band (Fig 3.A). The intensity of the band did not increase linearly with the amount of loaded proteins (Fig 3.A), phenomenon of ligand retention (8). Indeed, the quantity of BLM retained per μg of proteins increased with the quantity of proteins in the sample (Fig 3.A). An excess of non-radioactive CoBLM was able to extinguish the radioactive band, suggesting that the labelling of the protein was specific (Fig 3.B).

DISCUSSION

Our results suggest that BLM is able to bind to a DC-3F cell plasma membrane protein of an apparent approximated molecular weight of 250kDa. The existence of this protein and the saturable binding curves shown on Fig 1 are in favor of the presence of BLM-binding sites at the surface of the cells. According to the presence of this BLM-

binding protein, the [^{57}Co]BLM association profile would indeed describe the classical binding of a ligand with its binding-site, and consequently, the plateau value would give the direct number of binding sites per cell (140,000 to 400,000, depending on the number of *in vitro* passages of the DC-3F cells), and $K_{1/2}$ would be a direct estimation of the affinity of BLM for its binding site (5μM for both the "young" and the "old" cells).

Membrane binding sites have often been described for large cytotoxic molecules such as neocarzinostatin (9) and ricin (10), and they have also been recently found for smaller molecules like verapamil (11) and antisense oligonucleotides (12). The common feature of these molecules, except verapamil, is that they do not diffuse through the plasma membrane. It is commonly admitted that these membrane sites intervene in the internalization of cytotoxic molecules like ricin (10,13). The BLM-binding sites we isolated on the plasma membrane of DC-3F cells may then play a part in the internalization of BLM. This is in agreement with other results from our laboratory suggesting that BLM is unable to diffuse through the plasma membrane (3), and with the fact that the "old" cells, which possess less BLM-binding sites at their surface, are slightly less sensitive to BLM that the "young" cells (data not shown). This indicates that the cytotoxicity of the drug could directly depend on the binding of BLM to its membrane sites. The identification of this protein would be of great interest in the comprehension of the internalization pathway used by the bleomycin family of antibiotics.

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