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Biochemical and Microscopic Evidence for the Internalization of Drug-Containing Mast Cell Granules by Macrophages and Smooth Muscle Cells

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During mast cell degranulation the soluble component of the granule is released into extracellular fluid, whereas two neutral proteases and heparin proteoglycans form the extracellular granule remnants. These structures are negatively charged and bind with high affinity LDL and other basic molecules.

In this study we show that granule remnants expelled into extracellular fluid are able to bind the aminoglycoside antibiotic gentamicin and the anticancer agent doxorubicin in a dose-dependent manner. In addition, granule remnants loaded with the two basic substances are subsequently phagocytosed by macrophages. Indeed, when cells are incubated for 24 h with 1 mg/ml gentamicin, the intracellular concentration of the drug, which in basal conditions is extremely low, increases significantly in the presence of degranulating mast cells (from 5.1 ± 1.0 to 25.4 ± 2.5 μg/mg protein) and a good correlation between histamine release and gentamicin uptake is evident. The antineoplastic agent doxorubicin can penetrate cells by passive diffusion; however, when mast cells are added to macrophage monolayer, incubated for 30 min with 50 μM of the antineoplastic agent, a significant increase in intracellular doxorubicin concentration is observed (from 3.5 ± 0.2 to 4.7 ± 0.2 μg/mg protein).

Internalization of granule remnants carrying gentamicin or doxorubicin is also evident in smooth muscle cells of the synthetic phenotype. In particular, when smooth muscle cells are incubated for 24 h with 1 mg/ml gentamicin, addition of isolated granules increases the uptake from 2.4 ± 0.2 to 4.8 ± 0.4 μg/mg protein. Similar results are obtained in smooth muscle cells incubated for 4 h with doxorubicin 50 μM (from 3.3 ± 0.2 to 4.8 ± 0.5 μg/mg protein). Data are confirmed by microscopic experiments by means of fluorescence microscopy and electron microscopic studies.

The study demonstrates that basic substances can enter phagocytic cells when loaded to granule remnants. The phenomenon can be of particular interest for substances like the aminoglycosides that do not cross biological membranes; indeed, the storage of these antibiotics in phagocytic cells could have important consequences on their antibacterial activity *in vivo.* **Macrophages and smooth muscle cells can also act as a reservoir for doxorubicin. High concentrations of the antineoplastic agent in these cells could be responsible for toxicity, as well as play an important role in the transport of the drug to tumor cells.** © 2000 Academic Press

Key Words: **phagocytic cells; mast cells; granule remnants; gentamicin; doxorubicin.**

Mast cell activation and consequent extrusion of cytoplasmic granules is extremely important in a variety of pathological and physiological events, including inflammatory reaction, immediate hypersensitivity, cytotoxicity, angiogenesis, fibrosis, and atherosclerosis. During the degranulation process, the soluble components of the granule, i.e., histamine, chondroitin sulphate proteoglycans, and a fraction of their heparin proteoglycans are solubilized and released from the granules into the extracellular fluid. In contrast, two neutral proteases, chymase and carboxypeptidase A, and the major fraction of heparin proteoglycans remain tightly bound to each other, forming extracellular granule remnants. It has been previously shown that the granule remnants bind with high-affinity LDL (Kokkonen and Kovanen, 1987a) and this binding is mediated by ionic interactions between the positively charged amino acids and the negatively charged groups of the heparin glycosaminoglycan chains of the granule remnants. In addition to lipoproteins, exocytosed mast cell granules bind other molecules that show high affinity for heparin, such as fibronectin, lipoprotein lipase (Kokkonen and Kovanen, 1987a), platelet factor 4 (McLaren and Pepper, 1983), aminoglycoside antibiotics (Decorti *et al.*, 1997a), and doxorubicin (Crivellato *et al.*, 1997). As a consequence of exocytosis, granules expelled from mast cells are ultimately phagocytosed and degraded by scavenger cells such as macrophages (Lindahl *et al.*, 1979). In particular it has been shown (Kokkonen and Kovanen, 1987b; Kokkonen, 1989) that LDL-bearing granule remnants are taken up by nonspecific phagocytosis and that the rate of uptake of

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the granule remnants is the same whether or not they carry LDL.

The present study was designed to verify if granule remnants carrying other positively charged drugs could be taken up by phagocytic cells of the body. This mechanism of entrance into cells could be particularly important for those drugs that do not cross biological membranes and hence in normal conditions do not enter most cells.

The aminoglycosides are polybasic due to their amino side chains and thus are polycationic at physiological pH and do not cross biological membranes. Despite the introduction of newer, less toxic antibiotics, aminoglycosides remain a mainstay of therapy for serious gram-negative infections. The aim of our study was therefore to verify if gentamicin, the most widely used of these antibiotics, could be taken up by phagocytic cells when bound to granule remnants. The study was performed on mice peritoneal macrophages. In addition, the uptake of gentamicin-bound granules was studied also in smooth muscle cells obtained from the intima and media of the rabbit aorta, which are also able to phagocytate granule remnants (Wang *et al.,* 1995).

Another antibiotic that, due to its positive charge, binds to the granule remnants is doxorubicin (Crivellato *et al.,* 1997). Doxorubicin easily forms an ionic complex with heparin (Aoyama *et al.,* 1987; Cofrancesco *et al.,* 1980; Foa *et al.,* 1983), with one molecule of heparin binding 16 molecules of doxorubicin (Aoyama *et al.,* 1987); hence, it is not surprising that the drug binds to heparin proteoglycan of mast cell granules.

In this study, the possible phagocytosis of doxorubicin-carrying remnants was studied in peritoneal macrophages as well as in smooth muscle cells. Indeed, during therapy, the patient's vasculature is exposed to high levels of doxorubicin, and it has been suggested that, in addition to the effects on cardiac muscle, doxorubicin may have effects on vascular smooth muscle at doses that produce no sign of cardiotoxicity (Dalske and Hardy, 1988). Moreover, it is noteworthy that doxorubicin has been shown to induce a marked non-cytotoxic histamine release from rat peritoneal (Crivellato *et al.,* 1999; Decorti *et al.,* 1986) and cardiac (Decorti *et al.,* 1997b) mast cells.

The incorporation of drug-carrying remnants in phagocytic cells present in various tissues could represent a peculiar modality of internalization and intracellular repository of basic drugs. The phenomenon could lead to long-term toxicity and/or to a modified pharmacological effect.

MATERIALS AND METHODS

Animals. Wistar rats (200–400 g) and CD1 male mice (20–30 g) belonging to a local conventional breeding colony, and New Zealand white rabbits (4 to 8 weeks) obtained from Harlan, Italy, were used. Animals were housed at 20°C on a 12-h light/dark cycle and had free access to both food and water.

Cell isolation and culture. Macrophages were harvested from anesthetized CD1 male mice using the method of Kokkonen and Kovanen (1987b).

Arterial smooth muscle cells were isolated from rabbits using the method of Wang *et al.* (1995). To obtain smooth muscle cells of the synthetic phenotype, cells were seeded at a density of 10^5 cells/ml in medium A (RPMI 1640 culture medium containing 2 mM L-glutamine, 20% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) and at confluence were subcultured (1:2) for up to five to nine passages.

Rats were intraperitoneally injected with 20 ml of buffered saline solution at 37°C. The whole fluid was collected from the abdominal cavity and cells were suspended in balanced salt solution containing 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, and 10 mM Hepes, supplemented with 0.2% bovine serum albumin (BSSA). The solutions were adjusted to pH 7.4 with NaOH before use. Mast cells were purified from peritoneal fluid with the method of Lagunoff and Rickard (1987). The lavage from five or six animals was centrifuged at 150*g* for 8 min at 4°C, and the pellet of cells was resuspended in 4 ml of ice-cold BSSA The cell suspension was layered over 10 ml of Percoll suspension (7 ml Percoll, density 1.130 g/ml; 1 ml salt solution consisting of 1.54 M NaCl, 27 mM KCl, and 6.8 mM $CaCl_2$; 0.8 ml water; 0.14 ml Path-o-cyte 4, adjusted to pH 7.2 with 0.1 M KH_2PO_4) in a 40-ml conical glass centrifuge tube. The mast cells, sedimented to the bottom of the tube on centrifugation for 20 min at 225*g* at 4°C, were collected, the pellet was gently resuspended in 10 ml of BSSA, and the mast cells were sedimented at 150*g* for 5 min to remove Percoll. This wash was repeated once. The purity of the mast cells was greater than 90% in the final preparation, as determined by staining with toluidine blue. For isolation of mast cell granules, 1.5×10^6 mast cells were preincubated for 15 min at 37°C in BSSA, and then stimulated by addition of compound 48/80 (5 μg/ml), and incubation was continued for 15 min to allow completion of mast cell degranulation as described by Lindstedt *et al.* (1993). The supernatant containing the secreted granules was removed and centrifuged at 12,000*g* for 15 min to sediment the granule remnants. The concentration of granule remnants used in the experiments is expressed in terms of remnant proteins.

Uptake experiments. For experiments, macrophages were seeded at a density of 3×10^6 cell/ml in 1 ml of EBME medium containing 20% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, in 35-mm dishes, and incubated overnight under an atmosphere of 95% air and 5% CO_2. Smooth muscle cells at the sixth passage were seeded at a density of 10^5 cells/ml in 35-mm dishes in 2 ml of medium A, under an atmosphere of 95% air and 5% CO_2 at 37°C.

After 24 h (macrophages) or 72 h (smooth muscle cells), the dishes were washed twice with 4 ml of PBS at 4°C, and then 2 ml of medium containing the drugs was added in the presence or absence of intact mast cells (6×10^5 cells/well) or of granule remnants (20 μg/well) and the incubation continued for predetermined times at 37°C.

After incubation, the medium was removed, and dishes were rinsed three times with ice-cold PBS. The cells were scraped with a rubber policeman into 2 ml of ice-cold PBS. The dishes were then rinsed again with 4 ml of PBS to improve the recovery. The cells obtained from both procedures were centrifuged at 4°C for 5 min at 150*g*. The supernatants were aspirated and the cell pellet was resuspended gently in 6 ml of ice-cold PBS and centrifuged again. To evaluate gentamicin uptake, the final pellet was resuspended in 0.5 ml of PBS and the cells were homogenized with a sonicator. Gentamicin concentrations were determined in the whole homogenate by an immunoenzymatic technique (EMIT AMD, Bracco, Italy).

To evaluate doxorubicin uptake, the cell pellet was resuspended in 2 ml of 0.3 N HCl in 50% ethanol, mixed thoroughly in a vortex mixer, and centrifuged at 700*g* for 10 min at 4°C. Doxorubicin content in the supernatant fraction was determined fluorimetrically with the method of Bachur *et al.* (1970). Standard curves of doxorubicin dissolved in 0.3 N HCl/50% ethanol were used for computation of doxorubicin content.

Histamine was determined by the fluorimetric method of Shore *et al.* (1959). Protein concentration of samples was determined by the method of Lowry *et al.* (1951).

Statistical analysis. Averages ± SE of the means were calculated on data from three to six replicates from a single typical experiment; statistical eval-

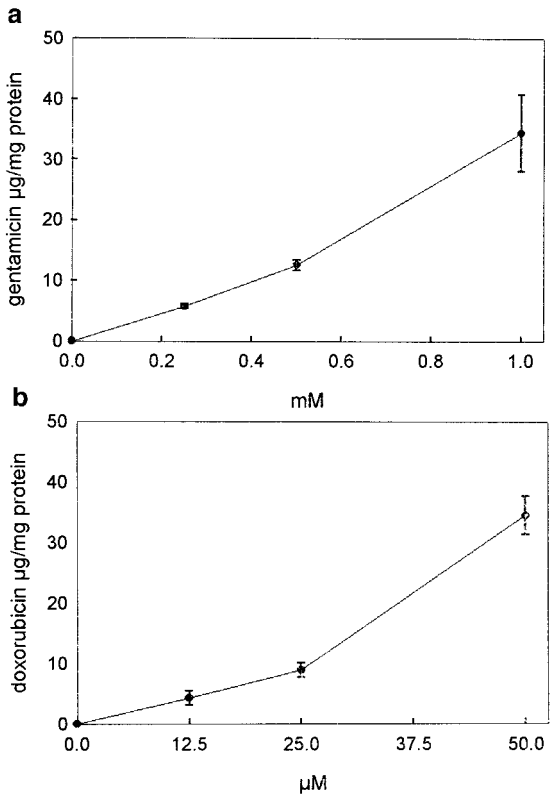

FIG. 1. Binding of basic molecules to extruded mast cell granules. Granules were obtained by stimulation of mast cells with 5 μg/ml compound 48/80 for 15 min at 37°C. The assay was performed in BSSA containing 20 μg/ml granules and different concentrations of gentamicin (a) or doxorubicin (b). After incubation for 10 min at 37°C, granules were centrifuged and the concentrations of the two drugs were assessed as described under Materials and Methods. Each point represents the mean ± SE of data from three to six determinations from a typical experiment.

uation of results was carried out using Student's t test for independent samples. All experiments were repeated at least three times. Values of $p < 0.05$ were considered significant.

Microscopic studies. Mouse peritoneal macrophages and rabbit smooth muscle cells were incubated for 4 h at 37°C with doxorubicin (100 μg/ml) in the presence of rat peritoneal mast cells or mast cell granules, respectively, and observed under a Zeiss Axioskop microscope. The instrument contained two illumination sources, a tungsten bulb for bright-field observation and a mercury lamp (Osram HBO 100 W, Berlin-München, Germany) for epifluorescence examination. The fluorescence setting was equipped with fluorescein/rhodamin optics. Cells were studied using a 100× Plan-Neofluar N.A. 1.30 objective at oil immersion, by alternate bright-field and fluorescence observations. At least 100 cells were examined for each experiment.

For electron microscopic investigations, the cells, treated as above, were fixed with 2.5% glutaraldehyde in phosphate-buffered solution, pH 7.4, at 4°C for 1 h and postfixed with 1% osmium-tetroxide at 4°C for 1 h. The pellet was dehydrated through graded ethanol, infiltrated with propylene oxide and epoxy resin, and embedded in Epon 812. Thin sections were counterstained with a uranyl acetate and lead citrate sequence and observed in a Philips CM12 electron microscope at 80 kV.

Chemicals. Doxorubicin, gentamicin, compound 48/80, avidin, and cytochalasin B were purchased from Sigma Chemical Co. (St. Louis, MO). Percoll and Path-o-cyte 4 were purchased from Pharmacia and Miles Scientific, respectively. All other chemicals were of analytical grade.

RESULTS

Mast cells were treated with compound 48/80 (5 μg/ml) to induce noncytotoxic degranulation; the granule remnants expelled into extracellular fluid accumulated gentamicin as well as doxorubicin in a dose-dependent manner (Figs. 1a and 1b). This is not unexpected, as it has been demonstrated that the major components of the remnants bind many different positively charged molecules by ionic interactions.

Figure 2 shows the intracellular gentamicin concentrations of macrophages incubated for 24 h with mast cells. In basal conditions intracellular gentamicin concentrations were, as expected, extremely low. When mast cells were induced to exocytate with compound 48/80, a significant increase in gentamicin intracellular concentrations was observed. Incubation with avidin, a compound with strongly positive charges that could compete with gentamicin for binding to the granule remnants, significantly reduced gentamicin uptake. When increasing concentrations of compound 48/80 were used, a good correlation between histamine release and gentamicin uptake was evident (Figs. 3a and 3b).

Similar results were obtained with the anticancer drug doxorubicin (Fig. 4). Basal doxorubicin concentrations were not, however, as low, as this drug can enter cells by passive diffusion. Addition of mast cells to macrophages caused a significant increase in the uptake. In this case, however, it was not necessary to add the secretagogue compound 48/80, as doxorubicin is by itself a histamine-releasing agent (Decorti *et al.,* 1986). Incubation of macrophages with cytochalasin B, an inhibitor of phagocytosis (Axline and Reaven, 1974), reduced doxorubicin concentrations to levels observed in samples without mast cells (Fig. 4).

To study the uptake of drugs by smooth muscle cells of the synthetic phenotype, the cells were used after they have been subcultured at least five times (Wang *et al.,* 1995). Indeed, the change from contractile to synthetic phenotype in smooth

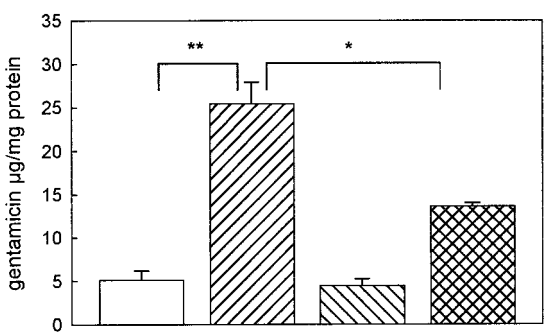

FIG. 2. Gentamicin concentrations in macrophages and mast cells incubated for 24 h with 1 mg/ml gentamicin (□), gentamicin plus 5 μg/ml compound 48/80 (▨), gentamicin plus 400 μg/ml avidin (▧), or gentamicin plus compound 48/80 plus avidin (▩). Each bar represents the mean ± SE of data from three to six determinations from a typical experiment. *$p < 0.05$, **$p < 0.01$, Student's t test for independent data.

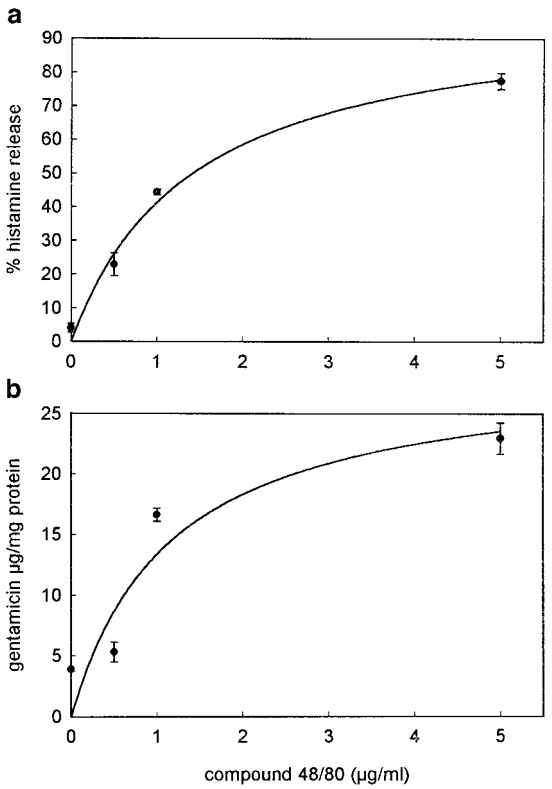

FIG. 3. Degranulation of mast cells (a) and gentamicin intracellular concentrations (b) as function of 48/80 concentrations. Compound 48/80 was added to monolayers of macrophages containing mast cells and 1 mg/ml gentamicin and incubated for 24 h. Each point represents the mean ± SE of data from three to six determinations from a typical experiment.

muscle cells has been found to be irreversible after subculturing aortic cells five times, corresponding to at least eight cell doublings (Chamley-Campbell and Campbell, 1981). Similar to macrophages, smooth muscle cells of the synthetic phenotype were capable of taking up higher concentrations of drugs

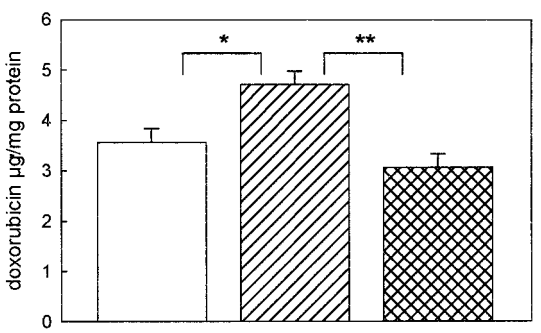

FIG. 4. Doxorubicin concentrations in macrophages incubated for 30 min with 50 μM of the antineoplastic agent (□), with doxorubicin and mast cells (▨), or with doxorubicin, mast cells, and 10 μg/ml cytochalasin B (▩). Each bar represents the mean ± SE of data from three to six determinations from a typical experiment. *$p < 0.05$; **$p < 0.01$, Student's t test for independent data.

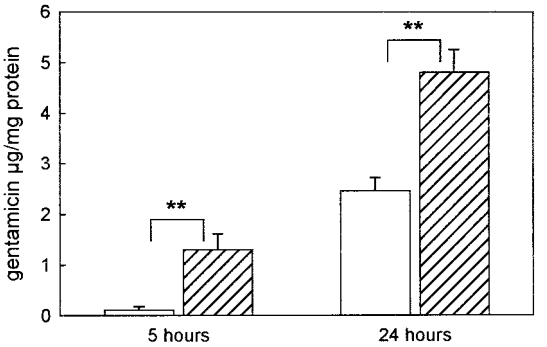

FIG. 5. Gentamicin concentrations in smooth muscle cells of the synthetic phenotype incubated for 5 or 24 h with 1 mg/ml gentamicin (□) or gentamicin plus isolated granules (▨). Each bar represents the mean ± SE of data from three to six determinations from a typical experiment. **$p < 0.01$, Student's t test for independent data.

when incubated with granule remnants. The phenomenon is evident for gentamicin (Fig. 5) as well as for doxorubicin (Fig. 6).

Data were confirmed also by microscopical observations. Indeed, when mouse peritoneal macrophages were coincubated with rat peritoneal mast cells, treated with doxorubicin, and observed by fluorescence microscopy, numerous spherical yellow-reddish intensely fluorescent structures, showing the same size and microscopical appearance of peritoneal mast cell granules, could be observed in the macrophage cytoplasm (Fig. 7a). Electron microscopy strongly suggested that these structures were phagocytosed mast cell granules (Fig. 7b); they still displayed the particulate pattern of mast cell granular matrices, despite advanced proteolytic digestion. This intracytoplasmic, membrane-bound material can be easily differentiated from other granular or vacuolar material because it retains many ultrastructural characteristics of released mast cell granules. Granule remnants exhibit a unique dotted and/or reticulate architecture, consisting mostly in a reticular framework made up of minute electron-dense particles joined together by fila-

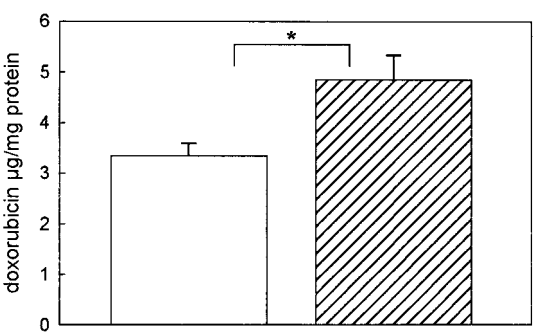

FIG. 6. Doxorubicin concentrations in smooth muscle cells of the synthetic phenotype incubated for 4 h with 50 μM doxorubicin (□) or doxorubicin plus isolated granules (▨). Each bar represents the mean ± SE of data from three to six determinations from a typical experiment. *$p < 0.05$, Student's t test for independent data.

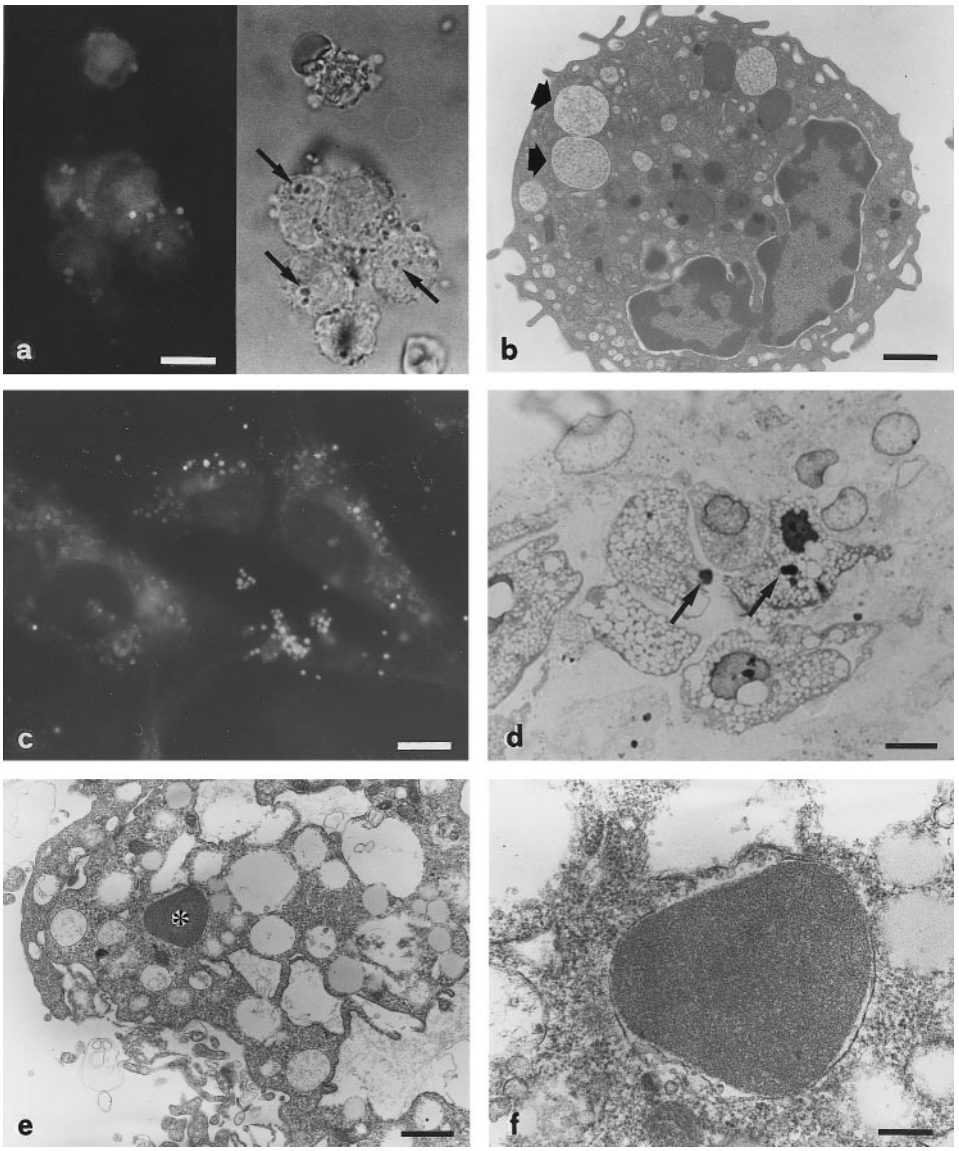

FIG. 7. Microscopical and ultrastructural appearance of mouse peritoneal macrophages and smooth muscle cells of the synthetic phenotype actively taking up doxorubicin-loaded mast cell granules. (a) Paired fluorescent and bright-field photographs showing fluorescent granules (arrows) phagocytosed by macrophages. (b) Electron microscopy allows recognition of typical mast cell granule remnants in the cytoplasm of this macrophage (arrows). (c) After exposure to doxorubicin-containing mast cell granules, smooth muscle cells present numerous fluorescent structures filling their cytoplasm. (d) Toluidine blue-stained semithin section that confirms the presence of metacromatic granules inside smooth muscle cell cytoplasm (arrows). (e) Electron microscopy allows identification of phagocytosed structures (asterisk) showing the characteristic texture of mast cell granules. The structure presented in f is a higher magnification of the granule in e. Bars represent 5 μm (a, c, and d); 1 μm (b, e); and 0.25 μm (f).

mentous material, which are peculiar for mast cell granules (Dvorak, 1991). The ultramicroscopic evaluation of 100 cells in the control group and 100 in the mast cell-treated group demonstrated the complete absence of granular structures in control cells and the presence of typical granular material in more than 90% of cells conincubated with mast cells, confirming, therefore, the nature of these inclusions.

The incorporation of drug-carrying remnants was also verified in smooth muscle cells of the synthetic phenotype by fluorescence microscopic and electron microscopic studies. When these cells were incubated with doxorubicin-loaded mast cell granules and examined by fluorescence microscopy, a great number of small, round, intensely fluorescent structures could be observed filling the cell cytoplasm (Fig. 7c). These structures showed the same dimension as well as the same fluorescence properties and microscopic appearance of rat peritoneal mast cell granules after exposure to doxorubicin. They could be found not only inside the cell cytoplasm but also apparently attached to the cell surface or free floating in the medium.

Examination by light microscopy of smooth muscle cell preparations revealed large polyhedrical or spindle-shaped elements, whose cytoplasm presented a vacuolated, foamy appearance. Exposure of these cells to mast cell granules caused the intracytoplasmic presence of 1–2-μm diameter, round to oval, metachromatic granules (Fig. 7d). Observed by electron microscopy, these granules exhibited the general ultrastructural characteristics of rat peritoneal mast cell granules (Fig. 7e). They were surrounded by an endocytic membrane and showed the typical features of loose-textured granular matrices (Fig. 7f). Similarly to macrophages, these granular structures were observed in more than 90% of granule-treated cells and were completely absent in control samples.

DISCUSSION

The present study demonstrates that rat peritoneal mast cells are able to promote accumulation of some basic drugs in mouse peritoneal macrophages and in rabbit smooth muscle cells *in vitro*. Under normal conditions, granules are located in the mast cell cytoplasm; after stimulation, and subsequent degranulation, the granules, which are negatively charged due to their heparin glycosaminoglycan chains, can interact with basic substances in the extracellular fluid. It has been shown (Kokkonen and Kovanen, 1987) that the granule remnants derived from extruded granules of stimulated mast cells bind LDL, and the binding sites are part of the heparin proteoglycan molecules. Phagocytosis of mast cell granule remnant-bound LDL by macrophages and smooth muscle cells of the synthetic phenotype results in the formation of typical foam cells, filled with cytoplasmic cholesteryl ester droplets (Wang *et al.,* 1995), a major cellular component of the early stage of atherosclerotic lesions. Besides LDL, exocytosed mast cell granules bind several other cationic molecules; indeed, it has been shown that fibronectin and lipoprotein lipase, two proteins with affinity for heparin, are also bound by granule remnants (Kokkonen and Kovanen, 1987). Immunoelectron microscopic studies have revealed the presence of platelet factor 4, a protein with clusters of positively charged amino acids, in mast cell granules in human skin and breast tissue (McLaren and Pepper, 1983). In previous studies we have shown that the aminoglycosides gentamicin and tobramycin (Decorti *et al.,* 1997a), as well as the antineoplastic agent doxorubicin (Crivellato *et al.,* 1997; Decorti *et al.,* 1989), bind to mast cell granules as a consequence of exocytosis. Doxorubicin is a basic compound which forms an ionic complex with heparin (Aoyama *et al.,* 1987; Cofrancesco *et al.,* 1980; Foa *et al.,* 1983), and it has been shown (Crivellato *et al.,* 1997) that the drug binds to the granule remnants extruded into the extracellular fluid, as well as to the granules remaining inside the mast cells. To stimulate mast cells and induce their degranulation, we used the well-known secretagogue compound 48/80. This compound was not used in the experiments with doxorubicin, as this anticancer drug is a secretagogue by itself, and, at the concentrations employed in this study, induces a significant histamine release. The histamine-releasing action of doxorubicin has many features in common with that induced by compound 48/80 and other basic mast cell secretagogues (Decorti *et al.,* 1986). Indeed, the release is not sustained by extracellular calcium but is largely dependent on intracellular stores of this cation, is blocked by extremes of temperature, and is very rapid and virtually complete within 10 s. A variety of stimuli, whether immunological or nonimmunological, are known to activate the mast cells and cause their degranulation; however, the process of mast cell degranulation is the same irrespective of the type of stimulus (Galli *et al.,* 1984). Any degranulating stimulus probably induces the same sequence of events that finally leads to the uptake of drug-loaded remnants by phagocytic cells.

This phenomenon seems of particular interest for the aminoglycosides; indeed, these antibiotics are highly polar cations and hence do not cross biological membranes and are largely excluded from most cells. The intracellular concentration of gentamicin was indeed extremely low when macrophages or smooth muscle cells were incubated for long periods of time with this agent and resting mast cells. However, when mast cells were stimulated to exocytate, the intracellular concentration of the aminoglycoside rose sharply. The storage for long periods of the aminoglycosides in phagocytic cells, where they normally cannot penetrate, could have important consequences on their antibacterial activity *in vivo*. On the other hand, the increase in intracellular delivery of gentamicin could improve the therapeutic effect on intracellular infections.

Phagocytosis has been also observed in several types of muscle cells. Garfield *et al.* (1975), comparing three types of muscle cells, found that the aortic smooth muscle cells of the contractile phenotype were more active in phagocytosing latex particles than skeletal or cardiac muscle cells. Blaes *et al.* (1982) have found that smooth muscle cells of the synthetic phenotype from rat aorta phagocytosed carbon particles more actively than fibroblasts derived from rat skin. Finally, Wang *et al.* (1995) have demonstrated that smooth muscle cells of the synthetic phenotype derived from rabbit aorta phagocytose mast cell granule remnants more actively than their contractile counterpart. Hence, smooth muscle cells of the synthetic counterpart were chosen in this study as, among muscle cells, they have the highest phagocytic capacity.

Mast cells are ubiquitous cells, are present in the intima of the human aorta (Kamio *et al.,* 1979), and are particularly abundant in areas of inflammation. In these areas, in addition to mast cells, which are often activated, i.e., degranulated, T lymphocytes and macrophages are also present, both of which secreted factors that can stimulate mast cells to degranulate. Exocytosed granules are quite large and remain in the microenvironment of their parent cells; hence, the granule-mediated phagocytosis of drugs must be a local phenomenon restricted to those tissues in which degranulation occurs. However, as smooth muscle cells are able to phagocytate doxorubicin-loaded granules extruded from mast cells located in the arterial

wall, the anticancer drug could accumulate at high concentrations in these cells and hence be responsible for toxicity. Cardiotoxicity is the main toxic effect of doxorubicin; however, besides the effects on cardiac mast cells, the drug acts on vascular smooth muscle even at doses that produce no sign of cardiotoxicity (Dalske and Hardy, 1988).

In addition, macrophages and smooth muscle cells can act as reservoirs for the antineoplastic drug. This should be particularly important for macrophages, as these cells seem to have an important role in tumor cell destruction and it has been demonstrated (Haskill, 1981) that macrophages loaded with cytoplasmic doxorubicin-containing inclusions play an important role in the transport of the drug to tumor cells.

These data demonstrate a peculiar modality of entrance of basic molecules into phagocytic cells by means of granule remnants extruded from activated mast cells. The phenomenon is particularly relevant for those substances charged at physiological pH and hence unable to cross biological membranes and could lead to long-term toxicity and/or to a modified pharmacological effect.

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REFERENCES

Aoyama, T., Horioka, M., and Nagamitus, S. (1987). Macromolecular complex of drugs. 1. Doxorubicin–heparin complex. *Chem. Pharm. Bull.* **35,** 808–813.

Axline, S. G., and Reaven, E. P. (1974). Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. Role of subplasmalemmal microfilaments. *J. Cell Biol.* **62,** 647–659.

Bachur, N. R., Moore, A. L., Bernstein, J. G., and Liu, A. (1970). Tissue distribution and disposition of daunomycin (NSC-82151) in mice: Fluorometric and isotopic methods. *Cancer Chemother. Rep.* **54,** 89–94.

Blaes, N., Crouzet, B., Bourdillon, M. C., and Boissel, J. P. (1982). Comparative phagocytosis in culture of aortic smooth muscle cells and fibroblasts from rat. *Proc. Soc. Exp. Biol. Med.* **170,** 453–458.

Chamley-Campbell, J. H., and Campbell, G. R. (1981). What controls smooth muscle phenotype? *Atherosclerosis* **40,** 347–357.

Cofrancesco, E., Vigo, A., and Pogliani, E. (1980). Anti heparin activity of Adriamycin. *Thromb. Res.* **18,** 743–746.

Crivellato, E., Candussio, L., Decorti, G., Klugmann, F. B., and Baldini, L. (1997). Adriamycin binds to the matrix of secretory granules during mast cell exocytosis. *Biotech. Histochem.* **72,** 111–116.

Crivellato, E., Candussio, L., Decorti, G., Klugmann, F. B., and Mallardi, F. (1999). Adriamycin induces exocytosis in rat and beige mouse peritoneal mast cells: An ultrastructural, morphometric and biochemical study. *J. Submicrosc. Cytol. Pathol.* **31,** 279–286.

Dalske, H. F., and Hardy, K. (1988). Effect of low dose doxorubicin on calcium content and norepinephrine response in rat aorta. *Eur. J. Cancer Clin. Oncol.* **24,** 979–983.

Decorti, G., Bartoli Klugmann, F., Candussio, L., and Baldini, L. (1986). Characterisation of histamine secretion induced by anthracyclines in rat peritoneal mast cells. *Biochem. Pharmacol.* **35,** 1939–1942.

Decorti, G., Bartoli Klugmann, F., Candussio, L., Furlani, A., Scarcia, V., and Baldini, L. (1989). Uptake of adriamycin by rat and mouse mast cells and correlation with histamine release. *Cancer Res.* **49,** 1921–1926.

Decorti, G., Candussio, L., Klugmann, F. B., and Baldini, L. (1997a). Binding of aminoglycoside antibiotics by degranulating mast cells. *Chemotherapy* **43,** 36–42.

Decorti, G., Candussio, L., Klugmann, F. B., Strohmayer, A., Mucci, M. P., Mosco, A., and Baldini, L. (1997b). Adriamycin-induced histamine release from heart tissue in vitro. *Cancer Chemother. Pharmacol.* **40,** 363–366.

Dvorak, A. M. (1991). *Basophil and Mast Cell Degranulation and Recovery: Blood Cell Biochemistry*, Vol. IV. Plenum Press, New York.

Foa, P., Cofrancesco, E., Lombardi, L., Colombi, M., Pogliani, E. M., and Polli, E. E. (1983). Non interference by heparin with the cytostatic effect of adriamycin: An in vitro study on a human promyelocytic leukemia cell line. *Br. J. Cancer* **48,** 735–738.

Galli, S. J., Dvorak, A. M., and Dvorak, H. F. (1984). Basophils and mast cells: Morphologic insights into their biology, secretory patterns, and function. *Prog. Allergy* **34,** 1–141.

Garfield, R. E., Chacko, S., and Blose, S. (1975). Phagocytosis by muscle cells. *Lab. Invest.* **33,** 418–427.

Haskill, J. S. (1981). Adriamycin-activated macrophages as tumor growth inhibitors. *Cancer Res.* **41,** 3852–3856.

Kamio, A., Taguchi, T., Shiraishi, M., Shitama, K., Takebayashi, S., Cleveland, J. C., and Kummerow, F. A. (1979). Mast cells in human aorta. *Arterial Wall* **5,** 125–136.

Kokkonen, J. O. (1989). Stimulation of rat peritoneal mast cells enhances uptake of low density lipoproteins by rat peritoneal macrophages in vivo. *Atherosclerosis* **79,** 213–223.

Kokkonen, J. O., and Kovanen, P. T. (1987a). Low-density-lipoprotein binding by mast-cell granules. Demonstration of binding of apolipoprotein B to heparin proteoglycan of exocytosed granules. *Biochem. J.* **241,** 583–589.

Kokkonen, J. O., and Kovanen, P. T. (1987b). Stimulation of mast cells leads to cholesterol accumulation in macrophages in vitro by a mast cell granule-mediated uptake of low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **84,** 2287–2291.

Lagunoff, D., and Rickard, A. (1987). Methods for the study of rat peritoneal mast cell secretion. In *In Vitro Methods for Studying Secretion. The Secretory Process* (A. M. Poisner, J. M. Trifarò, Eds.), pp. 13–28. Elsevier, Amsterdam.

Lindahl, U., Pertoft, H., and Seljelid, R. (1979). Uptake and degradation of mast-cell granules by mouse peritoneal macrophages. *Biochem. J.* **182,** 189–193.

Lindstedt, K. A., Kokkonen, J. O., and Kovanen, P. T. (1993). Inhibition of copper-mediated oxidation of LDL by rat serosal mast cells. A novel cellular protective mechanism involving proteolysis of the substrate under oxidation stress. *Arterioscler. Thromb.* **13,** 23–32.

Lowry, O. H., Rosenbrough, N. J., Fark, A. L., and Randell, L. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

McLaren, K. M., and Pepper, D. S. (1983). The immunoelectronmicroscopic localization of human platelet factor 4 in tissue mast cells. *Histochem. J.* **15,** 795–800.

Shore, P. A., Burkhalter, A., and Cohn, V. H. (1959). A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.* **127,** 182–186.

Wang, Y. F., Lindstedt, K. A., and Kovanen, P. T. (1995). Mast cell granule remnants carry LDL into smooth muscle cells of the synthetic phenotype and induce their conversion into foam cells. *Arterioscler. Thromb. Vasc. Biol.* **15,** 801–810.