Antigen-Induced, IgE-Mediated Degranulation of Cloned Immature Mast Cells Derived From Normal Mice

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Cloned, immature mast cells derived from normal mice were passively sensitized with mouse monoclonal IgE antibodies with specificity for DNP, and then stimulated to degranulate with DNP_{35}-HSA. Cells were fixed for transmission electron microscopy or recovered for quantitation of histamine release at various intervals up to 30 minutes after antigen challenge. The cloned mast cells rapidly extruded the contents of their immature granules (dense progranular material and membrane-bound vesicles) to the exterior via multiple openings in the plasma membrane. Degranulation was associated with striking activation of the cell surface, characterized initially by elongation of surface processes, as well as by close approximation of strands of rough endoplasmic reticulum to the cell surface and by the development of coated pits. At later times after stimulation, degranulated mast cells had released nearly all of their granules and exhibited angular surfaces lacking elongated processes. These findings demonstrate for the first time that cloned, immature mast cells, like their mature counterparts, can undergo classic morphologic release reactions involving exocytosis of granules. (Am J Pathol 1987, 126:535–545)

IN 1981, several groups, including our own, reported that cells with many of the features of mast cells developed in cultures of normal mouse hematopoietic cells maintained in media containing macromolecules derived from mitogen-activated T cells, cloned $Ly1^+2^-$ inducer T cells, or WEHI-3B tumor cells.[1–6] Hasthorpe had reported a similar finding in 1980,[7] although her mast cell-like cell line was derived from the spleen of a mouse previously given an injection of cell-free supernatant from Friend-virus–producing erythroleukemia cells. These cells had several features of mast cells, but contained C-type virus particles as seen by electron microscopy.[7]

It was clear from the beginning that mouse mast cells grown in suspension cultures differed in certain important respects from the best characterized cell type available for detailed comparative studies, mature peritoneal mast cells (reviewed in[8–10]). The cultured cells appeared immature by ultrastructure,[1,9–11] contained low levels of histamine,[1–3,6,9–13] and expressed fewer cell surface receptors for IgE immunoglobulin than did mature peritoneal mast cells.[10] In addition, we[10] and others[13,14] found that the cultured mast cells incorporated $Na_2{}^{35}So_4$ into granule-associated chondroitin sulfates. In contrast to the cultured mast cells, normal mouse peritoneal mast cells synthesized heparin.[10,14]

We[1,9–11] and several other investigators[5,13,15] noted that growth factor-dependent cultured mouse or rat mast cells expressed certain similarities to "T-cell–dependent" or "mucosal" mast cell (MMC) populations *in vivo* (reviewed in[8–10]). Although some investigators suggested that cultured mast cells might be committed to express the MMC phenotype, we[1,8–11] and Yung and Moore[16] argued that an alternative hypothesis could not be excluded: that many of the properties of cultured mast cells might reflect their immaturity. Attempts to induce further maturation of mast cells in suspension culture met with only limited success. The inducing agent sodium butyrate caused a marked inhibition of cultured mast cell proliferation, resulted in the increased storage of hista-

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mine and chondroitin sulfate, and favored partial maturation of cytoplasmic granules.[10,11] The cells did not appear fully mature by ultrastructure, however, nor did they synthesize detectable amounts of heparin.[10] By contrast, when growth-factor–dependent mouse mast cells were injected into genetically mast-cell–deficient WBB6F$_1$-*W/W^v* mice, they generated two phenotypically distinct mast cell populations; mast cells with the morphologic and histochemical features of heparin-containing connective tissue type mast cells developed in the skin, peritoneal cavity, and stomach muscularis; whereas mast cells with the features of MMC developed in the stomach mucosa.[17] These findings support the view that growth-factor–dependent cultured mouse mast cells represent an immature cell in the mast cell lineage.

In part because growth-factor–dependent mouse mast cells can be generated in large numbers *in vitro,* and in part because these cells are thought to share many similarities with MMCs, they have represented a popular model system for the analysis of mast cell mediator release.[11,18,19] Until now, this work has focused almost exclusively on the biochemistry and kinetics of the release reaction or on the nature of the mediators elaborated upon stimulation of the cells. We therefore performed ultrastructural studies of growth-factor–dependent cloned mouse mast cells that had been passively sensitized with a mouse monoclonal anti-DNP IgE and then stimulated to release histamine by exposure to specific antigen (2,4-dinitrophenylated human serum albumin [DNP$_{35}$–HSA]).

Materials and Methods

Cloned Mouse Mast Cells

The cloned mast cells (C1.MC/9) have been described in detail.[1,8–10,20] We showed previously that this clone appears immature by ultrastructure,[1,8–10] synthesizes ^{3}H-histamine from ^{3}H-histidine, and stores lower levels of histamine than do normal mouse peritoneal mast cells,[9–11] and expresses roughly half as many plasma membrane receptors for IgE than are present on normal mouse peritoneal mast cells.[10] We also showed that these cells bind IgE with an equilibrium constant very similar to that of normal mouse peritoneal mast cells[10] but synthesize chondroitin sulfate glycosaminoglycans, rather than heparin.[10] The cells synthesize serotonin and store larger amounts of this amine than do normal mouse peritoneal mast cells.[20] Clone MC/9 differs in surface structures and function from B cells, suppressor or inducer T cells, macrophages, and NK-like cells.[9,10] The cells used in the present study were strictly growth-factor ("IL-3"[21,22])–dependent and were maintained as previously described in medium supplemented with the supernatants of concanavalin A-activated BALB/c spleen cells.[1,9,10] Some cultures were supplemented with sodium butyrate (1 mM) for 4 days for induction of partial maturation of the mast cells.[10,11]

Stimulation of Degranulation

Mast cells were washed twice in "medium" (Hanks' minimal essential medium with Pipes buffer, 0.47 g/l, replacing $NaHCO_3$ [GIBCO]) and then were passively sensitized with monoclonal mouse IgE anti-DNP,[23] the generous gift of F.-T. Liu, D. H. Katz, and T. Ishizaka. The cells (1.0×10^7 in 1.0 ml "medium" supplemented with 10% fetal calf serum, pH 7.2) were incubated with 100 μg IgE/ml for 60 minutes at 37 C.[11] They were then washed three times in "medium" and incubated ($3–5 \times 10^5$ cells/1.0 ml of "medium," at 37 C) with antigen (DNP$_{35}$–HSA at 0.01 μg/ml[11]). Controls included cells incubated for 60 minutes at 37 C without IgE, which were then stimulated with antigen, and cells which had been passively sensitized with IgE but then incubated without antigen. Replicate samples were recovered at various intervals after the addition of antigen for ultrastructural studies (see below) or for measurement of histamine content. For analysis of histamine release, the cells and supernatant were recovered separately after centrifugation in a Brinkmann microfuge (2 minutes, room temperature) and were analyzed fluorometrically in an Autoanalyzer II (Technicon Instruments Corporation, Tarrytown, NY) equipped to detect histamine in the 0.5–10 ng/ml range.[24]

Transmission Electron Microscopy

Cells were fixed by the addition of a sevenfold excess volume of 1% paraformaldehyde, 1.25% glutaraldehyde, 0.025% $CaCl_2$ in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hour at room temperature.[25,26] The fixed cells were washed twice in 0.1 sodium cacodylate buffer, pH 7.4, and were centrifuged through soft agar in a microfuge.[25,26] The agar-embedded pellets were then processed for electron microscopy as previously described.[25,26] Some samples were exposed to cationized ferritin (2.0 mg/ml, Miles Laboratories, Kankakee, Ill) prior to spinning into agar blocks (after fixation) as previously described.[27,28] The agar-embedded pellets were dehydrated in a graded series of alcohols and then infiltrated and embedded in a propylene oxide–Epon sequence. Thin sections were cut with an LKB 5 ultramicrotome (LKB Instruments, Rockville, Md), placed on copper grids, stained lightly

with lead citrate, and examined in a Philips 400 electron microscope.

Results

Control Mast Cells

All of the control mast cells (cells not passively sensitized with IgE but then exposed to antigen or cells passively sensitized with IgE but incubated without antigen) appeared similar by transmission electron microscopy (Figure 1). In accord with our previous descriptions of the ultrastructural features of these cells,[1,9-11] the control mast cells appeared round, with a surface exhibiting regular short folds (Figure 1A). The nuclei appeared oval, lobular, or, in some planes of section, segmented, and exhibited variable condensation of chromatin. Actively dividing cells often contained fewer granules than did cells not undergoing division. The cells contained immature granules, consisting of round or oval membrane-bound cytoplasmic structures with variable amounts of dense progranular material and small membranous vesicles. After treatment with sodium butyrate for 4 days,

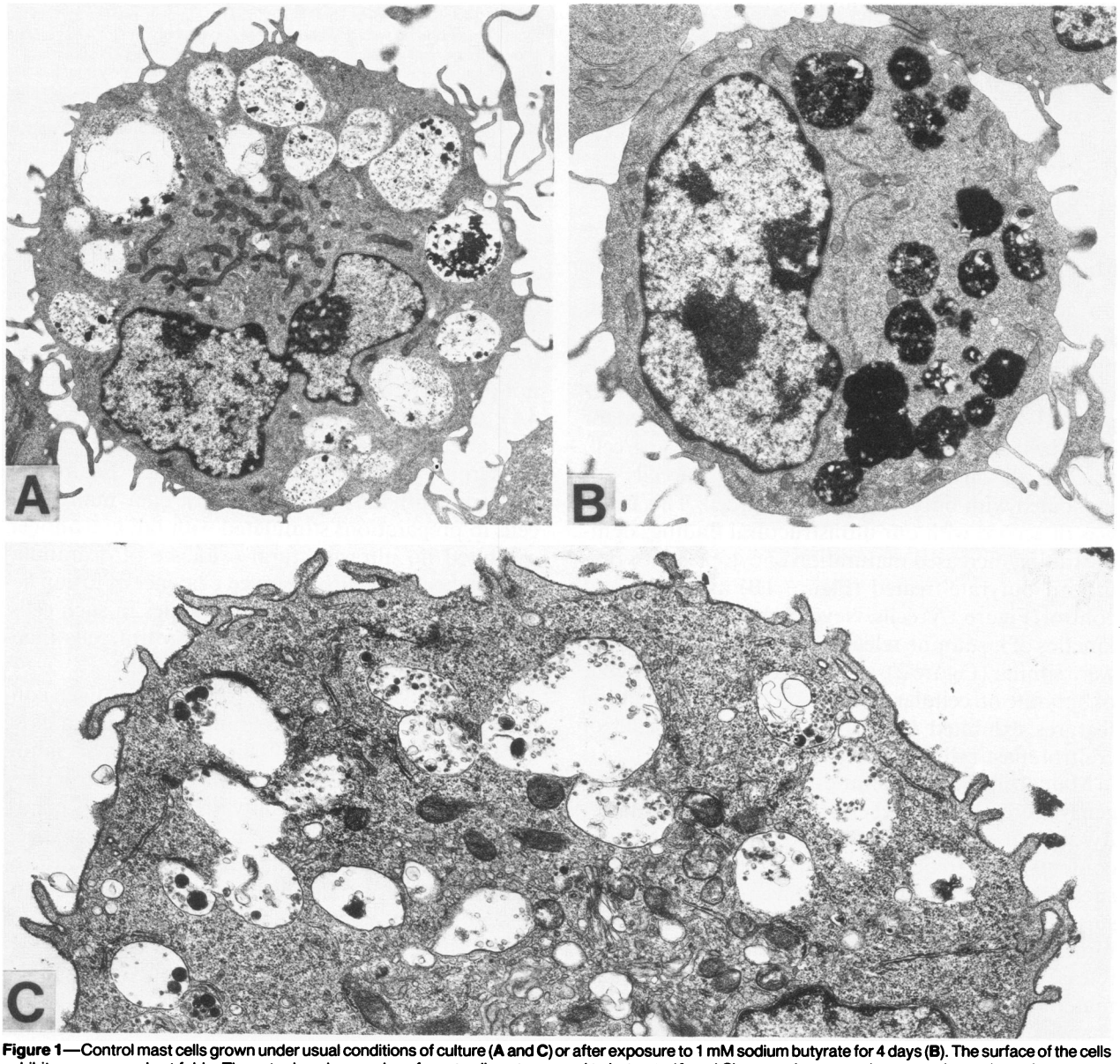

Figure 1—Control mast cells grown under usual conditions of culture (**A** and **C**) or after exposure to 1 mM sodium butyrate for 4 days (**B**). The surface of the cells exhibits numerous short folds. The cytoplasmic granules of mast cells not exposed to butyrate (**A** and **C**) appear immature: large membrane-bound structures containing many vesicles as well as a few dense progranular structures. After exposure to butyrate (**B**), the cytoplasmic granules of many cells are nearly filled with dense content. Occasionaly similar cells were also observed in preparations not treated with butyrate. The cell shown in **C** was exposed to cationized ferritin after fixation. The tracer stains the cell membrane uniformly but does not enter any of the immature granules. (**A**, ×5500; **B**, ×8500; **C**, ×15,000)

many of the cells contained granules with increased amounts of dense content and little if any recognizable vesicular material (Figure 1B). Some of the granules of such cells appeared homogeneously electron-dense. Control mast cells also exhibited nondilated rough endoplasmic reticulum (RER), free ribosomes, mitochondria, and, at the surface, coated pits. Few coated vesicles were observed in the cytoplasm. Deposits of cytoplasmic glycogen were not seen.

To demonstrate that the largely electron-lucent immature granules present in control mast cells did not communicate with the cell exterior, some cells were exposed to cationized ferritin after fixation (Figure 1C). Cationized ferritin uniformly labeled the plasma membrane of such cells but was never observed within the immature granules. This is an important point, because largely lucent immature granules represent a characteristic feature of cultured mast cells maintained under conditions similar to those in this report,[1,9-11] and these structures should not be interpreted as evidence of ongoing exocytosis.[29]

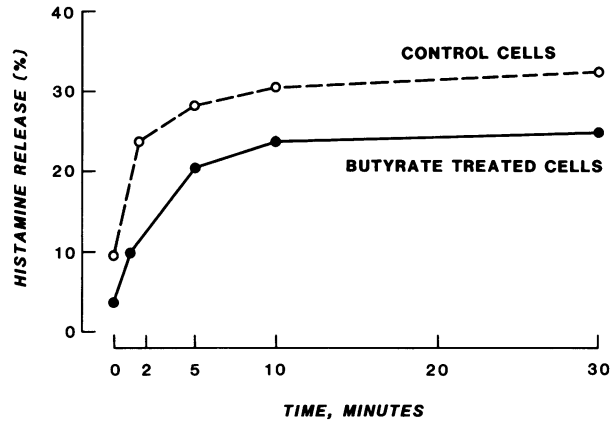

Figure 2—IgE-dependent, antigen-induced histamine release by cloned mast cells incubated with or without 1 mM sodium butyrate for 4 days. Data shown are histamine percentage of the total sample (cells plus supernatant) present in the supernatant of mast cells recovered immediately after passive sensitization with IgE for 60 minutes ("0" time), or at various intervals after addition of DNP_{35}–HSA. Controls included butyrate-treated or untreated cells incubated for 60 minutes without IgE and then for 30 minutes without antigen (3.6% and 5.9% release, respectively) or for 30 minutes with antigen (3.7% and 5.6% release, respectively) and butyrate-treated or untreated cells incubated with IgE for 60 minutes and then for an additional 30 minutes without antigen (7.7% and 7.9% release, respectively).

Mast Cells Stimulated With IgE and Specific Antigen

According to measurements of cell-associated and supernatant histamine, the extent and kinetics of mast cell degranulation in the cells used for ultrastructural analysis were similar to those previously reported.[11] The histamine content of cells incubated for 4 days with 1 mM sodium butyrate was 0.75 pg/cell, whereas the histamine content of control cells not incubated with butyrate was 0.35 pg/cell. This result was in accord with our ultrastructural findings demonstrating increased maturation of cytoplasmic granules in butyrate-treated (Figure 1B) as opposed to control (Figure 1A) cells. Nevertheless, the extent and kinetics of histamine release in these two populations were similar (Figure 2), and, apart from the influence of butyrate on cellular maturation, the ultrastructural features exhibited by activated butyrate-treated or control mast cells were identical.

Many cells fixed 2 minutes after exposure to antigen (Figure 3) exhibited exteriorization of the majority of their cytoplasmic granules. Extruded granule contents were frequently observed at the surface of such cells or in association with numerous elongated surface processes (Figure 3). Other mast cells fixed 2 minutes after activation by antigen exhibited focal release of granule contents (both dense progranular material and membrane vesicles) but no apparent change in the remainder of the cytoplasmic granules. The release of individual granules occurred at multiple sites in the cell surface of a single cell. Some cells exhibiting evidence of degranulation contained strands of RER in close association with and parallel to the plasma membrane surface (Figure 4A). Such strands of RER contained ribosomes on the membrane more distant from the plasma membrane but not on the membrane nearer the cell surface (Figure 4A). An additional feature of mast cells fixed 2 minutes after exposure to antigen was the presence of numerous coated pits on the cell surface (Figure 4B). In accord with the histamine release data, many mast cells in preparations stimulated with IgE and antigen exhibited no ultrastructural evidence of degranulation. We occasionally observed evidence of fusion between individual cytoplasmic granules in such cells, but no more frequently than in control cells incubated without IgE or antigen.

Cells fixed 5 minutes after exposure to antigen continued to exhibit extruded granule material in close approximation to the cell surface or in association with greatly elongated and complex surface structures (Figure 5). In addition, many activiated cells exhibited large clefts extending from the cell surface to deep within the cytoplasm (Figure 5A–C). Unlike the processes of control cells (Figure 1), many of the processes of activated mast cells were discontinuous with the cell surface in the plane of section examined (Figures 3–7). Some activated cells exhibited areas of the surface that were devoid of cell processes but were associated with abundant extruded granule contents (Figure 6).

At 10 minutes after stimulation with antigen (Figure 7), some mast cells continued to exhibit deep

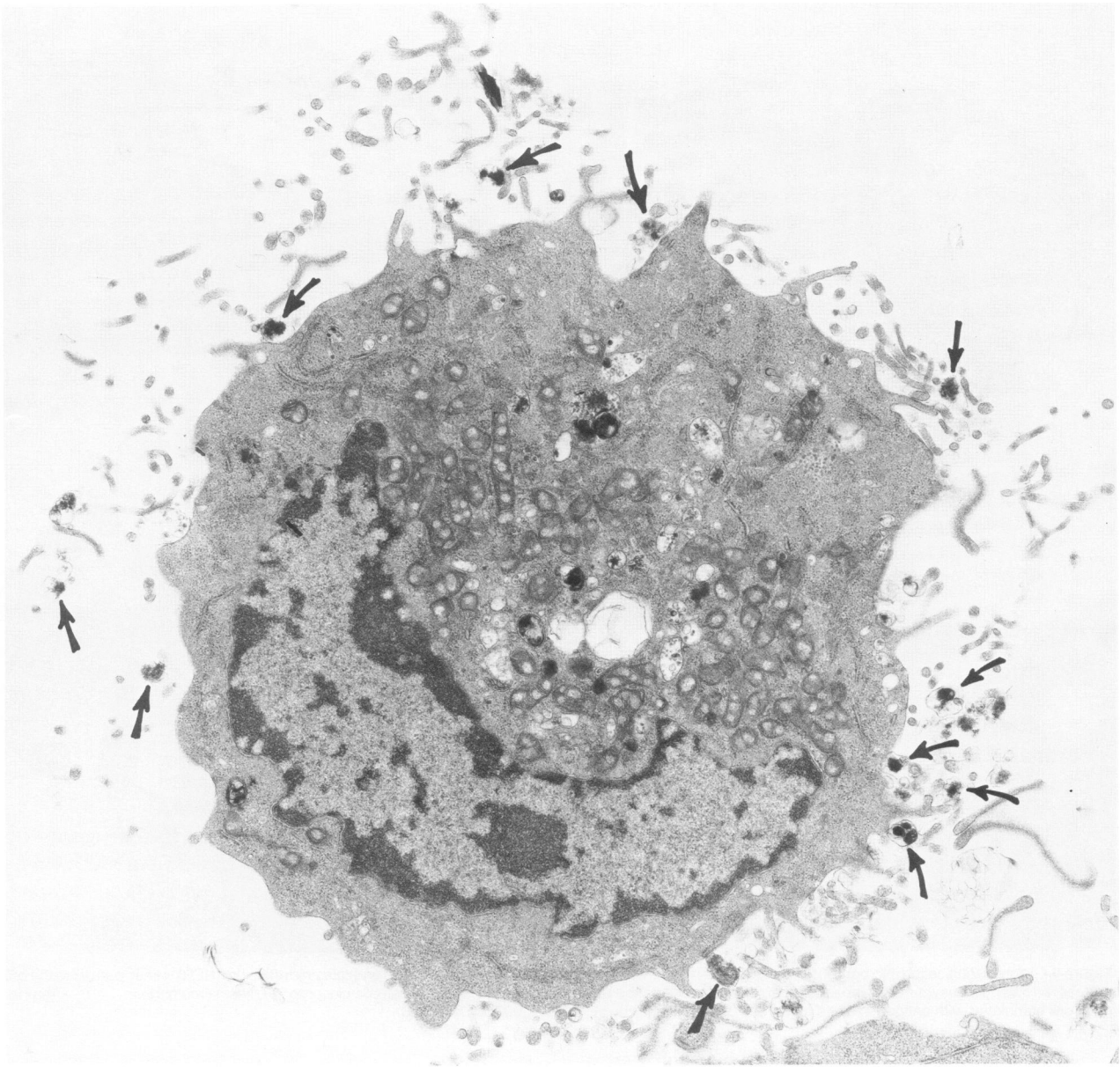

Figure 3 — At 2 minutes after exposure to antigen, this passively sensitized immature mast cell has extruded nearly all of the contents of the immature granules. Dense progranular structures (*arrows*) remain close to the cell surface, which exhibits elongated and complex surface processes. (×14,500)

clefts in communication with the cell surface, whereas other, smaller cells exhibited a scant cytoplasm and large areas of surface that had few or no points of attachment to elongated surface processes. Other cells contained a few remaining immature cytoplasmic granules, strands of RER extending to the cell surface, and numerous associated cell processes, many of which had no point of attachment to the cell surface. Similar cells were also observed in preparations fixed 30 minutes (Figure 7) after exposure to antigen, and some of these cells had an even less complex surface than those fixed at earlier intervals after stimulation. Studies of activated mast cells exposed to cationized ferritin after fixation indicated that none of the immature granules retained in the cytoplasm of these cells admitted the tracer, evidence that these structures did not communicate with the cell surface.

Discussion

In a previous study of the degranulation of uncloned, growth-factor–dependent mouse mast cells,

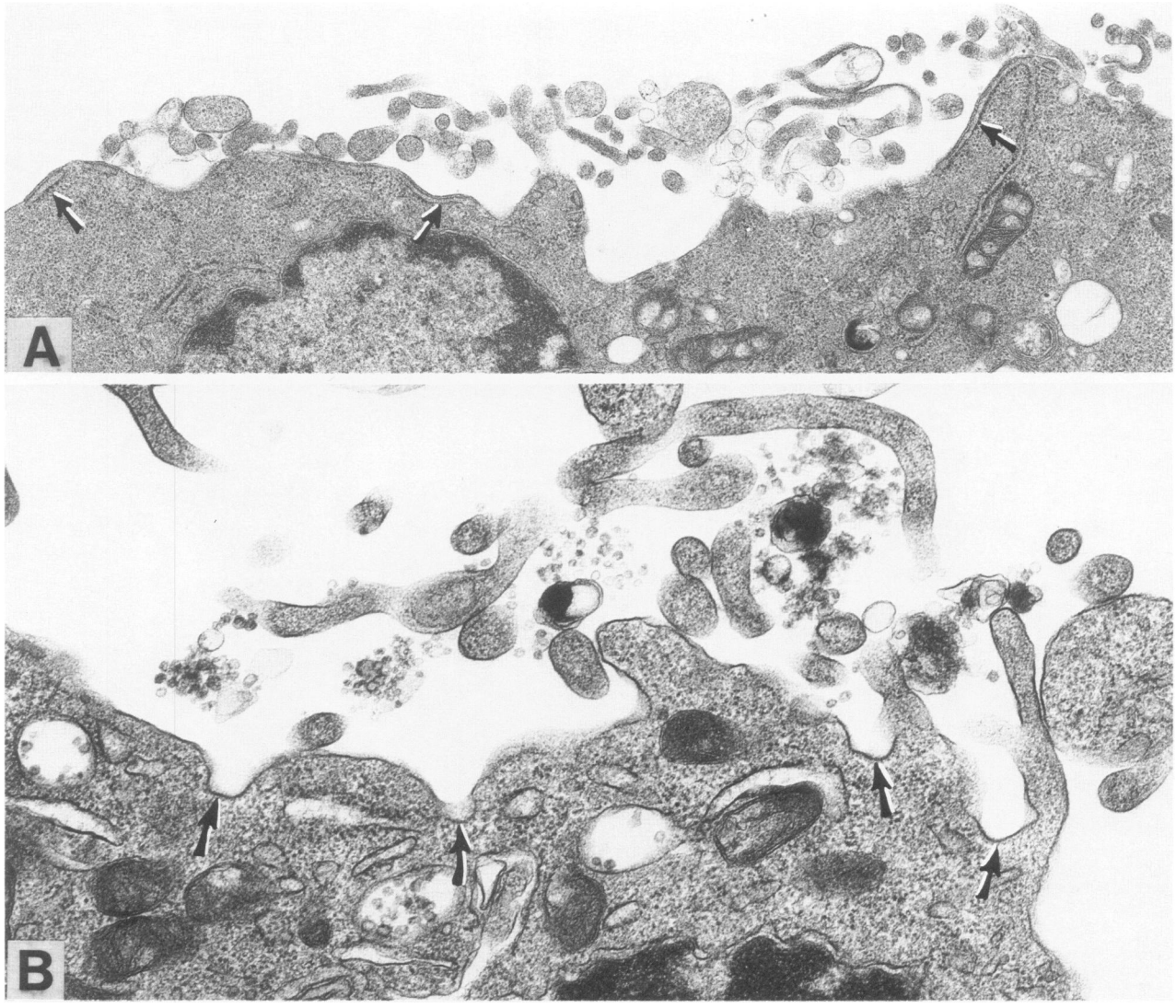

Figure 4—High magnification micrographs of a mast cell 2 minutes after stimulation with antigen. Rough endoplasmic reticulum (RER) is seen parallel to the cell surface (*arrows*). The asymmetric appearance of this RER reflects the absence of ribosomes on the lamella closer to the plasma membrane. **B**—Another mast cell stimulated with antigen exhibits numerous coated pits (*arrows*). (**A**, ×22,000; **B**, ×33,500)

the authors concluded that cells stimulated with IgE and anti-IgE or with the calcium ionophore A23187 showed evidence of fusion between granules but not "the obvious structural characteristics involved in exocytosis."[29] In the present study, we found that cloned mouse mast cells stimulated to degranulate in response to IgE and specific antigen extruded individual cytoplasmic granule contents to the exterior of the cell. This was accompanied by the development of clefts extending from the surface to deep within the cytoplasm of degranulating cells, as well as extraordinary activation of the cell surface. These features are very similar to those that have been described previously in degranulating human basophils[9,30–34] or in mast cells derived from a number of different sources.[9,26,30,35–42] Unlike mature basophils or mast cells, however, most of the granules in growth-factor–dependent mouse mast cells maintained under usual culture conditions exhibit heterogeneous contents consisting of a mixture of dense progranular material and membranous vesicles. Moreover, some of the granules in control cells appear virtually devoid of electron-dense content. Our studies with cationized ferritin indicate that these cytoplasmic structures, which we interpret as immature granules, do not communicate with the cell exterior and therefore should not be regarded as evidence of degranulation.

Our study demonstrates clearly that cloned mast

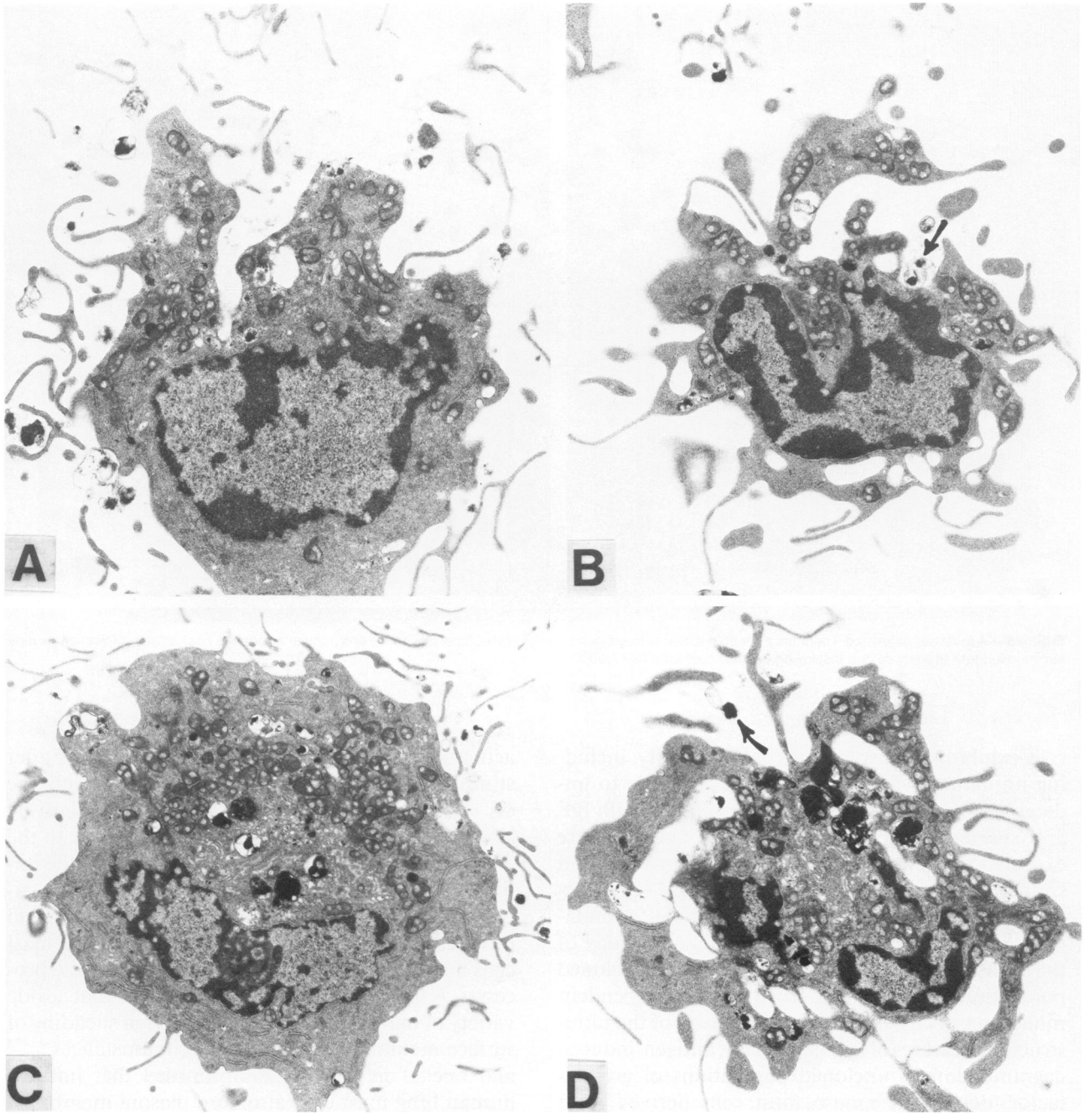

Figure 5—Immature mast cells fixed 5 minutes after antigen-induced degranulation showing large clefts extending from the surface to deep within the cells (**A**, **B**, and **D**). Extruded dense progranular structures (*arrows* in **B** and **D**) can be seen within some of these clefts. Two of the cells (**B** and **D**) exhibit irregular nuclear contours, and all contain numerous mitochondria. Many surface processes appear in discontinuity with the cell surface in the planes of section shown (especially in **A** and **C**). (**A**, ×10,000; **B**, ×9500; **C**, ×7000; **D**, ×9000)

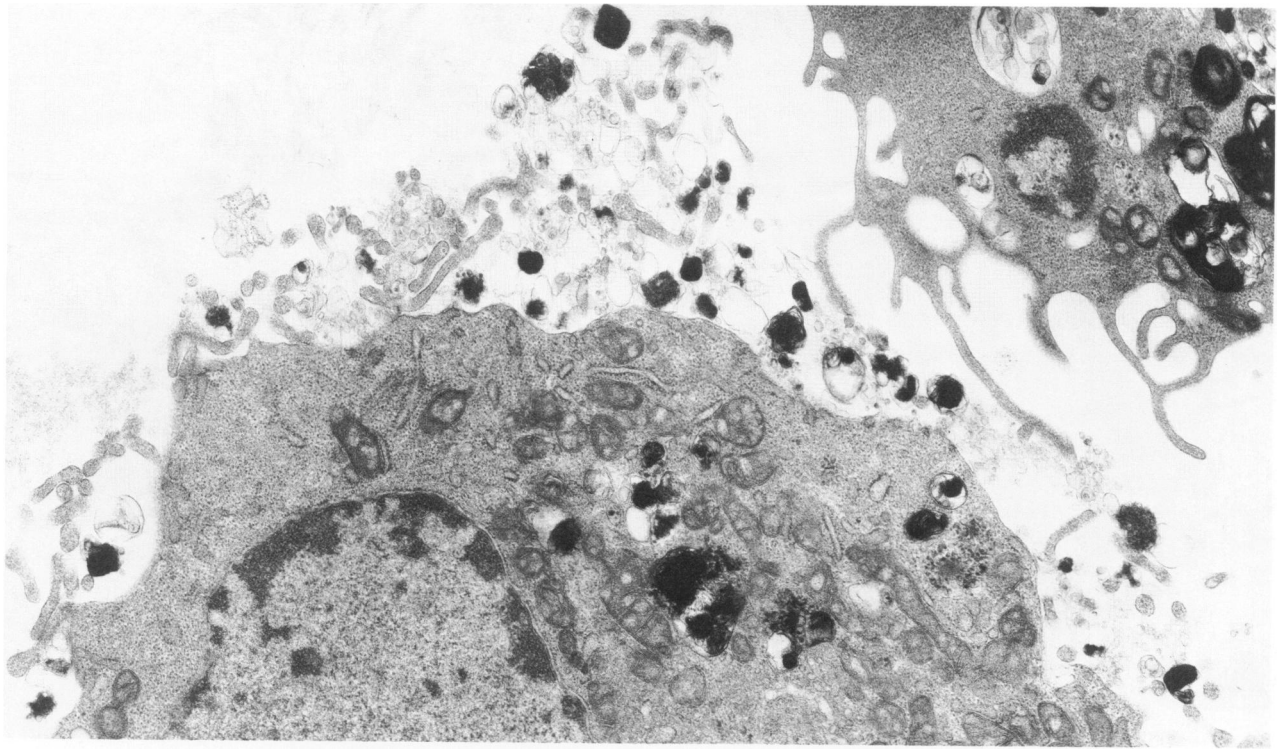

Figure 6—A mast cell examined 5 minutes after stimulation with antigen shows large numbers of dense progranular structures near an area of the cell surface lacking elongate processes and displaying angular contours. (×17,500)

cells exhibiting many features of immaturity, including immature cytoplasmic granules, are able to undergo exocytosis in response to stimulation with IgE and specific antigen. Although these findings were expected in view of previous reports that these and similar cell populations can release granule-associated mediators upon appropriate stimulation,[1,11,13,18,29] our report represents the first study of the ultrastructural features of degranulation in cloned populations of immature, growth-factor–dependent mouse mast cells. Furthermore, analysis of the ultrastructural features of IgE-dependent, antigen-induced degranulation in uncloned populations of growth-factor–dependent mouse mast cells derived from C57BL/6 mice gave results very similar to those obtained with clone MC/9 (data not shown).

As in other models of basophil or mast cell activation,[9,26,30–42] stimulation of degranulation in cloned mouse mast cells was associated with extraordinary activation of the cell surface. At later intervals after stimulation (10–30 minutes), the plasma membrane of many degranulated mast cells was relatively smooth, with few or no points of attachment to the elongated surface processes often observed in the immediate vicinity of the cells. Among the various possible explanations for this finding, we are attracted to the idea that degranulation of cloned mouse mast cells may be associated with shedding of surface processes. A large body of literature indicates that a wide variety of mammalian cells may undergo shedding of surface membrane under certain circumstances,[43–54] and one of us (AMD) has proposed that purified human lung mast cells also shed plasma membrane after stimulation with anti-IgE.[26] Large numbers of cloned mouse mast cells may be grown *in vitro,* suggesting that these cells may represent a good model system for the analysis of membrane changes associated with cell activation.

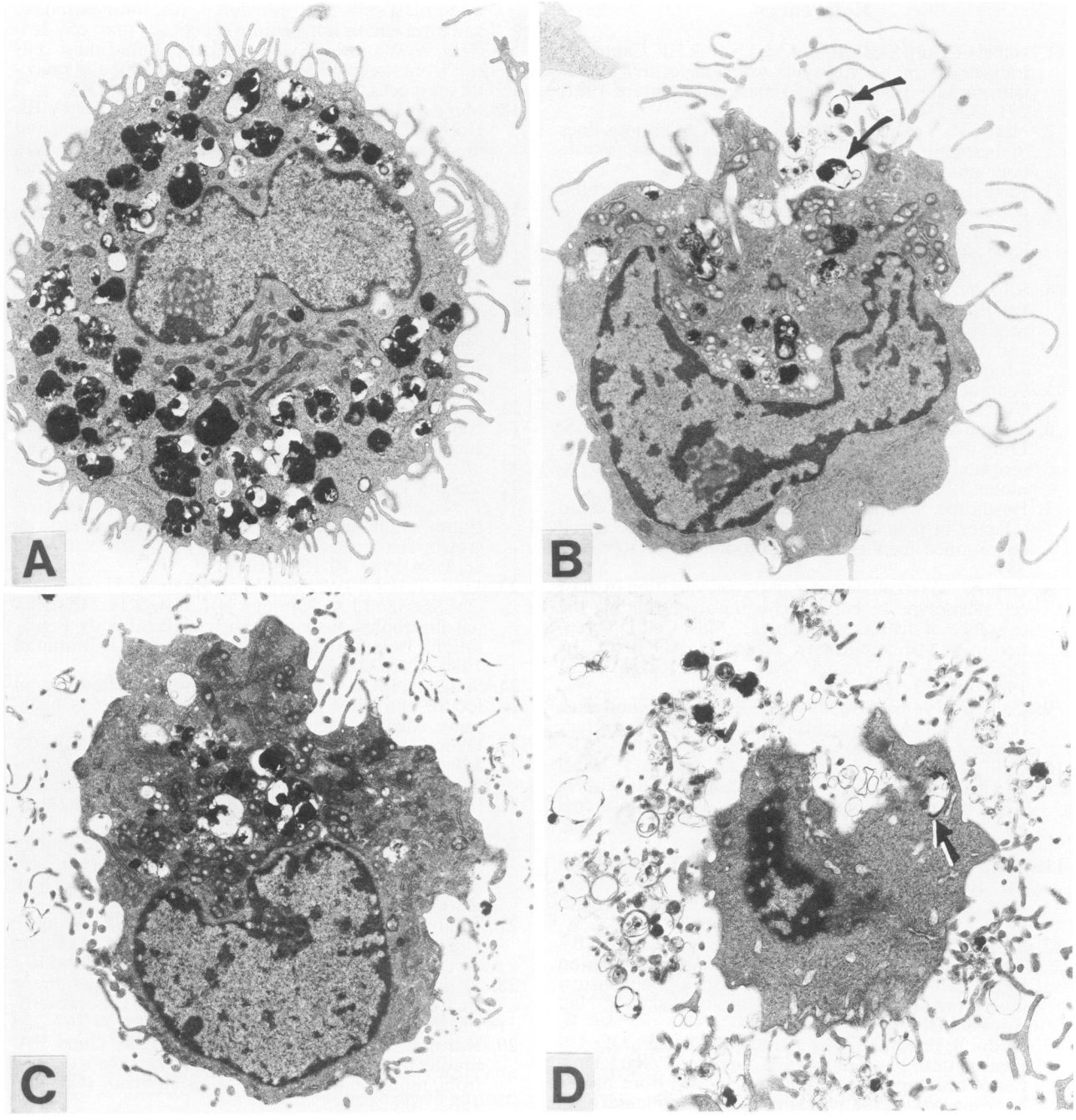

Figure 7—A panel of immature mast cells, all from the same experiment, illustrating the kinetics of IgE-dependent, antigen-induced degranulation. **A**—Control mast cell exhibiting numerous cytoplasmic granules and many short surface processes. **B**—One minute after exposure to antigen, dense progranular structures and vesicles (*arrows*) are present within multiple deep clefts open to the exterior. There is also marked elongation and irregularity of surface processes. **C**—By 10 minutes after stimulation, the surface processes frequently appear discontinuous with the cell surface. **D**—By 30 minutes after stimulation, many degranulated mast cells appear small (~ 7 μ) and contain no or few residual granules (*arrows*). The cells often are surrounded by dense progranular structures, vesicles, membranes, and the cross-sections of narrow surface processes. (**A**, ×7000; **B**, ×9000; **C**, ×7000; **D**, ×9000)

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