Comparison of Several Methods for Isolation of Rat Peritoneal Mast Cells.* (31631)

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Interest in the role of mast cells in various pathological and physiological phenomena has prompted the development of methods for isolating these cells. Mast cells have been isolated from rat peritoneal fluid (they constitute 2-8% of the peritoneal cell population) by density gradient centrifugation through concentrated solutions of sucrose(1), Ficoll (a high molecular weight polysaccharide)(2) or albumin(3). Although it has been claimed (2,3) that sucrose is inferior to albumin or Ficoll for isolating mast cells, there has been no detailed comparison of these 3 media. The experiments reported here were designed to provide a systematic comparison of sucrose, Ficoll and albumin solutions as isolation media for rat peritoneal mast cells.

Methods. Mixed peritoneal cells were harvested from young male rats (250-300 g) of the Wistar strain according to the method of Uvnäs and Thon(2). The cells from 3 or 4 rats were pooled for each experiment. The animals were anesthetized with ether and exsanguinated. A section of midline abdominal skin was excised, a small hole was cauterized in the abdominal wall through which a buffered isotonic salt solution was introduced (see below). The hole was closed and the abdomen massaged gently for 90 seconds. The fluid, containing several types of cells, was then aspirated through a midline incision and centrifuged at 200 × *g* for 5 minutes at room temperature. The supernatant was discarded, and the cells were resuspended in 16-18 ml of buffered salt solution. Aliquots of mixed cell suspension were taken for mast cell counts and for determination of histamine. Eight ml of cell suspension were layered over the dense medium in a centrifuge tube. Centrifugation separated the cells into two layers, one (mixed cells) at the interface of the light and heavy media and the other (mast cells) at the bottom of the tube. The upper layer of fluid was removed with a pipette, and the interfacial layer of cells was similarly removed and resuspended in fresh buffered solution. The cells of this layer were washed, and samples were taken for histamine assay and mast cell counts. The sides of the centrifuge tube were wiped gently at the level of the interface to remove adhering cells. The dense medium containing mast cells was diluted with 2 ml of fresh, buffered salt solution and centrifuged (200 × *g* for 5 min). The mast cells were washed twice in fresh, buffered salt solution and finally resuspended in 8-9 ml. Aliquots were taken for estimation of histamine release as described below. All wash fractions and the media removed from the cells were acidified and stored frozen until assayed for histamine.

Media. A buffered salt solution (pH 6.8-7.0) containing 150 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 3 mM $Na_2HPO_4 \cdot 2H_2O$, 3.5 mM KH_2PO_4, 5.6 mM dextrose and 0.1% human serum albumin (Fraction V Nutritional Biochemicals) was used for suspending the mixed peritoneal cells and the isolated mast cells.

The following dense media were used for isolating mast cells: Sucrose-A, a medium prepared according to the directions of Padawar and Gordon(1) which by our calculations contains 3.13 M sucrose, 91.6 mM NaCl, and 1.4% gelatin; Sucrose-B, the Padawar and Gordon medium plus 0.1% human serum albumin; Sucrose-C, a 50% solution of sucrose (1.46 M) in the buffered salt solution (described above); Albumin, a 37% solution of bovine serum albumin (Fraction V, Nutritional Biochemicals) in buffered salt solution; and Ficoll, a 35% solution of Ficoll (Pharmacia, Uppsala, Sweden) in buffered salt solution. All media were stored at −10°C. The specific gravities of the media ranged from

* Supported by grant HE 02953 from Nat. Heart Inst. Nat. Inst. Health. Publication 823 of the Division of Basic Health Sciences, Emory University.

† Predoctoral trainee of USPHS Graduate Pharmacology Training Grant 2T1 GM 179.

1.1053-1.3087. Separation of the cells was achieved by centrifugation for 15 minutes at 500 × *g* for Sucrose-A and Sucrose-B, 5 minutes at 200 × *g* for Sucrose-C, and 10 minutes at 250 × *g* for Albumin and Ficoll.

Histamine release experiments. Aliquots of all mixed cell suspensions and all isolated mast cell suspensions were incubated at 37°C for 10 minutes in the presence of either compound 48/80 (10 μg/ml) or antigen (0.1 ml horse serum). The cell- and supernatant-fractions were separated by centrifugation, acidified with HCl and frozen until assayed for histamine.

For experiments with sensitized mast cells 18 rats were given subcutaneous injections of 1 ml of horse serum (Colorado Serum Co.) and 0.5 ml of *Hemophilus pertussis* vaccine (Lederle) as adjuvant. Fourteen days later the animals were sacrificed and the cells prepared as described below.

Histamine assay. The amount of histamine in all fractions was assayed on atropinized segments of guinea pig ileum. Identification of the spasmogen in the fractions as histamine was based on the blockade of the contractions by pyrilamine (10^{-7} M) in selected experiments.

Mast cell counts. Estimates of the number of mast cells in the original mixed cell suspensions and in each cell fraction were obtained by counting the cells stained by 0.1% toluidine blue in a hemocytometer according to the method of Bray and Van Arsdale(4).

Results. The total recovery of mast cells (from the dense media and the interfacial layers) was greater than 85%. Recovery of mast cells from the dense media was in excess of 80% except from the sucrose-gelatin medium. Total histamine recovered was greater than 90% in all experiments. The histamine content of mast cells isolated through albumin or Ficoll solutions did not differ significantly from that of the mixed cell suspensions, but mast cells isolated through sucrose media lost more than 70% of the total histamine (Table I). Further loss of histamine occurred during incubation for 10 minutes (Table I, spontaneous release). Mean spontaneous release was 36% and greater from cells isolated through sucrose in contrast

TABLE I. Comparison of Rat Peritoneal Mast Cells After Isolation Procedures.*

	Mixed cells	Ficoll	Albumin	Sucrose-A†	Sucrose-B†	Sucrose-C†
No. mast cells × 10^6/8.0 ml of original mixed cell suspension	2.2 ± .9 (24)	1.6 ± .6 (7)	1.3 ± .6 (5)	1.4 ± .5 (6)	2.1 ± .3 (3)	4.5 ± .7 (3)
Total mast cells‡ recovered (%)	—	88.8 ± 6.3 (7)	98.2 ± 5.8 (6)	84.1 ± 4.2 (5)	90.1 ± 18.0 (3)	110.6 ± 22.7 (3)
Mast cells recovered from dense medium (%)	—	82.1 ± 7.2 (7)	93.8 ± 5.8 (6)	66.7 ± 2.2 (5)	80.1 ± 16.5 (3)	107.4 ± 21.6 (3)
Histamine, μg/10^6 mast cells	16.9 ± 1.7 (24)	18.8 ± 2.5 (7)	20.4 ± 4.8 (6)	4.5 ± 1.4‖(5)	1.8 ± 1.2‖(3)	3.6 ± .5‖(3)
Total histamine† recovered (%)	—	91.7 ± 4.1 (7)	102.7 ± 4.3 (6)	101.2 ± 4.9 (5)	95.9 ± 8.5 (3)	105.4 ± 10.0 (3)
Histamine recovered in isolated mast cells (%)	—	85.3 ± 3.6 (7)	98.2 ± 4.2 (6)	7.4 ± 2.2 (5)	8.8 ± 3.5 (3)	31.3 ± 9.6 (3)
Histamine release (%)						
Spontaneous	2.0 ± .4 (22)	1.2 ± .3 (8)	3.8 ± .8 (7)	37.8 ± 7.3‖(7)	56.9 ± 12.8‖(3)	36.0 ± 10.7‖(3)
48/80 (10 μg/ml)§	86.9 ± 1.4 (22)	78.7 ± 3.0 (6)	78.5 ± 5.2 (5)	17.9 ± 6.3‖(5)	−5.5 ± −2.5‖(3)	9.0 ± 5.0‖(3)
Antigen (0.1 ml horse serum)§	34.9 ± 7.5 (6)	22.5, 23.0 (2)	20.2, 20.3 (2)	10.3—.5 (2)	—	—
Toluidine blue staining	+	+	+	+	+	+

* Values are means ± standard errors. Numbers in parentheses indicate No. of experiments.
† Sucrose media described under *Methods.*
‡ Percent mast cells or histamine recovered included histamine recovered from washings and cells recovered from interface of isolation medium.
§ Corrected for spontaneous release.
‖ Values significantly different from corresponding values for mixed peritoneal cells ($p < .01$).

to less than 4% for cells isolated through Ficoll or albumin.

The amount of histamine released from mast cells in response to 48/80 or antigen was approximately equivalent for mixed peritoneal cells and for Ficoll- or albumin-isolated cells. Much less histamine was released from sucrose-isolated cells. Uvnäs and Thon reported that addition of serum or serum albumin to their mast cell media preserved the sensitivity of isolated mast cells to histamine releasing agents and decreased the amount of spontaneous release(2,5). In our experiments addition of 0.1% human serum albumin to the sucrose medium of Padawar and Gordon did not prevent loss of histamine from mast cells during isolation, nor did it decrease spontaneous histamine release (Table I). Cells isolated through this medium failed to release their remaining histamine in the presence of 10 μg/ml of 48/80. The presence of other salts such as calcium, potassium and magnesium (in the Sucrose-Cmedium) did not protect mast cells from damage during isolation.

Discussion. Ficoll and bovine serum albumin are superior to sucrose as dense media for the isolation of rat peritoneal mast cells. Cells isolated through these two media retain their histamine and their ability to respond to histamine releasing doses of compound 48/80 and antigen. In contrast, cells isolated through sucrose lose much of their histamine during isolation and subsequent periods of incubation in physiological medium. Sucrose-isolated cells respond less to histamine releasing agents and show other signs of damage such as clumping, sparse granulation, and less definite staining with toluidine blue.

Damage to the mast cells which occurred with 3 different types of sucrose media appears to be related to the presence of sucrose rather than to the presence or absence of any other constituents. A solution of 50% sucrose in our standard physiological medium damaged mast cells as much as the sucrose medium of Padawar and Gordon. Solutions of Ficoll and albumin in physiological medium which had densities comparable to those of sucrose solutions did not damage the cells. Addition of small amounts of human serum albumin to sucrose media did not protect the mast cells from damage despite the fact that the work of Uvnäs and Thon suggests that albumin in mast cell media decreases spontaneous histamine release.

The damage produced by sucrose is probably due to the hypertonicity of the medium, as suggested by Uvnäs and Thon(2). The low molecular weight of sucrose (342), compared with the high molecular weights of Ficoll (400,000)(6) and bovine serum albumin (69,000)(7), prohibits preparation of a dense but isotonic medium. Sucrose media that are dense enough for the isolation of mast cells cause crenation of erythrocytes, but Ficoll and albumin solutions of comparable density do not. Although both Ficoll and albumin media permit isolation of viable mast cells, Ficoll forms a colorless solution which facilitates visualization of cell layers during separation.

Summary. Several dense media were compared for suitability for isolation of rat peritoneal mast cells. Mast cells isolated in 35% Ficoll (a synthetic polysaccharide) or in 37% bovine serum albumin solutions did not lose histamine during the isolation procedure or during a short period of incubation in an isosmotic medium. They responded normally to the histamine releasing action of compound 48/80 or antigen. In contrast, cells isolated in three types of sucrose media lost much of their histamine during isolation and during subsequent incubation in an isotonic medium. Histamine release induced by compound 48/80 or antigen was less than in mixed peritoneal cell suspensions. It is concluded that concentrated sucrose solutions damage mast cells, probably as a result of the hypertonicity of the media.

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Received July 11, 1966. P.S.E.B.M., 1966, v123.

Action of Snake Venom Phospholipase A on Isolated Platelet Membranes.* (31632)

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Platelets play a significant part in the coagulation process by contributing a phospholipid (or more likely a phospholipoprotein) to the interaction of coagulation factors, which eventually leads to the formation of a prothrombin activator. This clot-promoting property of platelets is ordinarily present in latent form(1-3), but through an unknown mechanism the latency is lost as the coagulation process gains momentum. The physical form in which procoagulant material from platelets interacts with clotting factors is also unknown. Actual release from platelets has been suggested(4), and alternatively it has been proposed that the activity becomes available to the coagulation process as a catalytic lipoprotein surface on the plasma membrane of the platelet(5). These protein-lipoprotein interactions basically involve problems of cellular lipoprotein availability.

A further example of variations in the availability of cellular lipoproteins is revealed by the studies of Condrea and associates(6) and Kirschmann *et al*(7). These investigators have shown that, whereas the phospholipids of osmotically haemolyzed erythrocytes and intact platelets were hydrolzyed by *N. naja* phospholipase A, intact red cells were unaffected. Phospholipase A from Russell viper venom was also inactive against intact but active against haemolyzed red cells. Palestinian viper venom phospholipase A did not produce hydrolysis of phospholipids in intact platelets, intact erythrocytes, or osmotically haemolyzed red cells, and was only active against red cells disrupted by sonication. The results obtained with *N. naja* and Russell viper venom suggested similarities between lipid availability for the coagulation process and susceptibility to venom action. Specifically, all the cell preparations which resisted the action of venom were also inactive in *in vitro* coagulation systems. On the other hand, venom-sensitive cells were specifically those which were active in coagulation.

It therefore seemed pertinent to investigate the basis for the difference in venom sensitivity of intact platelets and erythrocytes. One of the principal hypotheses to be examined was the possibility that phospholipids of platelet membranes had a specific affinity for the venom enzyme. In line with this approach, the effect of venom phospholipases on isolated platelet membranes was studied and compared to the venom effect on intact platelets, isolated platelet granules, intact red cells, and plasma lipoproteins.

Methods. Washed platelets and isolated platelet membranes and granules were prepared from 500 ml citrated human blood as described by Marcus *et al*(8). Platelet-poor plasma was prepared by centrifuging platelet-rich plasma for 20 minutes at 5,000 × *g*. Red cells were washed 3 times in isotonic buffered saline, pH 7.4(9). The buffy coat was removed after each wash and the cells finally suspended in buffer to a concentration of 50%. Approximately 1 g wet weight of platelets was suspended in 10 ml of buffer. Subcellular platelet particles were dialyzed

* Supported by grants from Nat. Inst. Health (HE-09070-02), Veterans Administration, and New York Heart Assn.

† USPHS International Postdoctoral Fellow.