Uptake and Degradation of Mast-Cell Granules by Mouse Peritoneal Macrophages

By Ulf LINDAHL,* Håkan PERTOFT† and Rolf SELJELID‡

*Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences,
The Biomedical Centre, Box 575, S-751 23 Uppsala, Sweden,

†Department of Medical and Physiological Chemistry, University of Uppsala, The Biomedical Centre,
Box 575, S-751 23 Uppsala, Sweden,

and ‡Institute of Medical Biology, University of Tromsø, Tromsø, Norway

(Received 22 January 1979)

³⁵S-labelled mast-cell granules isolated from mouse mastocytomas were added to mouse macrophages *in vitro*. The granules were avidly phagocytosed, and subsequently the radioactivity was released to the medium as inorganic [³⁵S]sulphate. After pulse-labelling, a total of about 80% of the cell-associated radioactivity was thus released in the course of 24 h, indicating an extensive breakdown of the sulphated polysaccharides, mainly heparin, present in the granules. The uptake of the mast-cell granules caused pronounced, but reversible, spreading of the macrophages.

The glycosaminoglycan heparin occurs in a variety of animals, including species that are widely separated on the evolutionary scale (Cássaro & Dietrich, 1977). It has a number of striking biological effects, the best known of which is its ability to prevent the coagulation of blood [for a review, see Rosenberg (1977)]. However, these effects have invariably been observed as a result of adding exogenous heparin to a biological system, *in vitro* or *in vivo*. The biological function of endogenous heparin remains elusive.

Heparin is synthesized and stored in the mast cells, which are normal constituents of the loose connective tissues of the body. Under influence of certain immunological or pharmacological stimuli, heparin is released from the cells into the extracellular space. The fate of such extracellular endogenous heparin is unknown. In the connective tissue, the mast cells show a distribution similar to that of macrophages, which are specialized in recognizing and removing particulate matter. Recently, Fabian *et al.* (1978) showed that heparin was taken up and partly degraded by macrophages *in vitro*, and furthermore, that this uptake was greatly enhanced by basic polypeptides. They suggested that binding of heparin to a basic protein from eosinophils might similarly affect the interaction of heparin with macrophages *in vivo*. However, heparin already occurs as a complex with protein in the mast cell (the mast-cell granules), and observations by Yurt *et al.* (1977) indicate that this complex is maintained extracellularly after being discharged from the cell. The present study was therefore concerned with the

interaction between macrophages and mast-cell granules.

Materials and Methods

Materials

Macrophages were harvested from hybrid (DBA × C₃H) F₁ mice or from outbred NMRI mice by peritoneal lavage, essentially as described by Cohn & Benson (1965). Cold F 10 medium (2 ml) was injected into the peritoneal cavity of each mouse and the abdomen was massaged. The fluid was then withdrawn and heat-inactivated foetal-calf serum (Serva Feinbiochemica, Heidelberg, Germany) was added to a final concentration of 20% (v/v). Each mouse produced 4 × 10⁶–5 × 10⁶ cells. The cells were seeded in Linbro RM plastic plates (Linbro Scientific, Hamden, CT, U.S.A.) with glass coverslips at a density of 0.5 × 10⁶ cells/16 mm well or in 35 mm Falcon plastic Petri dishes (Gateway International, Los Angeles, CA, U.S.A.) at a density of 3 × 10⁶ cells/dish. After 2 h incubation at 37°C in an atmosphere of air/CO₂ (19:1), non-attached cells, about 50% of the total, were rinsed off and the adhered macrophages kept *in vitro* in F 10 medium with 20% heat-inactivated foetal-calf serum. The viability of the cells was better than 99% as judged by Trypan Blue exclusion. Experiments were always started with 1-day-old cultures. Mast-cell granules were added to the cultures without change of medium.

The density-gradient medium Percoll, Sephadex and Sepharose gels were obtained from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. DEAE-cellulose (DE-20) was a product of Whatman Biochemicals (Maidstone, Kent, U.K.). The heparin, chondroitin sulphate and papain used were as described previously (Jansson *et al.*, 1975). [^{35}S]sulphate (carrier-free) was a product of The Radiochemical Centre (Amersham, Bucks., U.K.).

Mast-cell granules were isolated from a mouse mastocytoma described previously (Ögren & Lindahl, 1971) by a modification of a procedure developed by S. Ögren & H. Pertoft (unpublished work). Two tumour-bearing mice were given 300 μCi each of inorganic [^{35}S]sulphate (carrier-free) by intraperitoneal injection. 3 h later the tumours (about 1 g of tissue/mouse) were removed and homogenized in 12 ml of ice-cold 0.25 M-sucrose with a Dounce glass homogenizer. All subsequent procedures were carried out in the cold ($+4^\circ\text{C}$). The homogenate was centrifuged at 800g (r_{av} , 24 cm; SW rotor) for 10 min and the pellet was discarded. The supernatant was collected and centrifuged at 40000g (r_{av} , 5.7 cm; 23.5° angle rotor) for 20 min. The pellet was resuspended in 4 ml of 0.25 M-sucrose and layered on top of two tubes each containing 8 ml of Percoll solution in 0.25 M-sucrose ($\rho = 1.065 \text{ g/ml}$). Centrifugation was performed for 30 min at 55000g (r_{av} , 7.9 cm; 14° angle rotor). The material was collected in fractions of about 0.5 ml each. Two peaks of radioactivity were obtained (Fig. 1) at buoyant densities of 1.04 g/ml and 1.08–1.10 g/ml respectively. The corresponding particulate material in the appropriate pooled fractions was washed twice in 0.25 M-sucrose and finally suspended in 0.4 ml of phosphate-buffered saline (0.15 M-NaCl in 50 mM-sodium phosphate buffer, pH 7.4). A 10 μl sample of the high-density material contained 10×10^3 – 15×10^3 c.p.m. of ^{35}S , 2–3 μg of uronic acid (corresponding to about 6–9 μg of polysaccharide) and 40–60 mg of protein. The low-density fraction usually contained about half as much ^{35}S and 6–10 times as much protein as the high-density fraction; the polysaccharide content of the low-density fraction could not be determined owing to anomalous brownish colour development in the carbazole reaction (probably due to excess amounts of protein or glycoprotein). The high-density fraction was used for all further experiments.

The ^{35}S -labelled polysaccharide was separated from protein by subjecting the granules to density-gradient centrifugation in caesium chloride/4 M-guanidinium chloride [loading concn. 40% (w/v) CsCl; $\rho = 1.45 \text{ g/ml}$], essentially as described previously (Jansson *et al.*, 1975). Analysis of the isolated polysaccharide by ion-exchange chromatography on DEAE-cellulose (Lindahl *et al.*, 1976) showed more than 90% of the ^{35}S label co-migrating with standard heparin, and, in addition, a small

Fig. 1. Isolation of mast-cell granules by density-gradient centrifugation

Density-gradient centrifugation in Percoll/0.25 M-sucrose of 40000g fraction from a homogenate prepared 3 h after injection of mastocytoma-bearing mice with [^{35}S]sulphate. Centrifugation was performed as described in the Materials and Methods section. Fractions were analysed for ^{35}S (○) and for density (●). Fractions 4–8 and 14–17 were pooled and the particulate material recovered as indicated in the Materials and Methods section.

amount of material in the elution position of chondroitin sulphate. After treatment with nitrous acid [the pH 1.5 procedure of Shively & Conrad (1976)] about 80% of the ^{35}S appeared in the elution position of disaccharides and inorganic sulphate on gel chromatography (Sephadex G-25). These results show that the labelled polysaccharide in the mast-cell granules was predominantly heparin. Gel chromatography of granule heparin on Sepharose 4B, as obtained either after centrifugation in caesium chloride or after papain digestion (Ögren & Lindahl, 1971) showed a polydisperse material, with a peak K_{av} of about 0.3.

Analytical methods

Uronic acid and protein were determined by the procedures of Bitter & Muir (1962) and Lowry *et al.* (1951) respectively. ^{35}S was measured in a Packard model 2409 liquid-scintillation spectrometer, with Insta-Gel (Packard Instrument Co., Downers Grove, IL, U.S.A.) as scintillation medium. Gel chromatography was performed on columns of Sephadex G-15 (1 cm $\times$ 140 cm) or G-25 (1 cm $\times$ 190 cm) eluted with 0.2 M- NH_4HCO_3 or on a column (1 cm $\times$ 94 cm) of Sepharose 4B eluted with 1 M-NaCl/0.05 M-Tris/HCl, pH 8.0. Densities of gradient media and granule fractions were measured in an organic-

density-gradient column (Oster, 1965) or by use of density marker beads (Pharmacia).

Morphological evaluation was performed in live cultures with a Leitz Diavert-type inverted microscope or in preparations made by fixation with 2.5% glutaraldehyde in 0.1M-phosphate buffer, pH 7.4, containing 0.1M-sucrose. Some of the preparations were mounted for phase-contrast microscopy, others were dehydrated, critical-point dried with a Hitachi HCP-1 apparatus (in CO₂), coated with gold (Polaron SEM coating unit E 5000), and examined with a Hitachi HHS/2R scanning electron microscope at 20kV and a tilt angle of 20°. Pictures were taken on Ilford FP4 film.

Results

Uptake of mast-cell granules by macrophages in vitro

Radioactively labelled granules were added to macrophage cultures in amounts corresponding to 2–3 µg of uronic acid/10⁶ cells. Cell-associated radioactivity reached a plateau corresponding to approx. 20% of total radioactivity after 1 h, and remained at the same value for the next 24 h (Fig. 2). The amount of radioactivity removable from the cell surface with trypsin (0.25% trypsin at 37°C in 20 min)

decreased gradually during the same period of time. The presence of 20 µg of soluble heparin (about three times the amount in the added granules) did not significantly affect granule uptake. Glass-associated radioactivity in dishes without macrophages was negligible. When the cells were first pulsed for 1 h with ³⁵S-labelled granules and then 'chased' for 4 h with unlabelled medium, trypsin treatment failed to remove significant amounts of radioactivity from the cells. These results suggest that the granules not only attached to the surface of the macrophages but were actually ingested by the cells.

In phase-contrast microscopy of living cultures the mast-cell granules could be seen shortly after addition as a finely granular material. In pulse-chase experiments very little granular material was seen in between the cells after 3–5 h of 'chase'. Scanning electron microscopy demonstrated that, 1 h after addition, granules were clearly discernible on the cell surfaces (Plates 1a and 1b). When non-associated granules were rinsed off and macrophages allowed to grow for an additional 2 h period in medium without granules, the number of visible granules had decreased, and after about 4 h no granules could be seen on the cell surface or elsewhere in the extra-cellular space (Plates 2a and 2b). About 1–4 h after the granules had been added, the macrophages spread extensively on the glass, and at the same time most of the protrusions normally found on the upper surface of these cells disappeared (Plates 2a and 2b).

Fig. 2. Uptake of mast-cell granules by macrophages in vitro

³⁵S-labelled mast-cell granules were added to macrophage cultures in amounts corresponding to 2–3 µg of uronic acid/10⁶ cells. At given time points the medium was removed, and the culture dishes were rinsed three times with warm phosphate-buffered saline (see the text). The cells were then incubated in 0.25% trypsin at 37°C for 20 min and rinsed another two times. They were subsequently lysed in 0.1M-NaOH. The trypsin-treated preparations (■) and cell lysates (□) were assayed for radioactivity. The results are expressed as a percentage of total radioactivity added to the cultures.

Fig. 3. Release of radioactivity from macrophages pulsed with ³⁵S-labelled mast-cell granules

Macrophages were incubated with ³⁵S-labelled mast-cell granules for 5 h, then rinsed and chased with medium without radioactivity for 24 h. Portions of the medium were assayed at the time points indicated and the results expressed as a percentage of total radioactivity in the cultures.

Fig. 4. Gel chromatography on Sephadex G-15 of ^{35}S -labelled material present in intact mast-cell granules (○) and released into the medium from macrophages pulse-labelled with mast-cell granules (●).

The pulse period was 5 h; medium was collected for analysis after 24 h of 'chase' incubation. Before gel chromatography both the intact granules and the chase medium were digested with papain, as described by Jansson *et al.* (1975).

Later, the macrophages tended to round up and the protrusions re-appeared. Cell death during the experiments was insignificant, and the number of macrophages detached was not appreciably greater than in control cultures where no mast-cell granules were added.

Degradation of mast-cell granules by macrophages

After incubation of macrophages with ^{35}S -labelled mast-cell granules for 24–48 h, more than 90% of the radioactivity was recovered in the fluid phase. These observations suggested that the granules taken up by the cells had been modified and that the polysaccharide component had been converted into products that were largely released into the medium. In a pulse-chase experiment the macrophages contained 40% of the added radioactivity after 5 h incubation with labelled granules. During subsequent chase incubation, 80% of this radioactivity was released from the cells over a 24 h period (Fig. 3). Analysis by gel chromatography (Fig. 4) and by high-voltage paper electrophoresis at pH 1.7 (1.6 M-formic acid; 40 V/cm) showed that the released labelled material was inorganic ^{35}S -sulphate; the near absence of ^{35}S -labelled polysaccharide in the chase medium indicated that cell death with subsequent release of intact granules did not take place to any significant extent. Similar analysis of a macrophage culture medium after 24 h continuous incubation

with ^{35}S -labelled mast-cell granules indicated inorganic ^{35}S -sulphate as the only radioactive component present in significant amounts. Control incubations performed in the absence of cells yielded no inorganic ^{35}S -sulphate; furthermore, the ^{35}S -heparin showed the same elution profile on gel chromatography (Sephadex 4B) as did the starting material. In a separate experiment, macrophages were cultivated for 24 h in F 10 medium with 20% foetal-calf serum but with no granules added. The medium from this culture was then removed and incubated for an additional 24 h with radioactive granules. Again, no inorganic ^{35}S -sulphate was formed, as shown by gel chromatography on Sephadex G-15. However, chromatography on Sephadex 4B showed that the polysaccharide had been to some extent depolymerized (probably by an endoglycosidase), eluting with a peak K_{av} of about 0.8. Thus under the conditions used, extensive degradation of heparin could only be performed by the intact macrophages and could not be effected by soluble factors released into the medium.

Discussion

Our experiments show that macrophages can phagocytose mast-cell granules *in vitro*. Since the two types of cells are close neighbours in the loose connective tissues of the body, it is highly likely that the same event takes place also *in vivo*. The fate of the ingested granules is unknown, especially as regards the protein components. However, the heparin constituent is obviously degraded; the extensive formation of inorganic sulphate suggests, by analogy with the mechanisms known to operate in the degradation of heparin sulphate (Neufeld, 1974), that the polysaccharide is not only desulphated but also depolymerized to monosaccharides. This degradation of heparin is far more efficient than any analogous process previously observed in a mammalian system (see Fabian *et al.*, 1978). Of particular interest are the results of Fabian *et al.* (1978), who studied the uptake and degradation of artificial N - ^{35}S -sulphoheparin-protein complexes by mouse peritoneal macrophages. Although the amount of heparin added per cell was only about one-third of that given in the present study, the maximal amount of ^{35}S -sulphate released never exceeded 6% of the total radioactivity. The mast-cell granule appears to be an efficient vehicle for the uptake and degradation of heparin by macrophages.

What is then the functional significance of the granule uptake? One obvious possibility would be that the macrophages simply act as scavengers and remove superfluous granules and thereby heparin. However, it is also possible that mast-cell granules may have a role in the regulation of some macrophage function. It is known that heparin accelerates

EXPLANATION OF PLATE 1(a)

Scanning electron micrograph of macrophage after 1 h incubation with mast cell granules

Granules corresponding to 2–3 μg of uronic acid/10⁶ cells were added as described in the Materials and Methods section. The cell shows the typical outline as well as surface morphology of a macrophage. On the cell surface, numerous granules and small aggregates of granules can be seen. No granules appear to be attached to the glass. The bar represents 5 μm.

EXPLANATION OF PLATE 1(b)

Higher magnification of same preparation as in Plate 1(a)

Numerous mast-cell granules attached to the macrophage surface are clearly seen. The bar represents 1 μm.

EXPLANATION OF PLATE 2(a)

Scanning electron micrograph of macrophage after 1 h incubation with mast-cell granules and 4 h of 'chase' without granules

The macrophage is extremely spread out with few surface projections. No mast-cell granules can be seen. The bar represents 5 μ m.

EXPLANATION OF PLATE 2(b)

Higher magnification of same preparation as in Plate 2(a)

The macrophage surface is conspicuously smooth, with few and small projections. No mast-cell granules can be seen. The bar represents 1 μ m.

the inactivation of certain serine proteinases by interacting with antithrombin III (Rosenberg, 1977). It has previously been suggested that antithrombin III may participate in the regulation of cellular inflammatory reactions by affecting serine proteinases at the macrophage cell surface (Remold & Rosenberg, 1975). Although speculative, it is tempting to envisage granule uptake as an effective and specific means of inhibiting enzyme systems in the macrophage without affecting susceptible enzymes elsewhere, e.g. in the blood-coagulation system. Our observation that macrophages spread markedly after granule uptake could actually indicate that they are affected by the engulfed substances in a non-trivial way, leading to a change in the functional properties of the cells.

The expert technical assistance of Miss G. Bäckström and Mr. J. Bögwald is gratefully acknowledged. This work was supported by grants from the Swedish Research Association (nos. 2309 and 13X-04), the Faculty of Veterinary Medicine (Swedish University of Agricultural Sciences), Konung Gustaf V:s 80-årsfond, Svenska livförsäkringsbolags nämnd för medicinsk forskning and Kabi A.B., Stockholm.

References

- Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
Cássaro, C. M. F. & Dietrich, C. P. (1977) *J. Biol. Chem.* **252**, 2254–2261
Cohn, Z. A. & Benson, B. (1965) *J. Exp. Med.* **121**, 153–169
Fabian, I., Bleiberg, I. & Aronson, M. (1978) *Biochim. Biophys. Acta* **544**, 69–76
Jansson, L., Ögren, S. & Lindahl, U. (1975) *Biochem. J.* **145**, 53–62
Lindahl, U., Jacobsson, I., Höök, M., Bäckström, G. & Feingold, D. S. (1976) *Biochem. Biophys. Res. Commun.* **70**, 492–499
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Neufeld, E. F. (1974) *Prog. Med. Genet.* **10**, 81–101
Ögren, S. & Lindahl, U. (1971) *Biochem. J.* **125**, 1119–1129
Oster, G. (1965) *Sci. Am.* **213**, 70–77
Remold, H. G. & Rosenberg, R. D. (1975) *J. Biol. Chem.* **250**, 6608–6613
Rosenberg, R. D. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 10–18
Shively, J. E. & Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
Yurt, R. W., Leid, R. W., Spragg, J. & Austen, K. F. (1977) *J. Immunol.* **118**, 1201–1207