THE REACTION OF RAT MAST CELLS TO POLYLYSINE

JACQUES PADAWER

From the Department of Anatomy, The Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

The effects of a single intraperitoneal injection of polyamino acids (lysine, glutamic, aspartic) on mast cells of the rat are described. In vitro interaction of mast-cell components with these polyamino acids is also explored. Poly-DL-lysine (but not the acidic amino acids) has both immediate (minutes-hours) and long-term (days-weeks) effects on mast cells. At the dosage selected, some cells evidence rapid fusion of granules and degranulation, but without concomitant swelling; most display intracellular changes only. Neither degranulation nor granule fusion appears to be lethal. Rather, these spur the cell to greater synthetic activity which involves first the Golgi apparatus and subsequently also the endoplasmic reticulum. Early involvement of macrophages and eosinophils is described. Sequential studies after polylysine injection support the following concepts: (*a*) mast-cell granules exist as "physiological sets," several being confined to a common membranous "sac;" (*b*) each set can respond independently to applied stimuli; (*c*) each set can connect directly to the extracellular milieu; (*d*) poly-DL-lysine binds directly to the granules and stabilizes them; it is not readily digested; (*e*) mast-cell granules are produced directly; they do not arise by intake of exogenous polysaccharides. It is hypothesized that mast-cell granules are topologically outside the cell while held intimately within extensive cytoplasmic folds and recesses. Mast cells may function by causing intercellular connective tissue fluids to percolate over their granules which may process this fluid in some as yet undefined way(s).

INTRODUCTION

The cytoplasm of mast cells is replete with characteristic granules. On the basis of experiments in which the distribution of colloidal thorium dioxide taken up by mast cells was followed as a function of time, I recently suggested (46) that mast-cell granules are not individually invested by the membrane, but rather that groups of granules share a common membranous sac which holds them, frankfurter link-fashion, within a branching system of communicating cytoplasmic channels. These sacs show a functional polarity, and may open to the extracellular milieu distally through pores in the cell membrane.

These concepts are supported and extended by experiments described in this paper which, in addition, demonstrate the ability of well-differentiated mast cells to regenerate new granules after experimentally induced degranulation or granule alteration (incapacitation?)

MATERIALS AND METHODS

Animal Experiments

Sprague-Dawley male rats (Charles River Breeding Laboratories, North Wilmington, Mass.) of two age and weight ranges were used, namely, 35 and 51 days old, weighing 110–145 g and 200–279 g, respectively. At the start of the experiments, each animal was injected intraperitoneally with a single dose of test substance. Saline injections were used as con-

trols. Animals were killed at various times thereafter. Some of the peritoneal fluid obtained was used for air-dried preparations (light microscopy), and some was fixed and processed for electron microscopy as previously described (46). Air-dried preparations were: (*a*) stained with 0.5% aqueous toluidine blue in 0.1 M phthalate-HCl buffer pH 2.8, containing 10% formalin, or (*b*) stained by a nonaqueous toluidine blue method (40), or (*c*) treated with alkaline alcoholic dansyl chloride and observed by fluorescence microscopy, a method specific for lysine residues (54), or (*d*) fixed with methanol and then treated with polylysine solution prior to processing through methods (*a*) to (*c*).

Model (Test-Tube) Experiments

The ability of the polyamino acids to bind purified mast-cell constituents and some related substances was explored with test-tube experiments. In these experiments, compounds in solution (1 mg/ml in saline) were mixed at room temperature. The polyamino acids were of the same lots as for in vivo experiments. Polylysine binding to the acidic substances was explored to see (*a*) whether they would precipitate with polylysine, (*b*) whether they could elicit metachromasia of toluidine blue in dilute solution, or (*c*) whether they would affect the binding of heparin to toluidine blue (orthochromatic shift).

The following test substances and sampling times were used:

Poly-DL-lysine hydrobromide, Gallard-Schlesinger Chemical Mfg. (Yeda) Corp., New York, mol wt 17,000, degree of polymerization (D.P.) = 120, Lot No. LY 78. Injected dose: 0.4 ml of a 1.0 mg/ml solution in saline, intraperitoneally, per rat. Peritoneal fluid samples were obtained at 5, 15, 30 min, 1, 4, 24, 48 hr, 5 days, 2, and 3 wk after injection.

Poly-L-glutamic acid, Mann Research Laboratories, New York, mol wt 61,000, D.P. = 475, Lot MA P 1704. Injected dose: 0.4 ml of a 1.0 mg/ml solution in saline, intraperitoneally, per rat. Peritoneal fluid samples were obtained 3 hr later.

Poly-L-aspartic acid, Mann Research Laboratories mol wt 20,000, D.P. = 174, Lot MA L 2332, was dissolved by addition of NaOH to saline solution. Injected dose: 0.4 ml of a 1.0 mg/ml saline solution per rat, intraperitoneally. Peritoneal fluid was sampled 3 hr later.

Heparin. Mann Assayed, USP, 114 U/mg, 5.9% moisture, Lot G2627.

Toluidine blue O. Matheson, Coleman, and Bell (Cincinnati, Ohio), 66% dye, certification No. CU3. A 0.005% (w/v) solution was used.

N-acetyl neuraminic acid. Sialic acid, Sigma Chemical Co., St. Louis, Mo., Lot No. 97B-2420. For this substance, the dye solution was neutralized first with NaOH.

Colominic acid, ex E. coli. "Pure, lyophilized"; Pierce Chemical Co., Rockford, Ill., lot No. 111240. Colominic acid is a high molecular weight polysialic acid (2).

The ability of acidic polyaminoacids to precipitate with pertinent substances was explored with the following compounds: *α-Chymotrypsin.* Worthington Biochemical Co. (Freehold, N. J.) CDI. 8LK, 53 U/mg, 99% protein. *Serotonin creatinine sulfate.* Calbiochem, lot 5661-10071. *Histamine dihydrochloride.* Fisher catalogue H 295, Lot 74332.

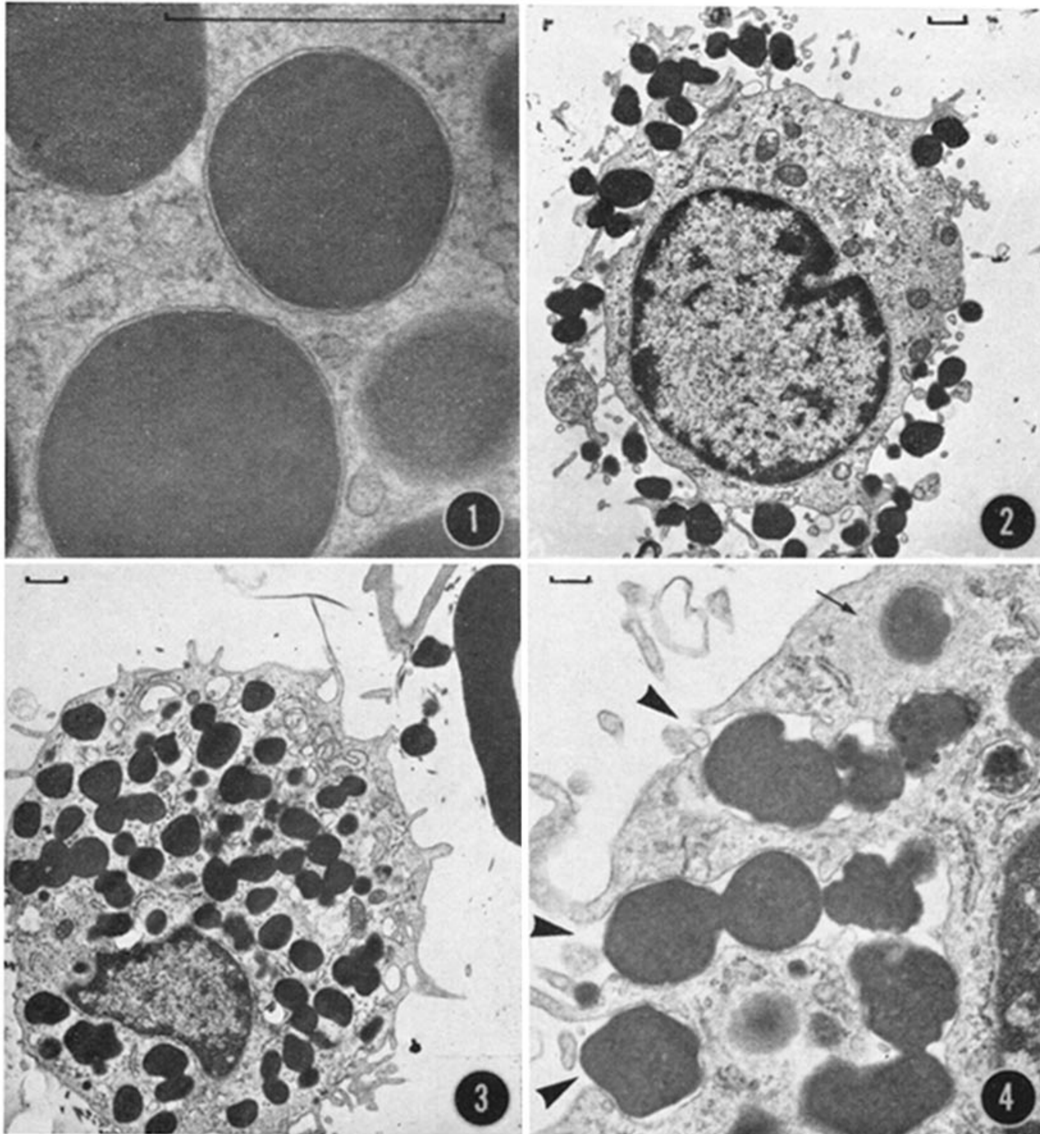
RESULTS

Animal Experiments

Polyglutamic and polyaspartic acids have no visible effects on mast cell cytology or staining properties, but both substances mobilize neutrophilic leukocytes into the peritoneal cavity. Polylysine, on the other hand, affects the various cell types of peritoneal fluid in specific ways. At the light microscopic level, extensive mast cell degranulation is evident in early samples. There is a general involvement of macrophages and eosinophils in taking up shed mast cell granules. The granules stain in the normal metachromatic hue whether still within mast cells, shed, or whether they have been ingested by macrophages or eosinophils for some time. At the electron microscopic level, additional immediate as well as long-term cytological changes in mast cells are evident. These, detailed below, involve the cell surface, the granules, the Golgi apparatus, and the rough endoplasmic reticulum. Only the effects on mast cells will be described in detail. Effects on other cells will be dealt with here only insofar as they relate to changes in the mast cells.

EFFECTS ON MAST CELLS: The characteristic granules of differentiated rat mast cells, at high magnifications, are seen to be bounded by a closely apposed membrane. A narrow gap of about 100 A separates the granule from the membrane (Fig. 1).

Injection of polylysine has an almost immediate effect on peritoneal mast cells. The first samples, obtained as rapidly as manageable 5 min after injection, reveal that some mast cells are partially or almost totally degranulated; some cells are devoid of cytoplasmic granules, but are surrounded by mast cell granules and have ultrastructural features (nucleus and other organelles) which suggest they may be fully degranulated mast cells (Fig. 2). Apparently unaffected mast cells also are encoun-



Marker in all illustrations represents approximately 1 μ.

FIGURE 1 Mast-cell granules from untreated control rat. Note regular 100 A gap separating the granule from the enveloping membrane. Lead citrate stain. × 45,000, approximately.

FIGURE 2 Peritoneal fluid cell, 5 min after injection of poly-DL-lysine. Characteristic mast-cell granules are closely associated with the cell periphery. Nuclear morphology, chromatin distribution, paucity of mitochondria and of endoplasmic reticulum all suggest that this is a fully degranulated mast cell which has just released the nearby granules. Yet, no notable changes are apparent in the ground cytoplasm and, were it not for the nearby granules, this cell would be classified as an "undifferentiated mononuclear." Uranium acetate and lead citrate stain. × 5000, approximately.

FIGURE 3 Peritoneal mast cell 5 min after polylysine injection. Note that most of the granules in this section profile are fused into branched and twisted sets and that such groups of fused granules can occur in close proximity to each other without fusion of the perigranular sacs. Uranium acetate and lead citrate stain. × 5000, approximately.

FIGURE 4 Peritoneal mast cell 5 min after injection of polylysine. Most granules are fused into characteristic sets through small areas of contact. In several areas, the perigranular membrane has lifted and clearly reveals that it is a continuous sac associated with each set of fused granules as a whole rather than with each granule individually. The granules are compact and dense as in uninjected animals or saline-injected controls (compare with Fig. 19 of reference 46 and with Fig. 22 of this paper, respectively). Note also the fibrillar condensations about some granules (arrow). In three areas (arrowheads), continuity between the granular channels and the external milieu is evident. Uranium acetate and lead citrate stain. × 5000, approximately.

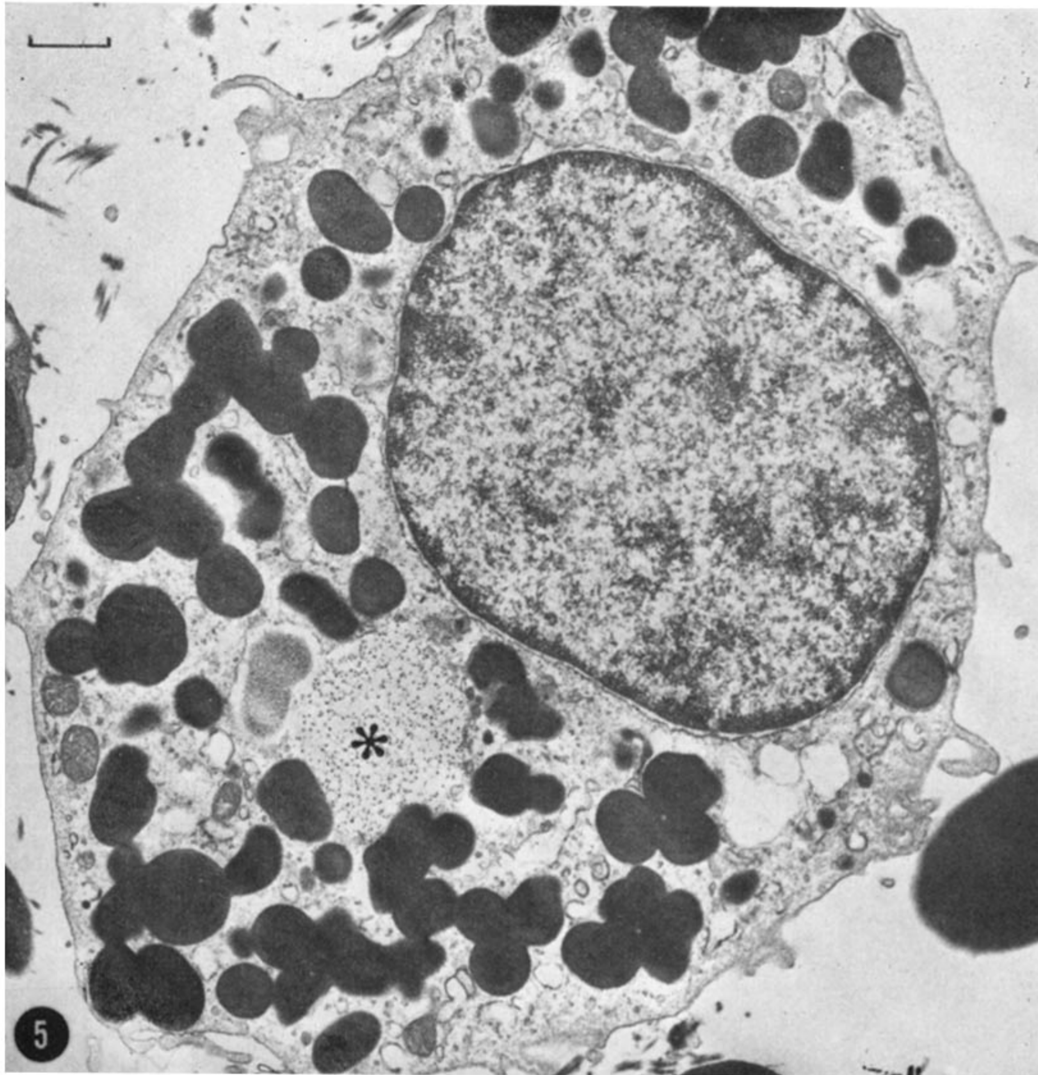
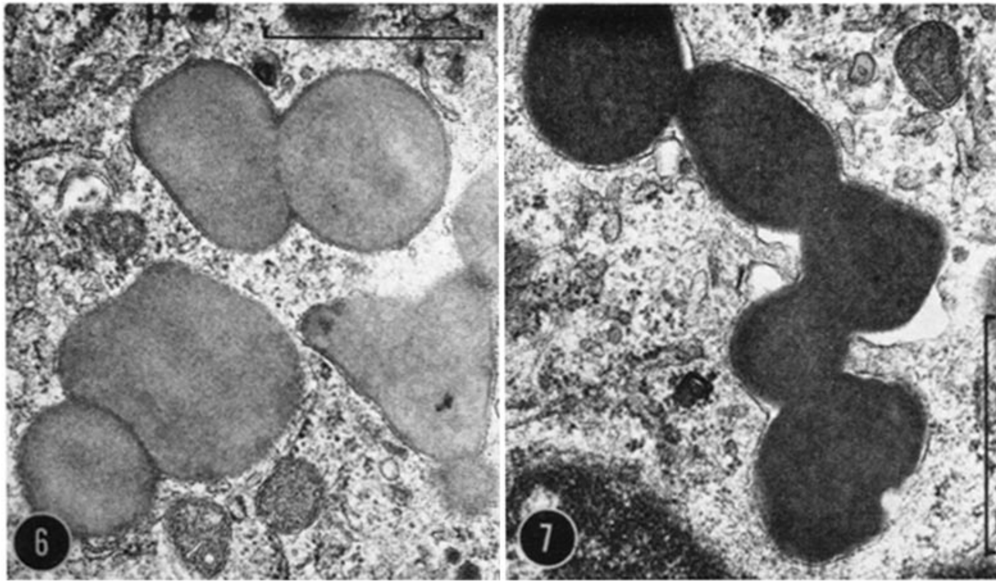
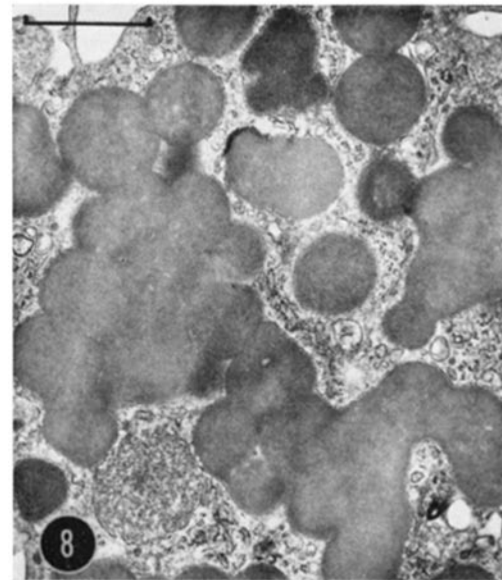


FIGURE 5 Peritoneal mast cell 5 min after polylysine injection. Cell-surface activity appears depressed: villi and outfoldings are fewer and less developed than normal. Since mitochondria and nucleus are satisfactorily preserved, this cannot be ascribed to poor fixation. Note that one granule has swollen (*); it has the characteristic texture and size of the fully disrupted granules that one encounters with damaged or with poorly fixed cells of untreated animals or with those disrupted by Compound 48/80. Double stain. × 10,300, approximately.

tered occasionally. At the dosage used, however, most of the mast cells remain granulated and display groups of granules which have characteristically fused to each other in open, branching, serpentine patterns (Figs. 3, 5, and 7). More compact clusters (Fig. 8) and condensed groupings occasionally also are seen. The membranous investment shared by these granules often is revealed to advantage as it lifts off and as the gap that normally separates it from the granules widens (Figs. 4 and 7). The affected granules are not swollen, and they retain the high electron opacity characteristic of normal granules. Fusion of granules appears to involve appreciable forces: the normally





FIGURES 6–8 Fused granules, 5 min post polylysine injection.

FIGURE 6 Note the rather broad area of fusion between granules. A denser boundary marks the granular domains. The perigranular membrane has not lifted, and a normal gap sets it off from the granular substance; it is not insinuated between the apposed granular faces. × 28,600, approximately.

FIGURE 7 Five granules fused rosary-fashion. The perigranular sac has lifted in several places. × 31,000, approximately.

FIGURE 8 Part of a cluster of 55 granules (in this profile) cross-fused with each other. Denser domain boundaries are present in several areas of the cluster. The perigranular sac is closely disposed to the cluster and the usual gap is displayed. Double stain. × 16,400, approximately.

spherical granules become deformed in areas of contact, thus greatly increasing the areas of apposition which are sometimes accented by denser boundary lines (Fig. 6).

Mast cells with fused granules in the process of being shed are encountered readily (Fig. 9); in these, the granules, still unswollen, are disgorged between well-delineated lips of peripheral cytoplasm (Figs. 4 and 9). Groups of such fused unswollen granules, like so many lengths of welded chain links, often are seen extracellularly in the vicinity of these cells (Figs. 2 and 9).

Polylysine appears to activate the mast-cell surface. Cells that are only slightly affected (as gauged by effects on the granules) show increased surface folds and invaginations and an enhanced pino-

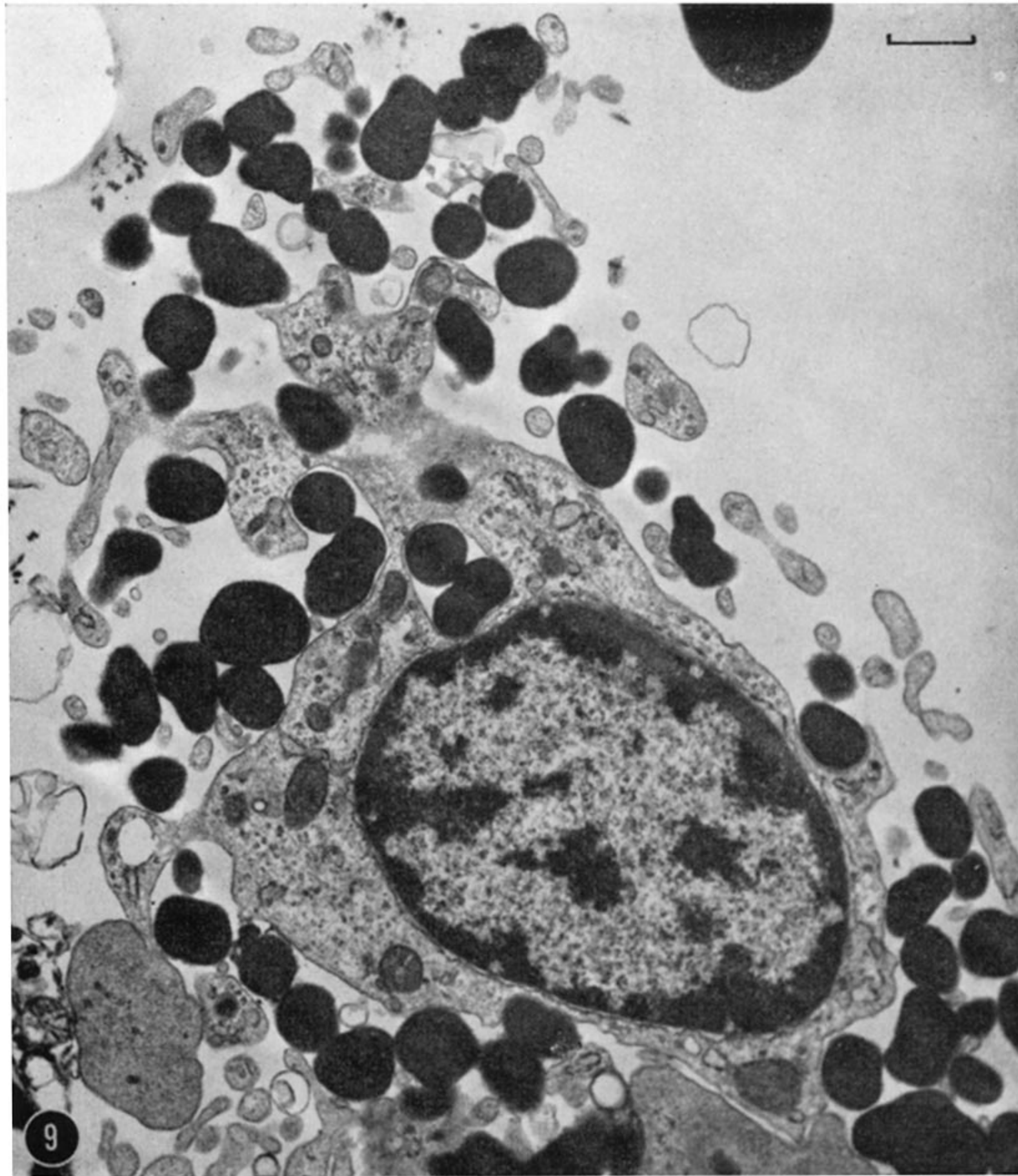


FIGURE 9 Degranulating mast cell, 5 min after polylysine injection. The channels in which mast cell granules are held have expanded greatly. They remain vaguely delineated by thin cytoplasmic partitions. The granules have remained unswollen, and many are being disgorged as fused sets. The two-Simensional nature of thin sections does not allow a statement about those granules that appear singly. dtained with uranium acetate and lead citrate solutions. × 11,900 approximately.

cytotic activity (Fig. 10). Markedly affected cells, on the other hand, usually display a diminution of these surface features (Fig. 5).

Since at the dosage used most of the mast cells are not degranulated, a time study to examine the evolution of these early effects proved possible.

Withing 30 min after injection of polylysine, the perigranular membranous sac which had lifted

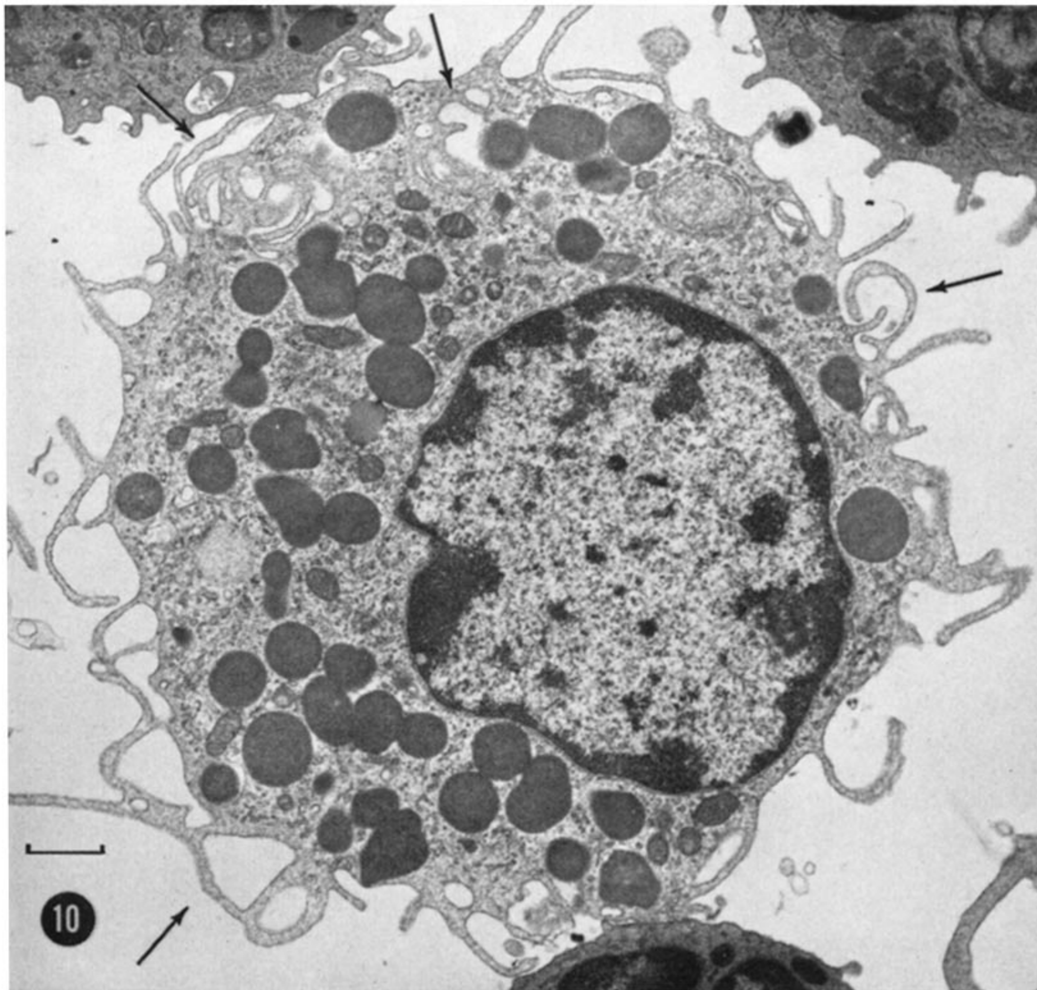
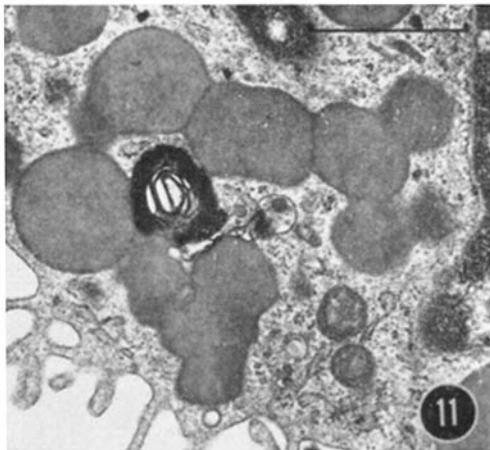


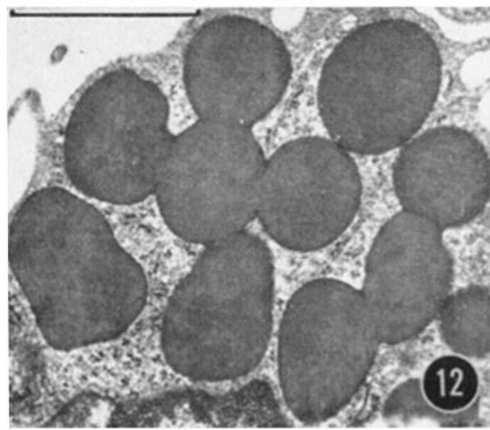
FIGURE 10 Peritoneal mast cell 5 min after polylysine injection. Cell surface is activated: outfoldings are longer and more complex than in untreated animals, and unusual surface invaginations are present (arrows). Fused granules indicate that this cell is affected by the polylysine. Doubly stained with uranyl acetate and lead citrate solutions. × 10,000, approximately.

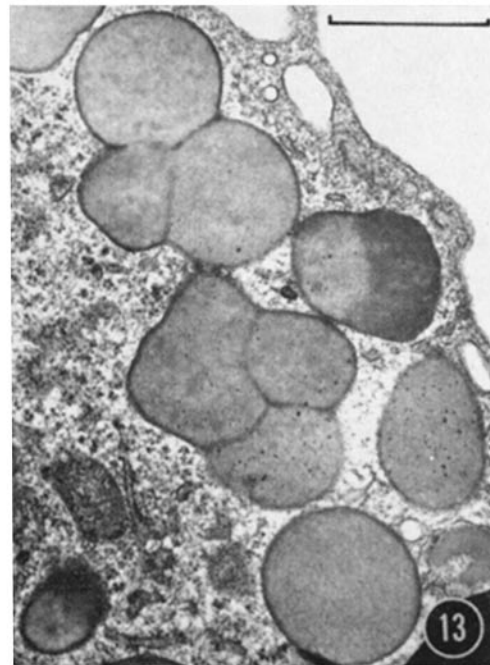
initially is seen to collapse or tighten around the fused granules. Granule apposition within the fused clusters seems more intimate than at earlier stages, and areas of intergranular contact become flattened so that the denser boundary line often appears straight (Fig. 11).

From 1 to 24 hr post injection, these features become progressively more evident, and fewer cells are seen in intermediate stages: free, shed granules are observed progressively less frequently as are cells in the process of degranulation. From 4 to 24 hr, the linear and cross-linked patterns of fused granules predominate, but as time elapses there is a definite tightening, and apposition of granules involves a more substantial portion of their surface areas (Figs. 12 and 13). Young granules are only rarely fused with more mature ones.

By the 5th day following injection of polylysine, several additional cytological changes are evident. Although mast cells with fused granules are still common, they often simultaneously display single granules which are surrounded by the usual gap (Fig. 14). The perigranular sac, which earlier had so tightened about the granules as to abolish the







perigranular gap, begins to lift again, and the trend toward compact clusters of granules now prevails (Fig. 14): the granules are fused through much, if not most of their surface area, and many are completely embedded within the clusters; the denser material delineating what may be termed the original granular domains now often is barely discernible (Fig. 15). Some dense material accumulates near small angular indentations on the surface of the clusters. The Golgi area of affected cells is particularly prominent at 5 days and contains more vesicular components than usual (Fig. 16); some of these vesicles have a dense periphery, and all of them are generally empty. Ribosomal clusters in increased numbers also are found, especially in the neighborhood of the Golgi area (Fig. 16).

2 wk after injection, the effects of polylysine still remain evident. Fused granules are still found in most cells, but their matrix now has acquired a spongy texture (Fig. 17). Much material of great electron opacity is plastered over the surface of the clusters and insinuated between granular masses. Granule boundaries within the clusters are still faintly resolvable as seams of slightly greater opacity. Other granules are dense and irregularly eroded; they are reminiscent of a mulberry and

FIGURE 11 Fused granules 30 min after polylysine injection. The perigranular sac is tightly apposed to the granules. Contact faces between granules are flat, as if compressive forces were involved. A denser boundary line is present at granular interfaces and is suggested at other peripheral granular areas. Note that mitochondrial and perinuclear membrane systems are apparently unaffected. Myelin figure is unusual but sometimes encountered in untreated cells as well; it could represent phagosome formation or cell injury. Stained with uranium acetate and lead citrate solutions. × 20,600, approximately.

FIGURE 12 Fused mast cell granules 4 hr post polylysine injection. The perigranular membrane is tightly apposed to the granules, leaving no gap. Doubly stained with uranyl acetate and lead citrate solutions. × 24,800, approximately.

FIGURE 13 Fused mast cell granules, 24 hr after polylysine injection. Fusion boundaries involve a substantial portion of the granular surfaces, thus resulting in compaction of the clusters. Domain boundaries, and especially outer limits of granular masses, stand out as denser lines of rather constant thickness. Close apposition of perigranular membrane to the apparently single granules suggests that these, too, are affected—perhaps as part of cluster(s) out of the section plane. Doubly stained. × 20,900, approximately.

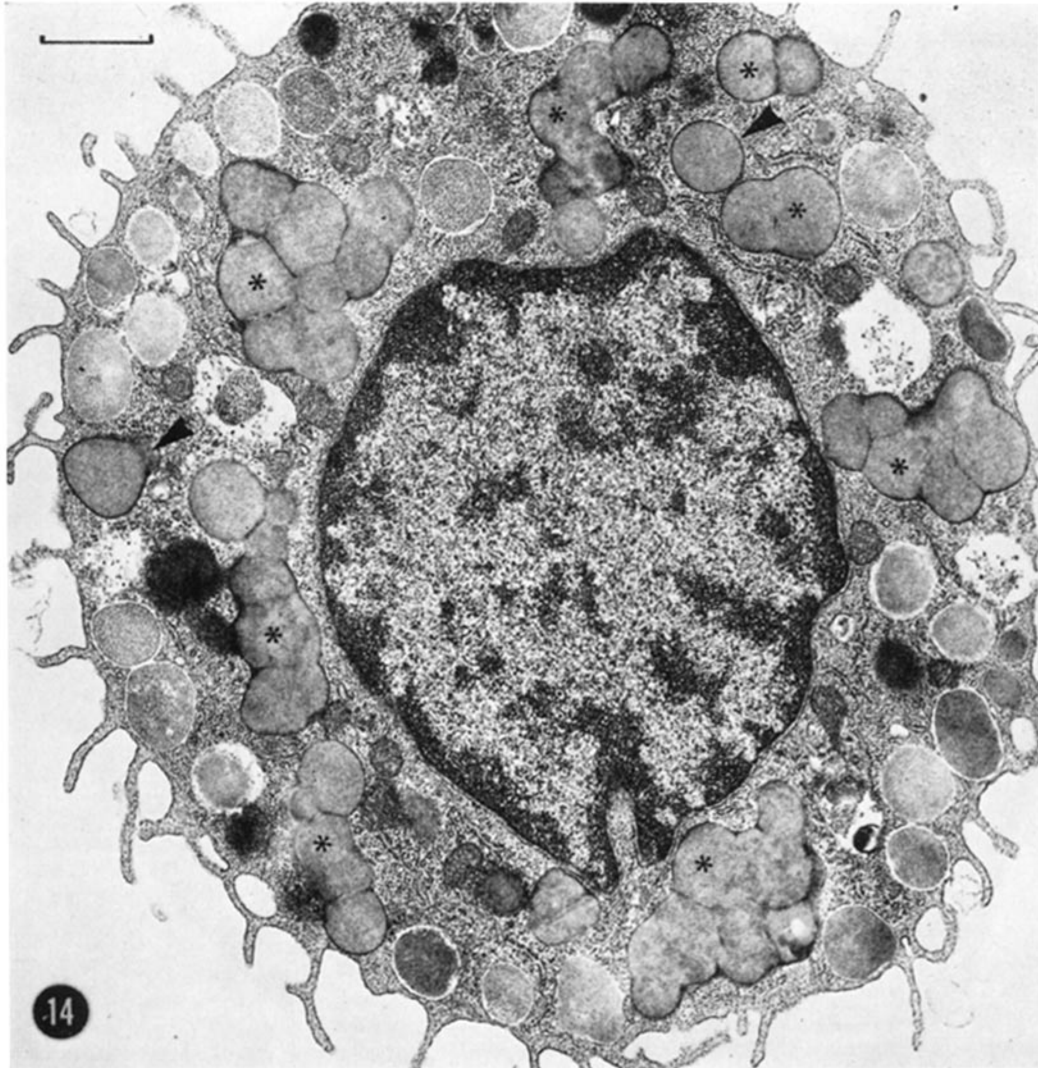


FIGURE 14 Peritoneal mast cell at 5 days post polylysine. The presence of several fused granular masses (*) proves that this cell was affected by the polylysine. These fused granules are even more compacted than at 24 hr, and they do not have a perigranular gap. Two single granules have a dense boundary (arrowheads), indicating that they are affected. There are also several single granules which do exhibit a perigranular gap (in fact, it is somewhat wider than normal). Double stain. × 14,900, approximately.

appear to consist of evenly sized spherules with a somewhat lucid core. This type of granule usually is held loosely in a large, ill-fitting vesicle. The Golgi area of affected mast cells is still prominently vesicular, but many of the vesicles contain a progranule (Fig. 18). A few developing granules (usually in early stages of progranule aggregation) are seen in an occasional section profile (Fig. 17).

In the last sampling, secured at 3 wk after polylysine injection, fused clusters of granules are no longer encountered. The dense, mulberry-textured granules in loosely fitting vesicles prevail and are the basis for deciding that the cell had been affected. This is a pertinent point because many of the peritoneal fluid mast cells appear essentially

normal at this time. The affected cells often display fewer mature granules than normal and an unusually large number of developing granules at the aggregating progranule stage. Fig. 19 shows a mast-cell profile with ten such granules in early stages of development; dense, eroded granules are still present. Some mast cells now display much well-developed rough endoplasmic reticulum (Fig. 19). This amount of rough endoplasmic reticulum is unusual for mast cells of 8-wk-old untreated rats, and would be even more unexpected for mast cells of the older group (10½ wk) of animals.

Polylysine does not visibly affect other membrane systems: mitochondrial, perinuclear, endoplasmic reticulum, and Golgi membranes all appear normal. No organelle fusion occurs.

The acidic polyaminoacids do not have these effects: mast-cell granules remain unfused after injection of either polyglutamic (Fig. 20) or polyaspartic (Fig. 21) acid. For comparison purposes, a typical mast cell from a saline-injected control animal (1 hr) is shown in Fig. 22.

Some Effects on Other Cells

Degranulation of mast cells by polylysine serves as a powerful and rapid stimulus for both macrophages and eosinophils, normally present in peritoneal fluid, to converge on the degranulating mast cells. Even in samples taken 5 min after treatment, many cells of both types already contain ingested mast cell granules, some single and some apparently taken up as fused sets. The avidity of these cells for the granules is remarkable in that several of them often are seen clustered around an affected mast cell, insinuating their pseudopodial processes into the enlarged channels of the mast-cell cytoplasm and literally plucking out the mast-cell granules from their berths before their complete release. An example of this common phenomenon is shown in Fig. 23.

Polylysine elicits surface changes indicative of increased pinocytotic activity in both macrophages and eosinophils. However, cellular organelles appear normal. Eosinophil granules, which were given special scrutiny, remain unconnected with one another not only after polylysine injection, but also after administration of polyglutamic or polyaspartic acid.

Neutrophils invade the peritoneal cavity in large numbers after either polyglutamic or polyaspartic acid is injected. The granules of these invading neutrophils are not fused at 3 hr. Polylysine, in the present experiments, did not mobilize neutrophils into the peritoneal cavity.

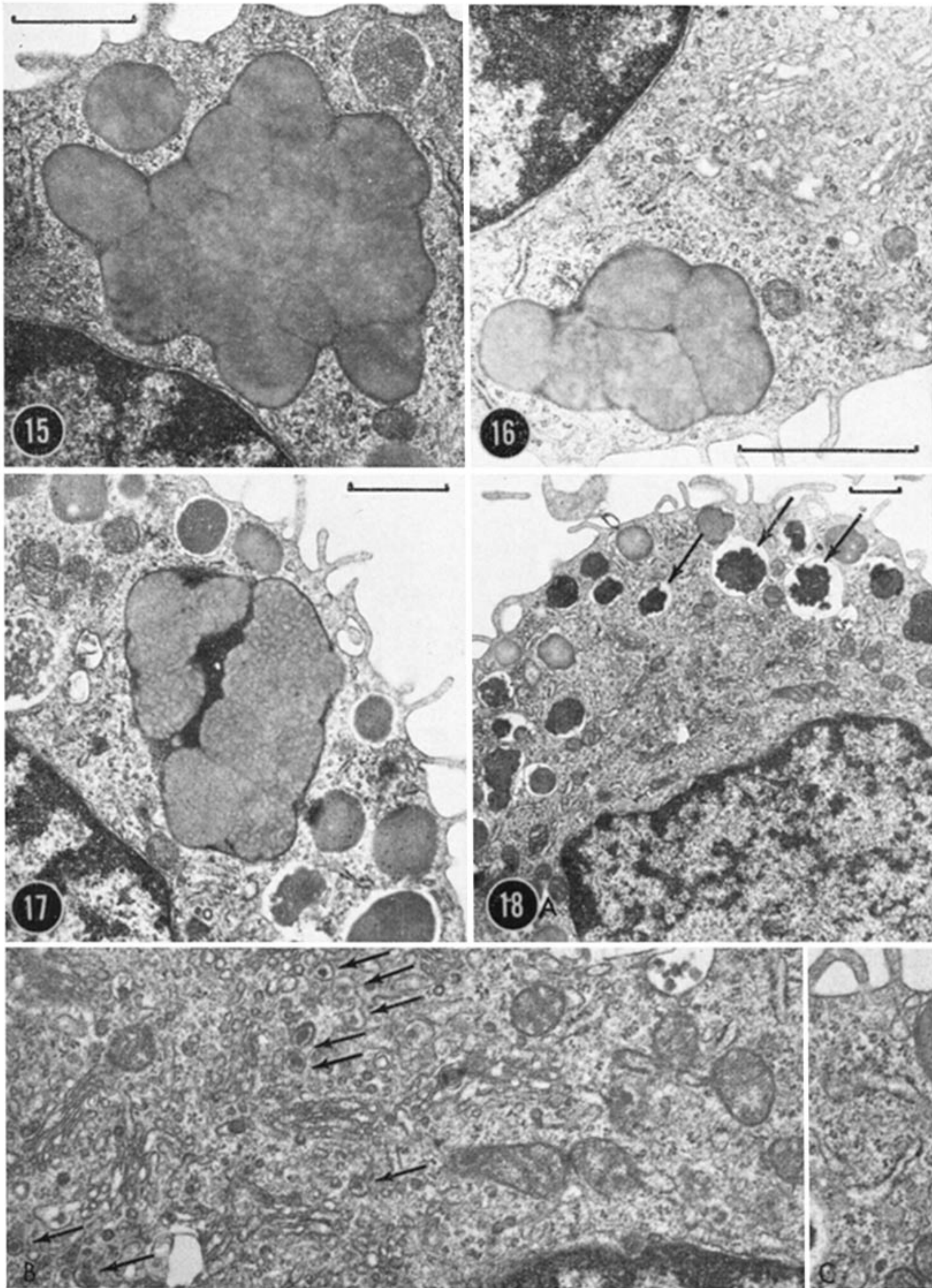
Model Experiments

Data obtained from test-tube experiments are summarized in Table I, in which interaction of various substances is revealed either by color shift of a dye-polysaccharide complex or by a precipitation reaction. It is evident that, because of their poly-electrolyte nature, polyaminoacids may complex with macromolecules even if of similar charge. It should be noted that the heparin-toluidine blue complex is not very stable; it can be dissociated not only by α-chymotrypsin or polyamino acids, but also by all of the small molecules tested. It is clear that complexing need not result in precipitation, a fact that must be remembered when extrapolating from Table I.

Whereas heparin elicits strong metachromasia when added to dilute solutions of toluidine blue, *N*-acetyl neuraminic acid does not, even in slightly alkaline dye solutions. The colominic acid–toluidine blue complex is slightly metachromatic. Surprisingly, only very slight metachomasia results when an acidic polyamino acid is added to toluidine blue solutions. Whereas colominic acid yields an immediate precipitate with polylysine, greater concentrations were needed to equal the heavy flocculation obtained with heparin.

Neither polyglutamic nor polyaspartic acid forms insoluble complexes with chymotrypsin, histamine, or serotonin. Polylysine does not precipitate when mixed with chymotrypsin. It must be stressed that the selection of a standard concentration by weight for all the substances tested, except, for the dye and heparin-dye complex solutions results in grossly different molar concentrations, and thus Table I must be extrapolated cautiously.

In vivo treatment is startingly ineffectual with respect to both staining and histochemical reactions. The normal toluidine blue metachromasia of mast cells is noted in air-dried, methanol-fixed peritoneal fluid preparations from either experimental or control animals. The nonaqueous method, although excellent for mast cells of control rats, causes gross postfixation disruption and rearrangement of granules in mast cells of polylysine-treated animals, but staining is virtually normal. No dansyl chloride fluorescence is detectable if treatment is limited to several hours; after 3 days of in vitro exposure, faint fluorescence of


15
16
17
18
A
B
C

mast-cell granules is seen in smears of both controls and injected animals.

In contrast, polylysine treatment of air-dried, methanol-fixed peritoneal fluid preparations markedly affects stainability: it abolishes uptake of toluidine blue by nuclei of all cells present and by mast-cell granules. Prolonged staining (1 hr instead of the standard 1 min aqueous dye schedule) barely overcomes this effect: nuclei and mast-cell granules stain faintly, the latter remaining orthochromatic. Polylysine binding in vitro is readily demonstrated by the dansyl chloride method, as both nuclei and mast-cell granules fluoresce brightly after even a short treatment (15 min) in the reagent.

DISCUSSION

For clarity of exposition, polylysine effects will be discussed under three headings: namely, (*a*) effects on the mast cell granules proper, both immediate and long-term, with attention to relevant structural correlates between them and the ground cytoplasm; (*b*) compensatory responses pertaining to a higher order of cellular activity which are detected after some delay, and (*c*) some effects on other cells, selected observations chosen for comparison purposes.

Effects on the Mast Cell Granules

The nature and rapidity with which fusion occurs, and the continuing alterations in fused granule patterns after polylysine injection support the concept, derived from Thorotrast uptake studies (46), that sets of mast cell granules are held within interconnecting channels and that they remain readily accessible to the extracellular environment.

Mast cells are readily degranulated by a variety of positively charged substances, especially macromolecules. Polylysine should represent an extreme case of this cationic effect. Polybasic substances cause "degranulation" following rapid swelling of the granules (4, 5, 53), with resultant bursting of the cell, possibly by osmotic influx of water upon sudden release of histamine (39). Granule swelling is so marked that only a sparse amorphous matrix remains, and extensive swelling of the granules rends the cell so violently that ultrastructural detail in the intergranular areas is lost (6, 22, 62, 64). Contrary to other degranulating agents, polylysine does not cause swelling of the granules—in fact, it stabilizes them. With this substance, interaction is swift: virtually all the granules of a mast cell can be affected within minutes. That polylysine interacts directly with the granules is suggested by the rapid and efficient in vitro blocking of toluidine blue

FIGURE 15 Fused granule cluster, 5 days post polylysine. The outer boundary is clearly delineated by a denser line and the granular domains are still suggested. Compaction is extreme: the granules are deformed to achieve complete packing. Double stain. × 17,900, approximately.

FIGURE 16 Golgi area of affected mast cell, 5 days post polylysine. Abundance of vesicular components and numerous polyribosomal units are noted. The vesicles are empty. Cluster of fused granules denotes that this cell was present at time of injection. There is some accumulation of dense material at angularities of the granular mass, and the granules have a spotty texture. Double stain. × 26,000, approximately.

FIGURE 17 Fused granule cluster in a mast cell, 2 wk post polylysine. The texture of the granular matrix is foamy and is less electron-opaque than at earlier stages. The outer boundary is outlined by a dense material that accumulates at angularities of the surface and penetrates the mass; at high magnification, this material appears coarsely particulate. Granule domains are still visible, if barely so. This fused granule clump was the only one present in this profile, which also contained several dozens of single granules. Double stain. × 14,700, approximately.

FIGURE 18 Golgi area of a mast cell 2 wk post polylysine (A). The well-developed vesicular components contain numerous progranules (B, arrows). Polyribosomal clusters (C) are unusually numerous, as are elements of rough endoplasmic reticulum. Many of the granules (arrows) are denser than others and are loosely held in a membrane-bounded vesicle; their matrix is unevenly textured, as if comprised of evenly sized spherules, and recalls the appearance of a raspberry; they display a ragged outline. These are believed to be granules affected by the polylysine. Doubly stained with uranium acetate and lead citrate solutions. × 7,000, approximately.

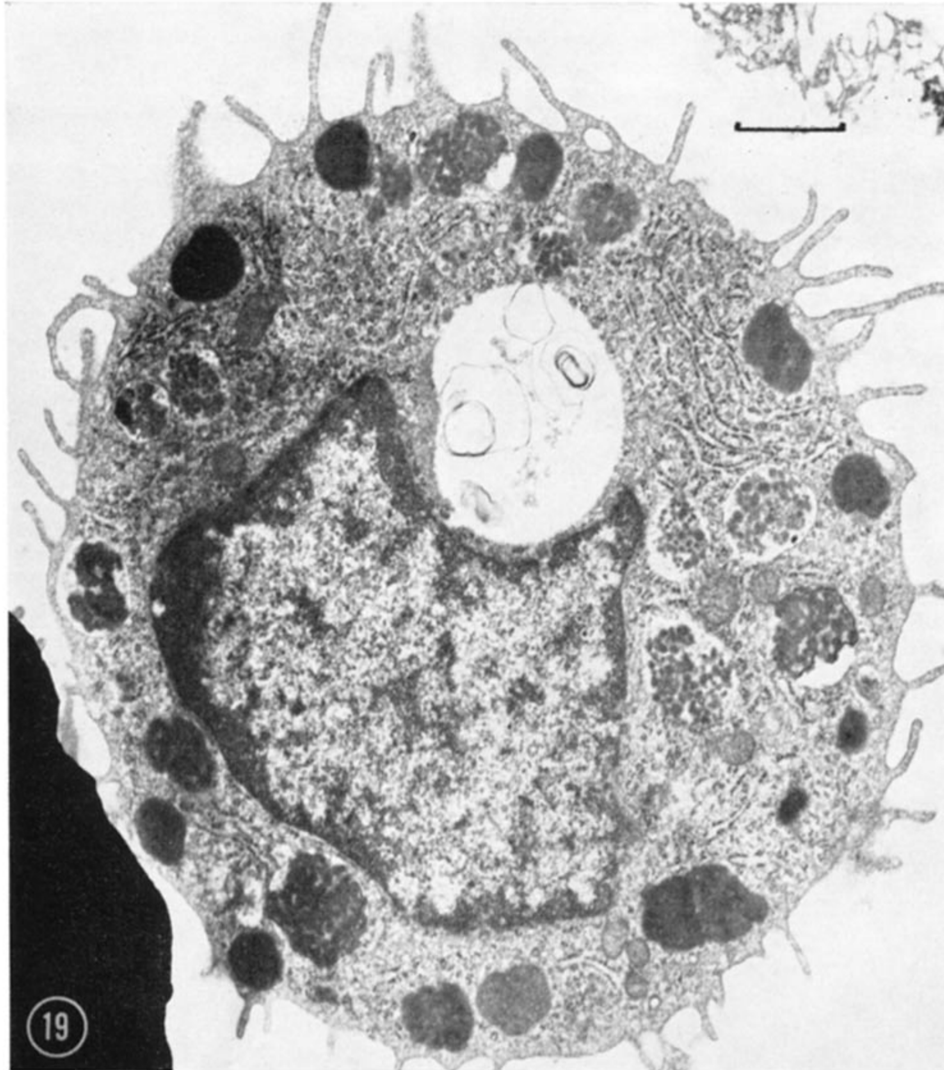


FIGURE 19 Peritoneal mast cell 3 wk after polylysine administration. The cytoplasm is replete with polyribosomes, there is an abundant rough endoplasmic reticulum, and several developing granules (condensing progranules stage) are present. There are also a few mulberry-textured granules of the type seen commonly at 2 wk. The large central area is of unknown nature; it does not seem to be membrane bounded, yet its contents suggest that it is not artifactitious. Likely derived from a degenerating Golgi complex, it is not a common feature after polylysine injection. Double stain. A, × 14,400, approximately; B and C, × 21,000, approximately.

binding by fixed mast-cell granules and by the fact that enough polysine is then bound to yield a strong dansyl chloride reaction.[1] Lack of granule swelling, especially after release, also argues for direct contact because granules generally swell in

[1] Chemical reactions of fixed cells may differ radically from those of live ones. The findings that metachromatic staining of mast-cell granules with toluidine blue is not abolished after polylysine injection and that no fluorescence develops on subsequent dansyl chloride treatment, whereas the opposite is true after in vitro treatment, are not necessarily paradoxical

presence of sodium. This stabilizing effect appears analogous to the condensation of the granular gel seen in vitro with basic dyes or with Compound 48/80 both in vivo (63) and in vitro (42). This latter hypothesis is attractive because it might explain the darker condensation lines seen in areas of contact and at granule periphery (Figs. 6, 11–14).

Fusion of the granules may result from binding of polylysine molecules to the surface of two or more adjacent granules simultaneously, thus bridging them effectively. Nevo et al. (38) have proposed a similar mechanism for polylysine aggregation of erythrocytes. The polylysine used in this study could span a distance[2] of more than 430 A (at body pH [29] the extended form of the molecule would prevail). The charge density on both polylysine and heparin would cause the polylysine bridge to become "reeled in" from both ends, so to speak, against the granular surfaces. Because the width of the polylysine molecule is some 10–15 A, apposition of the granules will be so intimate as to effect complete fusion at the electron microscope level. Normally, mast-cell granules may lie more than 430 A away from each other, but several observations suggest that shuffling of the granules may occur. Thus, time-lapse cinematography (49) reveals coordinated saltatory granular movements. Furthermore, fibrillar material is seen in the intergranular cytoplasm (45, and Figs. 4, 6, and 7 of this communication), and colchicine elicits powerful cytoplasmic movements which lead to marked churning of the granules (43). This could be explained best by a back-and-forth peristaltic shuttling of granules within their common membranous channel(s). Such a process could bring granules temporarily in virtual contact, thus insuring ample opportunities for the postulated interactions between polylysine and heparin of the granules.

The granules are normally nearly spherical; they become flattened in areas of apposition (Figs. 11–15). Therefore, the apposing forces are large or, alternatively, granular substance is readily deformable. Perhaps the polylysine bridge might effect a strong enough pull to deform a plastic granular matrix. Erythrocytes are similarly deformed (30) although there are no forces pushing them together.

The earliest effect of polylysine ranges from mere enlargement of the perigranular gap (Figs. 4 and 7) to extensive degranulation (Figs. 2 and 9). Variability of response from cell to cell must stem in part from heterogeneity of the mast-cell population. In larger measure, however, it must also derive from the fact that the injected material is not instantaneously diluted by the peritoneal fluid. The amount of fluid injected is only about twice the normal peritoneal fluid volume (41). Mast cells will be exposed randomly to a decreasing concentration of polylysine as binding and dilution of the polymer occur; hence, the spectrum of the re-

because only positive findings are admissible in histochemistry. Rather, this could indicate that, in vivo, relatively few heparin sites are involved in polylysine bridging—perhaps only those at the granular surface, and this would not noticeably affect subsequent staining of fixed preparations in which dye-binding involves the entire granule, not just its surface. The relatively smaller amount of polylysine bound in vivo may no longer react with dansyl chloride because all its reactive sites are bound; the stoichiometry in vitro may be such as to leave some available sites. Hence, the data pertaining to mast cells exposed to polylysine in vivo do not conflict with either the slide or test-tube experiments.

[2] This description is admittedly oversimplified, because polylysine molecules could bind to mast-cell granules in several ways: (*a*) the entire molecule could be bound to a single granule, (*b*) the molecule is shaped like a ribbon that binds by its edges and it might lie flat, on edge, or twist irregularly; even if lying "flat," it could still bind both from above and below the peptide plane since the side-chain carbons and the ϵ-amino bonds are free to rotate, and (*c*) it might meander on the granule surface and loop away from it, thus reducing the average molecular reach well below the calculated maximum. These modes may even be interconvertible. This would not alter the proposed mechanism, however, and might, in fact, explain the rates at which changes occur as well as the continuing rearrangements and tightening in patterns of fused granules for several days postinjection.

The shape of the molecule is affected by various factors. At body pH, the random coil conformation prevails, but the extended form is a permitted variant of the random coil. In aqueous ionic medium, and particularly where hydrogen bonding is possible, the extended beta configuration should be favored (29, p. 472).

No direct evidence is available as yet to prove that polylysine is actually bound to the granules in vivo. Although an indirect phenomenon involving the cell surface is theoretically admissible, the inferential evidence does not recommend such a mechanism at this time.

sponse. The enlarged perigranular gap might reflect osmotic inflow of water in response to amine release, as suggested by Norton for Compound 48/80 (39). This distention pressure could suffice for enlarging the aperture where the perigranular sacs meet the cell surface (Fig. 4) and for expelling the aggregated granules into the extracellular environment (Figs. 2 and 9). Thus, the mechanical pressure of swelling granules is not a prerequisite to degranulation. The gap around the granules is again reduced shortly after injection, and by 24 hr it is absent and the membrane becomes intimately apposed to the granular surface. The delay in this interaction suggests that membrane binding sites are normally too few and/or too weak to allow membrane-to-granule cross-linking to occur. Within hours, negatively charged binding sites must be either unmasked or synthesized, and these may then effect cross-linking of the perigranular membranous sac to the granule-bound polylysine. I have suggested (47) that the negative binding charges of the perigranular sac might be associated with sialic acid. Model experiments (Table I) do not negate this hypothesis: colominic acid, a polysialic acid (2), complexes efficiently with polylysine.[3]

Cytoplasmic forces must continue to effecs changes in the disposition of the fused granulet because the initial serpentine patterns (Figs. 3, 5, and 10) are slowly converted to progressively more condensed aggregates (Figs. 11–15).

The long-lasting effects of poly-DL-lysine suggest that it initiates irreversible effects or that it is not readily metabolized by mast cells. In vitro, enzymatic degradation of poly-L-lysine may occur readily, slowly, or not at all, depending on the enzyme involved (32, 63). Chymotrypsin digestion proceeds slowly (26, 37, 60, 61),[4] a finding of special interest because this enzyme is a granular constituent (3, 7, 34). Although both the L and the DL isomers of polylysine have essentially similar anticlotting effects (55), the DL isomer is more active (12). In time, the affected granules are resorbed or eliminated. Resorption may involve selective leaching or digestion of some normal binding substance. Eventually, only dense irregular masses remain loosely held within large vacuoles. Perhaps these are autophagic vacuoles. The dense material that accumulates around affected granules (Figs. 16 and 17) may be involved with these changes, but whether it contains enzymes (e.g., acid phosphatases) remains to be established.

I have recently hypothesized that several sets of granules are present in each mast cell and that these might function as independent physiological units (44). That suggestion is supported by the fact that several clusters of fused granules can be widely distributed in the cytoplasm, that adjoining clusters are not coalesced even when very close to each other, and, more compelling, that aggregates coexist with unaffected granules (Fig. 14). Admittedly, it is impossible to appreciate the three-dimensional continuity of structures from single thin sections, but where interaction has occurred the loss of a perigranular gap within a few hours after injection permits such an interpretation even when only an isolated granule profile is involved, and the statement that unaffected granules are present is defensible.

Early stages of granule formation are not usually drawn into the fused aggregates, suggesting that they may not bind polylysine as readily as older granules do. It is known that the polysaccharide component of younger granules is not as acidic when tested histochemically as that of older ones (10, 36, 52). Alternatively, they may be confined to a less accessible cytoplasmic compartment, al-

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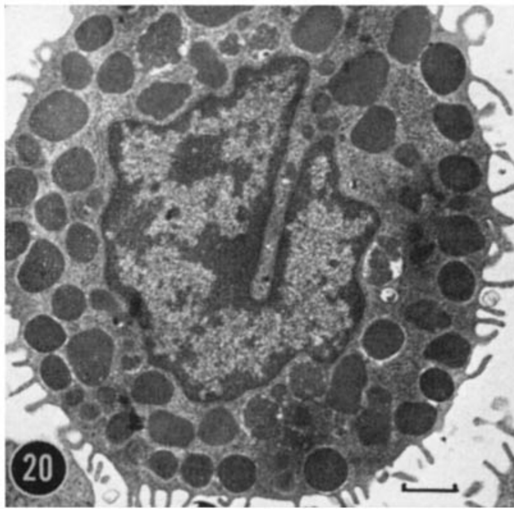
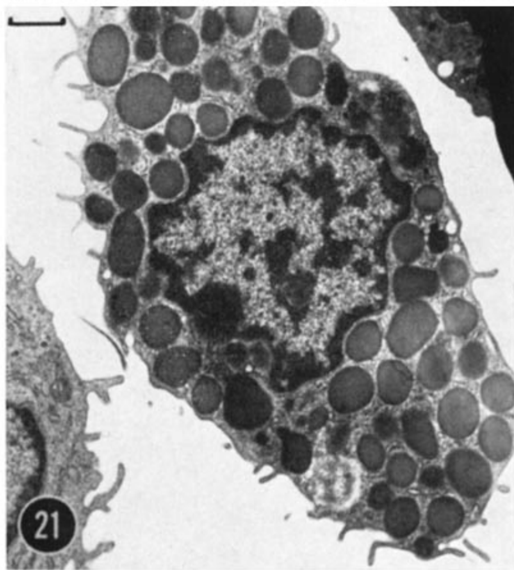
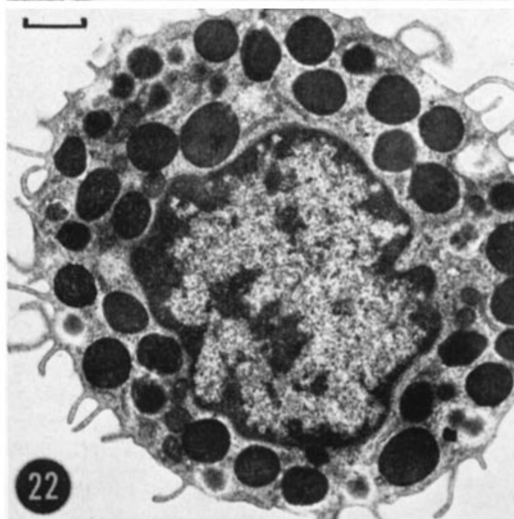
[3] This is not meant to imply that colominic acid is a component of mammalian cell membranes generally, nor of the mast-cell plasmalemma in particular. Rather, it is taken as a type compound, in recognition that neuraminic (=sialic) acids are appreciable components of mammalian membranes in general and of the plasmalemma in particular, and that these apparently contribute substantially to the surface charge of some cell types (4).

[4] Early statements regarding the slow degradation of poly-L-lysine by chymotrypsin (59) seem repudiated by a more recent assertion that "... so far, no homopolyamino acid has been reported to be susceptible to chymotryptic action" (29). Yet, digestibility by chymotrypsin is admitted in the latest review by the Israeli workers (60). As expected, whereas poly-L-lysine is readily digested by trypsin, the D isomer is not (quoted by 27) and a similar stereo-specificity is anticipated for chymotrypsin. The poly-DL-lysine used for the present studies was a random polymer, and thus some digestion (of the few short L segments that might be expected statistically) could occur without materially altering the situation either chemically or ultrastructurally. Further studies with D- and L-polylysines of various molecular weights are indicated.







though this possibility would seem at variance with Thorotrast uptake studies in which particles taken up by mast cells became associated with younger granules before being rapidly carried to more mature ones (46).

Fusion of granules to each other, perhaps as a prelude to secretion (a form of degranulation?), has been demonstrated for serous acinar cells (1, 24), and it has been suggested that microtubules and microfibrils effect a similar response in pancreatic islet cells (32). Whether these are analogous to the mast-cell situation is not clear at this time, but the possibility that they are must be entertained despite the fact that mast-cell granular substance remains mostly insoluble whereas these other secretory products are dispersed or solubilized upon exocytosis. However, previous studies with Thorotrast (46) argue for a basically different process in which perigranular membranes would be permanently interconnected and for which the fusion concept would thus be irrelevant.

Absence of granule fusion after injection of acidic polyamino acids deserves some comment. The granules contain much strongly basic protein, as evidenced by their stainability with Biebrich Scarlet at high pH (66). Bridging of positively charged granular components of adjacent granules by the negatively charged polyaminoacids might have been expected, in analogy to the polylysine effects. It did not occur, and one must infer either (*a*) that these complexes are not stable under conditions prevailing in the cell, (*b*) that perhaps side-chain carboxyl groups of the polyamino acids are not sufficiently ionized at body pH to favor binding, or (*c*) that the acidic polyamino acids cannot compete with the acid mucopolysaccharides present within the granules. It will be noted that no precipitate is formed (Table 1) upon in vitro mix-

FIGURE 20 Peritoneal mast cell, 3 hr after polyglutamic acid injection. There is no discernible difference between this cell and mast cells of untreated rats. Note extent of surface features. Stained with uranyl acetate and lead citrate solutions. × 7,000, approximately.

FIGURE 21 Peritoneal mast cell, 3 hr after polyaspartic acid injection. Cell appears no different than those from untreated animals. Uranyl acetate and lead citrate stain. × 7,100, approximately.

FIGURE 22 Peritoneal mast cell from saline-injected control rat (1 hr). This cell is essentially identical with cells of untreated controls. Note extent of surface features. Double stain. × 8,100, approximately.

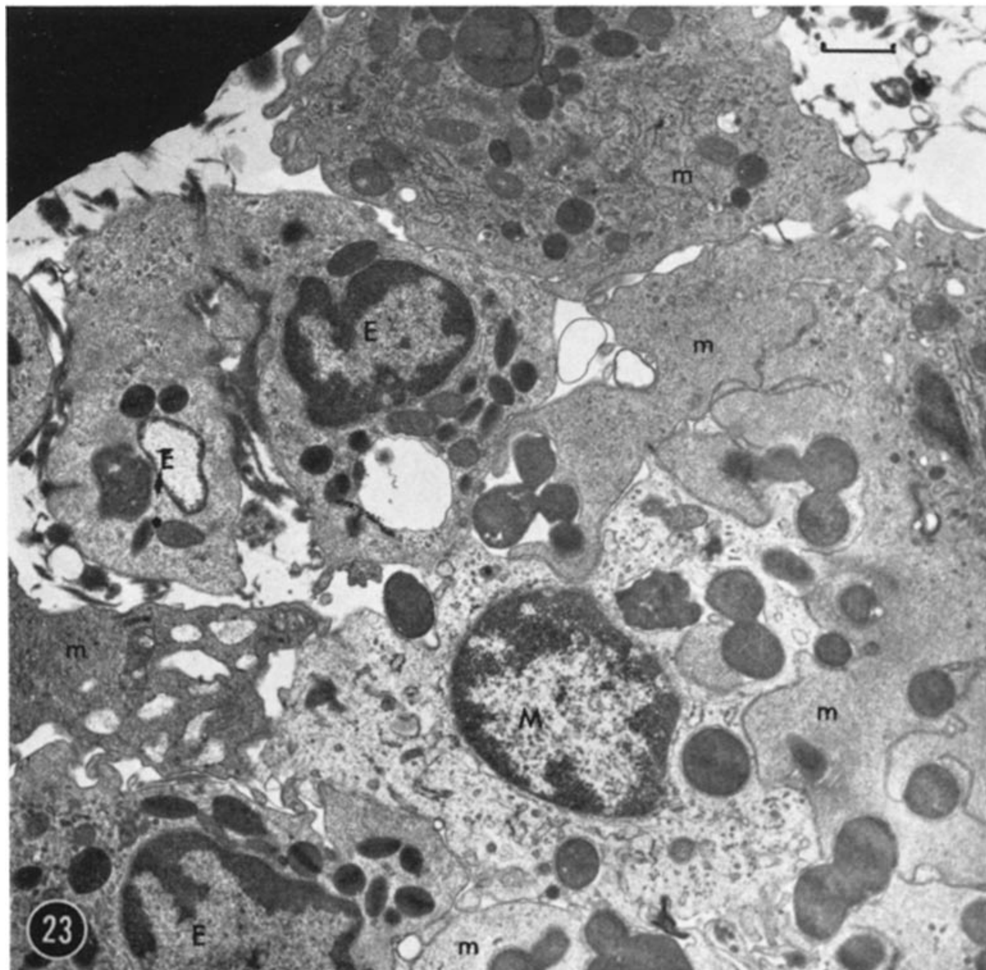


Figure 23 Chemotactic response of macrophages (*m*) and eosinophils (*E*) toward polylysine-affected mast cell (*M*) 5 min after injection. At least five macrophage and two eosinophil profiles surround the mast cell from which some granules are being released. The thin ground cytoplasm of the mast cell contrasts well with the denser ground cytoplasm of the macrophages and eosinophils. In several areas, isolated profiles of macrophage pseudopods are seen reaching into the mast-cell cytoplasmic recesses and are in intimate contact with small groups of fused granules. Note that eosinophil granules appear normal and are not fused to each other. Both macrophages and eosinophils contain ingested mast-cell granules. Double stain. × 9,400, approximately.

ing of polyglutamic or polyaspartic acid with a purified chymotrypsin which displays physicochemical properties not too different from the mast cell chymase (3, 11, 34, 35).

Compensatory Responses

Polylysine stimulates plasmalemmal activity of mast cells (Fig. 10). This is in agreement with work on other cell types (for review, see 56). It also stimulates uptake of particulates by leukocytes (13) and by mast cells (47).

The progressive hypertrophy of the Golgi apparatus and of the endoplasmic reticulum of mast cells after polylysine injection commands attention. Clearly, there is renewed or at least increased granule-synthetic activity following degranulation and/or granule incapacitation. This suggests that

TABLE I
*Interaction of Substances in Test Tube Experiments (Visual)**

	TB 0.001%	Hepar-TB cplx (meta)	PolyLys	PolyGlu	PolyAsp
Tol. bl.			No ppt. Ortho.	V. sl. meta	V. sl. meta
Heparin	meta		Immed., heavy ppt.	No ppt	No ppt
Hepar-TB cplx (meta)			Ortho	Ortho	Ortho ++++
Sialic acid	No ppt; ortho	Ortho	Sl. ppt	No ppt	No ppt
Colominic acid	No ppt; V. sl. meta	Ortho	Immed. ppt	Immed. opalesc.	Immed. opalesc.
α-Chymotrypsin		Ortho	No ppt	No ppt	No ppt
Serotonin		Ortho	No ppt	No ppt	No ppt
Histamine		Ortho	No ppt	No ppt	No ppt

* The substances named in the headings were added to those listed in the first column. The resultant effect is described, using the following abbreviations: meta = metachrcmatic; ortho = orthochromatic; ppt = precipitate; V.sl. = very slight; immed. = immediate; opalesc. = opalescence; Hepar. = heparin; cplx = complex; T.B. = Toluidine blue.

mast cells, by virtue of their granules, perform a steady function and that a feedback mechanism operates to increase their activity when it falters. Interestingly, feedback operates on granule production within existing cells. Cellular proliferation may also occur, but is neither demonstrated nor ruled out by the present study. Several papers in the literature bear on this point (14, 23), but these are subject to criticism or to alternate explanations (17). Activation of the Golgi and ER systems in preparation for regranulation indicates that the granules are products of the mast cells rather than accretions derived from extracellular macromolecules. This further delineates the difference between true and quasi- (18) mast cells and suggests that uptake of preformed polysaccharides by connective tissue cells (8, 16) is unrelated to mast cell differentiation (8).

Temporally, the Golgi apparatus hypertrophies, then becomes active some time before morphological activation of the endoplasmic reticulum. This suggests that granular components may be synthesized sequentially, or that different organelle systems are involved in the various maturation stages of components (i.e., sulfation of already formed polysaccharide?).

These observations demonstrate that degranulation of mast cells, under some conditions at least, is not lethal to the cell. "Regeneration" of mast cells (14, 51, 57, 63) requires several weeks. Some authors invoke mitosis of mast-cell precursors (14, 23); others rule this out (57). The timing is in good agreement with the present findings. Recently, Johnson and Moran (25) have published biochemical evidence which suggests that cytoplasmic integrity is not compromised even by degranulation with Compound 48/80.

The foregoing discussion leads one to ask: are the granules actually *in* the cytoplasm of the mast cells or are they *outside*? If one envisages this topological isolation as more than a membrane-bounded subcellular compartment such as, say, lysosomes, this admittedly is a startling if not heretical query. Yet much of the evidence supports the concept that the granules might be topologically outside the plasmalemma, i.e., in a more rigorous sense than are organelles. Specifically, one must ask whether mast cells secrete their granules into complex invaginations or folds of their plasmalemma and keep them confined within extracellular domains whose ionic composition they control. There are precedents for such a protected system. For instance, the sustentacular cells of the testis display such a relationship toward the developing spermatids. If cells can insulate other cells, could some not similarly insulate nonliving

components? Evidence that the granules continue to mature and grow, long after they arise, need not conflict with this concept. Again by analogy, collagen fibers continue to grow in diameter, and to age chemically as well, although they reside within the extracellular spaces. Mast-cell granules could perhaps represent a specialized glycocalyx, one that loses its intimate molecular attachment to the cell membrane and aggregates into granular masses that retain a looser anatomic (and functional?) relationship to the plasmalemma.

This concept of mast-cell structure could explain many observations in the confused mast-cell literature and could reconcile a few paradoxical ones. Only the briefest discussion is allowed, and only two implications will be considered. First, one might think of the granules as specialized "inverse lysosomes," in that they contain insolubilized enzyme to which the cell brings substances gleaned from the environment for possible processing—a biological analogue of the chemist's resin-bound enzyme column.

If so, the granules may remain associated with any given mast cell for a long time (50). Experiments in progress indicate a minimum of 8 months (48), a finding which supports the idea that mast-cell function is independent of degranulation. Spontaneous mast-cell degranulation (i.e., in urticaria pigmentosa) could perhaps be analogous to demyelination (i.e., in neuropathology). Second, it would mean that the perigranular "sac" is really a plasmalemmal infolding analogous to the intracellular canaliculi known to exist in some cells (e.g., gastric parietal cells). This concept would resolve some of the reservations regarding "degranulation" which have long troubled workers in the field. Thus Högberg and Uvnäs (20), as far back as 1957, suggested that shedding of granules may not cause mast-cell membrane destruction, but that the granules may actually be "secreted" through the intact membrane; this seems to occur even *before* granule swelling takes place (68), a process which, together with histamine release, appears to depend on contact with extracellular ions (34, 68). Histamine release from sucrose-isolated granules by low concentrations of salt in vitro prompted Lagunoff to argue that maintenance of mast-cell granule integrity in vivo "... calls for a mechanism that isolates the granules from the ionic milieu of the cell sap" (33, p. 92). Clearly, the ultrastructural organization of mast cells proposed in the present communication would satisfy these conditions by isolating the granules from both the intra- and the extracellular milieus.

As a closing cautionary note, it must be emphasized that the work presented here refers specifically to mast cells of the rat, and supportive literature has been selected mostly with this in mind. It is imperative that these observations be extended to other species—especially since there are major differences in granule composition and ultrastructure from species to species—before the concepts introduced above be assumed to apply to mast cells in general. However, excellent electron micrographs of degranulating human mast cells published recently (31) suggest that the present observations may have general significance for other species.

Effects on Other Cells

The effect of polylysine on macrophages and on eosinophils is not unexpected. Indeed, positively charged macromolecules greatly stimulate pinocytotic processes in a variety of cells (56). They also favor phagocytosis of starch grains by leukocytes (38) and of latex microspheres by mast cells (47). Isolated mast-cell granules are themselves avidly phagocytized by connective tissue fibroblasts (19), and macrophages will readily ingest mast-cell granules that have been shed in response to Cpd 48/80 or distilled water treatment (65). Chemotaxis for eosinophils after Cpd 48/80 -induced degranulation, on the other hand, has been denied by some (65) while affirmed by others (70), suggesting the affinity is not marked. The mast cell–polylysine complex, on the other hand, is evidently powerfully chemotactic for both macrophages and eosinophils without eliciting an influx of neutrophilic leukocytes into the injection site. Such an influx is noted upon injection of the acidic polyamino acids, however. This is at variance with the report of Stein et al. (67) which showed that both basic and acidic polyamino acids elicited diapedesis of neutrophils into the injected site. The reasons for this discrepancy are not clear but, in view of Fruhman's studies on neutrophil mobilization (15), greater attention to possible contamination with bacterial polysaccharides will be required before emphasizing the discrepancy. Fruhman reported that whereas small doses of lipopolysaccharides elicit neutrophil diapedesis, larger doses are inhibitory in that they delay the response. Polylysine can agglutinate bacteria (28) and thus might also bind and neutralize bacterial products;

the stoichiometry of the interaction thus may determine the response.

Despite the presence of strongly basic components in eosinophil granules (as indicated by their affinity for acid dyes), neither of the acidic polyaminoacids provoked their fusion or release. Neutrophil granules also contain basic substances (9, 58), but these granules, too, remained unfused. This contrasts with effects of polylysine on mast cells.

The immediate recruitment of several cell types in the sequelae to mast cell degranulation with polylysine emphasizes that mast cells may, at times, shift the ecological balance of connective tissues. The rapid and vigorous response of mast cells to positively charged polymers should alert us to possible mast-cell functions involving physiological basic macromolecules (i.e., protamines, histones, lysozyme) or basic peptides.

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