Vesicular Uptake of Eosinophil Peroxidase by Guinea Pig Basophils and by Cloned Mouse Mast Cells and Granule-Containing Lymphoid Cells

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Guinea pig basophils, cloned mouse mast cells, and cloned mouse granule-containing lymphoid cells were found to utilize a vesicular transport system to internalize eosinophil peroxidase (EPO) added *in vitro*. Kinetic analysis indicated that EPO internalization involved the binding of EPO to the plasma membrane, the formation of complex surface invaginations, and the movement of EPO-laden vesicles, tubules, and vacuoles toward the center of the cells. EPO became associated with multivesicular bodies in granule-containing lymphoid cells and mast cells, with immature granules in mast cells, and with mature granules in basophils. In other cells, the endogenous production of granule peroxidases (neutrophils and eosinophils) or the prior uptake of exogenous peroxidatic substances (some basophils) precluded cytochemical analysis of granules for EPO. Vesicular transport of EPO provides a possible explanation for the variable detection of peroxidase activity in mast cells or basophils. It also provides a mechanism for sequestration of this potentially toxic material or for its storage for possible future use. (Am J Pathol 1985, 118:425–438)

IMMATURE EOSINOPHILS and neutrophils synthesize endogenous peroxidases, which can be visualized by ultrastructural cytochemistry in the rough endoplasmic reticulum and Golgi apparatus and in certain granule populations.[1-3] The neutrophil peroxidase (myeloperoxidase, MPO) is packaged in azurophil granules, which develop in the promyelocyte stage, but not in specific granules, which are produced later in the myelocyte stage of development. The eosinophil peroxidase (EPO) is present in immature granules and in the matrix of mature specific granules; EPO is absent from the central major basic protein-containing crystalloid core of these granules. MPO and EPO differ in primary structure, in the nature of their heme prosthetic group, and in the reactions which they catalyze, although they have in common the ability to increase the rate of peroxidatic reactions many orders of magnitude. Both peroxidases, when combined with H_2O_2 and a halide, form powerful oxidants that are toxic to microorganisms and mammalian cells and can inactivate certain soluble mediators.[4] EPO, and to a lesser degree MPO, are strongly basic proteins and as a result bind avidly to negatively charged surfaces. Among these are the surfaces of microorganisms,[5-7] helminths,[8] mammalian cells,[9] and mast-cell granules.[10]

Despite our extensive knowledge of the peroxidase content of neutrophils and eosinophils, very little is known regarding the third blood granulocyte population, the basophil, or the histamine-containing tissue cell, the mast cell. Peroxidase activity has been described in the cytoplasmic granules of rat[11] and human[12] basophils and of hamster,[13] rat,[14] and mouse[15] mast cells. However, in unpublished data, one of us (A.M.D.) has noted marked variability in the peroxidase cytochemical reaction of human basophils and mast cells. Most cells were completely negative, whereas other, often adjacent, cells varied from those containing a sin-

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gle positive granule among many negative ones to cells in which all granules were peroxidase positive. Mast cells with peroxidase-positive granules were often associated in tissues with eosinophils, cells whose granules are characteristically rich in peroxidase activity. For example, in inflammatory bowel diseases, morphologic evidence of eosinophil and mast-cell degranulation[16,17] was associated with focal mast cell granule reactivity for peroxidase (A.M.D., unpublished data). However, peroxidase activity was never seen in the secretory apparatus (rough endoplasmic reticulum, Golgi complex) of these mast cells.

Previous studies[18,19] suggested a possible explanation for these findings. Horseradish peroxidase (HRP) injected into guinea pigs or added to guinea pig basophils *in vitro* bound to the basophil surface and was transported in pinocytotic vesicles to the granules, where membrane fusion resulted in discharge of the peroxidase into the intragranular space. The release of the accumulated HRP from the basophil granules by vesicular transport also was observed. In these studies, some basophils contained a variable number of peroxidase-positive granules even without exposure to HRP (background). This was thought possibly to reflect the uptake of hemoglobin by vesicular transport,[19] because the cells were exposed to hemoglobin *in vivo* during induction of basophilia by daily sheep red blood cell injections[19] and *in vitro* during lysis of erythrocytes in the purification procedure.[19,20] With regard to the latter, considerably greater background peroxidase activity was observed in basophils *after* red blood cell lysis, as compared with *before* lysis. In these studies, the rough endoplasmic reticulum and Golgi apparatus remained peroxidase-negative, both in control cells exhibiting "background" reactivity and in basophils exposed to HRP.

Because eosinophils are commonly seen in tissue reactions also containing basophils,[17,21,22] we initiated a study to determine whether eosinophil peroxidase, like HRP, could be internalized by basophils. The uptake of EPO by a number of other granule-containing cells of mouse or guinea pig origin was also investigated. We found that all of the cells tested internalized EPO by vesicular transport.

Materials and Methods

Guinea Pig Basophils, Neutrophils, and Eosinophils

Bone-marrow cells were obtained from Strain 2 guinea pigs (300–400 g) primed to develop a bone-marrow and peripheral-blood basophilia as previously described.[20] Briefly, the guinea pigs received 10 daily intraperitoneal injections of either sheep blood (SRBCs, 2 ml of a 1:1 (vol/vol) mixture of whole sheep blood and Alsever's solution) or ovalbumin (OA) (50 mg in 2 ml phosphate buffered saline [PBS], pH 7.3). The animals were killed by ether anesthesia followed by exsanguination, and bone marrow was removed from the femurs and tibias with a spatula and/or by flushing with Eagle's minimal essential medium (GIBCO, Grand Island, NY) containing 0.1% gelatin (DIFCO Laboratories, Detroit, Mich) at 20 C. The bone-marrow cells were gently resuspended, filtered through gauze, washed two times in PBS at 20 C, and resuspended in PBS at $\sim$1.2 $\times$ 10^7 cells/ml; and the number of basophils and eosinophils was counted.[20] For the experiments reported here, we used bone-marrow cells from 1 animal primed with SRBCs and from 1 primed with ovalbumin. While most of the granulocytes were neutrophils, the preparation from the SRBC-primed guinea pig also contained 5.2% basophils and 0.6% eosinophils. The preparation from the animal primed with ovalbumin contained 4.0% basophils and 2.1% eosinophils.

Cloned Mouse Mast Cells

We have previously described[23,24] the isolation, cloning, and long-term *in vitro* growth of mast cells derived from normal mouse hematopoietic tissue. Similar cells have been isolated and characterized in several other laboratories.[22] Although the precise relationship of these cells to mast-cell populations *in vivo* remains to be fully defined, under the usual conditions of growth *in vitro* the properties of these cells most closely resemble those thought to characterize immature mast cells and/or T-cell–dependent mast-cell populations ("mucosal mast cells"). Our cells resemble immature mast cells by ultrastructure,[23–25] not basophils.[26] They also express plasma membrane receptors ($Fc_\epsilon Rs$) that bind IgE antibody with an affinity similar to that of the $Fc_\epsilon Rs$ of normal mouse peritoneal mast cells,[24] synthesize and store both histamine[23,24] and serotonin (S. J. Galli, G. Weitzman, and I. Hammel, unpublished data), and degranulate and release histamine upon passive sensitization with IgE and challenge with specific antigen.[25] Cloned mast cells also degranulate and cause marked augmentation of local vascular permeability after passive sensitization *in vitro*, intracutaneous injection into W/W^v mast-cell–deficient mice,[27] and intravenous challenge with specific antigen (S. J. Galli and L. Wiberg, unpublished data). However, their ultrastructure differs from that of mature peritoneal mast cells,[22,24] and they express fewer $Fc_\epsilon Rs$[24] and contain less histamine than mouse peritoneal mast cells.[22–24] In addition, the cells synthesize chondroitin-6-sulfate rather than heparin[24] (J. A. Marcum and S. J. Galli, unpublished data) and are dependent for their continued *in vitro* proliferation on a macromolecule that can be elaborated by inducer T-lymphocytes[23,24,28] or certain neoplastic cells of hematopoietic origin.[22,24,29]

For the studies reported here, cloned mast cells (Cl. MC/9)[23-25] were maintained as previously described in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with, from GIBCO, L-arginine (11.6 mg/dl), L-asparagine (3.6 mg/dl), folic acid (0.6 mg/dl); and, from M. A. Bioproducts (Walkersville, Md), sodium pyruvate (1 mM), nonessential amino acids (10 mM, of each, catalog number 13-114A), essential vitamins (10 mM of each, catalog number 13-607C), L-glutamine (2 mM); as well as with 2-mercaptoethanol (5×10^{-5} M) and 20% (vol/vol) supernatants from concanavalin A-activated BALB/c spleen cells (Con A-CM[23,24]). The MC/9 cells used for the experiments reported here were from a population that has expressed viral particles in the rough endoplasmic reticulum by electron microscopy since early 1983. However, these cells remain strictly growth-factor-dependent. For most experiments, cells were washed two times in PBS and resuspended in PBS for incubation with EPO. In some experiments, EPO was added to the cells maintained either in their usual conditioned medium (containing a final concentration of 10% fetal calf serum and 20% Con A-CM) or in DMEM containing 20% Con A-CM but only 0.8% fetal calf serum (final concentration).

Cloned Mouse Granulated Lymphoid Cells

The cloned murine cell line Cl.Ly1$^-$2$^-$NK-1$^+$/11 (J11) initially was reported to express a Thy1.2$^+$Lyt1$^-$2$^-$Ly5$^+$Qat4$^+$5$^+$NK-1$^+$ surface glycoprotein phenotype and "NK-like" cytolytic function.[30,31] Subsequently, this clone was discovered to express large numbers of $Fc_\epsilon Rs$ that bound monoclonal mouse IgE with an affinity similar to that of the $Fc_\epsilon Rs$ of mouse cloned or peritoneal mast cells.[32] The J11 cells also contained numerous prominent cytoplasmic granules that resembled those of basophils by ultrastructure.[32,33] However, unlike basophils, this clone (designated Cl.Ly1$^-$2$^-$/11 in Nabel et al[23]) neither synthesized nor contained histamine, nor did it degranulate upon passive sensitization with mouse IgE antibody and challenge with specific antigen *in vitro* (S. J. Galli, A. M. Dvorak, and T. Ishizaka, unpublished data). Recent examination of additional cloned murine cells with cytolytic activity indicated that the expression of large numbers of $Fc_\epsilon Rs$ probably represented an extremely uncommon characteristic of cultured cells with "NK-like" or cytolytic T-cell activity (S. J. Galli, C. G. Brooks, A. M. Dvorak, and T. Ishizaka, submitted for publication). But prominent cytoplasmic granules appear to represent a common feature of murine lymphoid cells maintained in long-term culture in media rich in growth factors, in that granules similar to those of J11 have been reported in other cloned murine cells with NK-like function, as well as in clones with antigen-specific suppressor or cytolytic T-cell function (reviewed in Galli et al[22]).

J11 cells were maintained in continuous culture in modified DMEM supplemented with 20% (vol/vol) Con A-CM as described above and were harvested from their tissue cultures flasks by gentle agitation for 5–10 minutes in a solution of 0.01 M ethylenediaminetetraacetic acid (EDTA), 0.025M tris(hydroxymethyl)aminomethane, 0.05% human serum albumin (Miles Laboratories, Inc.), 0.7% NaCl, 0.037% KCl, pH 7.6, at 20 C. Cells were taken up from the flasks in an excess volume of DMEM with 10% fetal calf serum and then were washed two times in PBS and resuspended in PBS for incubation with EPO.

Eosinophil Peroxidase

The horse eosinophil peroxidase used in these studies was prepared as previously described and had a 415/280-nm ratio of >0.9 and a specific activity of ~700 guaicol units/mg protein.[34] The stock preparation was in 0.05 M sodium acetate buffer, pH 4.7–1.0 M sodium chloride at a concentration of 893 guaicol units/ml. Either 20 or 40 μl (18 or 36 units, respectively) were added to the reaction mixture in the uptake experiments described below.

EPO Uptake and Release

$Six \times 10^6$ guinea pig bone-marrow cells suspended in 0.5 ml of PBS, pH 7.3 prewarmed at 37 C were incubated with 18 units of EPO for varying intervals (1, 5, 15, 30, and 60 minutes at 37 C. Duplicate aliquots of reaction mixtures with and without EPO were taken at 30 minutes for fluorometric determination of cell-associated and supernatant histamine as previously described.[24] Experiments with cloned cells employed $\sim 5.0 \times 10^5$ cells in 0.5 ml PBS and 18 or 36 units of EPO. Controls without added EPO were included in each experiment. In release experiments, cells that had been incubated with EPO were washed twice and suspended in EPO-free PBS at 37 C and incubated for varying intervals up to 1 hour. Reaction was stopped by addition of 3 ml of dilute Karnovsky's fixative consisting of 1% paraformaldehyde, 1.25% glutaraldehyde, and 0.025% $CaCl_2$ in 0.1 M sodium cacodylate buffer pH 7.4[35]; and fixation was continued for 10 minutes at room temperature.

Transmission Electron Microscopy

Following fixation, cells were washed twice in 0.5 M tris-HCl buffer, pH 7.6, and reacted cytochemically for peroxidase as previously described.[18,19] The cells were postfixed in dilute Karnovsky's fixative for 1 hour at

room temperature, washed in 0.1 M sodium cacodylate buffer, pH 7.4, spun through molten agar in a microfuge, and processed for electron microscopy as previously described.[19] Random thin sections were either stained lightly with lead citrate or left unstained, and examined in a Philips 400 electron microscope.

Results

Guinea Pig Basophils

Some basophils contained a small proportion of peroxidase-positive granules in the absence of exposure to EPO (background), whereas the majority of basophils not exposed to EPO exhibited no peroxidase activity (Figure 1A). As previously reported,[18,19] the background was higher with basophils from guinea pigs primed with SRBCs (whose hemoglobin has pseudoperoxidase activity) than with those from OA-primed animals. No peroxidase-positive vesicles, vacuoles, or tubules were seen in these cells, nor was peroxidase evident in the secretory apparatus, ie, rough endoplasmic reticulum or Golgi-related structures.

Basophils incubated with EPO appeared to transport plasma membrane-bound peroxidase into the cell interior predominantly via smooth membrane-bound structures, here referred to as vesicles (smaller round to ovoid structures), vacuoles (larger structures otherwise similar to vesicles), and tubules (elongate structures) (Figure 1B–D). Occasional coated vesicles were also seen, some of which contained EPO. With long periods of exposure to EPO, positive vesicular structures increased in number and appeared to move from the periphery to a more central location in the cytoplasm. Positive structures were routinely seen in the vicinity of the Golgi area after 5 minutes of EPO uptake. At 15 minutes, multivesicular bodies were EPO-positive, as were cytoplasmic granules (Figure 1B). Granules first developed positive EPO reactivity around their periphery and later throughout their matrix. As in the previous study with HRP,[19] some basophils (approximately 5%) had only negative granules, whereas in many cells (approximately 20%) all of the granules were peroxidase-positive. However, most basophils exhibited variable granule activity, with some granules uniformly positive and others positive only at their periphery. Although positive vesicles were often seen in the vicinity of negative granules (Figure 2) we only rarely observed peroxidase-positive vacuoles attached to negative granules. Some basophils exhibited membrane-free granules within cytoplasmic degranulation sacs, a finding also observed in basophils stimulated to degranulate in response to antigen or Con A.[36,37] Granules within degranulation sacs bound EPO avidly. Membrane-free extruded granules with abundant EPO activity were also seen in the extracellular space, either attached to basophil surfaces or undergoing phagocytosis by neutrophils. However, evidence of degranulation was observed only rarely in cells incubated with EPO, and the amount of histamine present in the supernatant of cells incubated with EPO for 30 minutes (1.9% of the total) was no greater than that in the supernatant of control cells incubated for 30 minutes without EPO (2.4%).

In washout experiments, basophils were incubated with EPO for 30 minutes, washed free of unbound enzyme, and then incubated in medium lacking EPO in order to follow the release of previously internalized peroxidase. In contrast to earlier studies with HRP, in which a substantial proportion of the peroxidase was lost from the cell during the second incubation,[19] granules containing EPO remained positive even at late washout times (1 hour). Numerous positive vesicles were seen, in the absence of residual cell-surface-bound EPO, at all time periods studied.

Guinea Pig Neutrophils and Eosinophils

Endogenous peroxidase activity was present in the rough endoplasmic reticulum and Golgi apparatus of immature cells in the neutrophil and eosinophil series, in the matrix of eosinophil specific granules, and in neutrophil azurophil granules. The smaller and more pleomorphic specific granules of neutrophils were negative. Vesicles, tubules, vacuoles, and multivesicular bodies were never positive in cells not exposed to exogenous EPO. By contrast, numerous cytoplasmic vesicles contained peroxidactic activity in neutrophils and eosinophils incubated with EPO.

Mouse Mast Cells

Cultured immature mouse mast cells were peroxidase-negative when tested for endogenous enzyme activity (Figure 3A). These mast cells avidly took up EPO, which first adhered to the plasma membrane and then was internalized within vesicles and tubules, often in focal aggregates (Figure 3B–D). Positive coated vesicles were also internalized, although this accounted for a minor fraction of EPO uptake. Figure 3 also shows extracellular aggregates of peroxidase positive material (Figure 3C and D). We reported previously that monomer EPO readily forms aggregates in solutions lacking added detergents,[34] and that aggregates of EPO may be visualized on the surface of cells incubated with this enzyme *in vitro*.

Uptake of EPO appeared to increase between 1 and 5 minutes (Figure 4) and then remain stable throughout

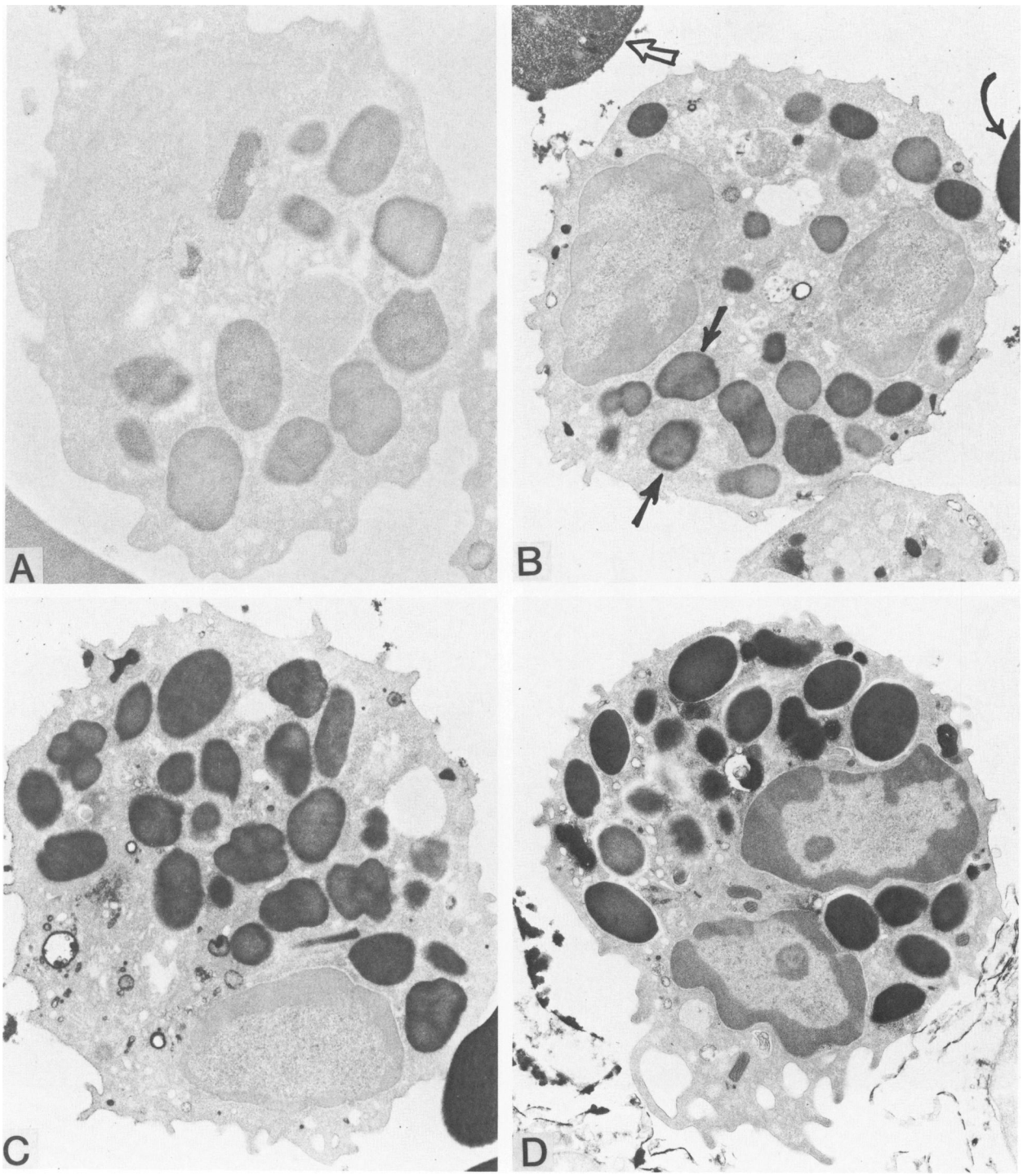
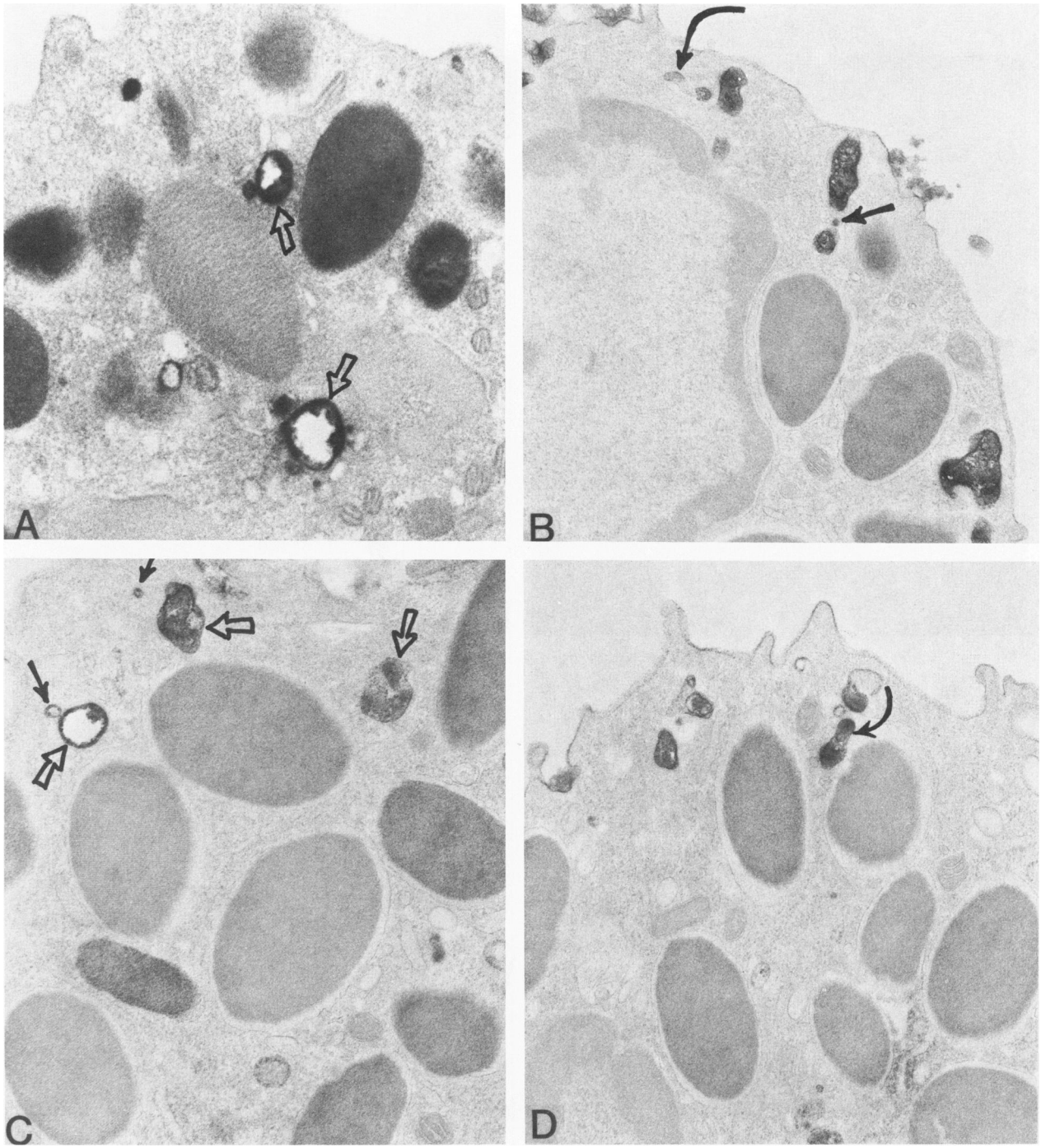


Figure 1—Guinea pig basophils stained for endogenous peroxidase activity (**A**) or reacted for peroxidase activity following exposure to EPO for 5 minutes (**B**), 15 minutes (**C**), and 60 minutes (**D**). In the absence of prior exposure to EPO (**A**), no peroxidase activity is detected in granules, vesicles, or any other cytoplasmic structures. By contrast, cells incubated with EPO exhibit peroxidase activity in vesicles and vacuoles (**B–D**) and in granules. Granule reactivity is seen first at the periphery of individual granules (*solid arrows* in **B**) but increases progressively with time of incubation until, in some cells, most granules appear uniformly and intensely positive. **B** also shows part of a peroxidase-positive reticulocyte (*open arrow*) and part of a peroxidase-positive erythrocyte (*curved arrow*). (**A**, ×14,500; **B**, ×9500; **C**, ×10,000; **D**, ×10,000)



the duration of the experiments (60 minutes). Many cells internalizing EPO appeared undamaged. However, other cells incubated with EPO in PBS for 5 minutes or longer were dead, as evidenced by ultrastructural criteria, including the diffuse labeling of the cytoplasm by EPO. When the experiment was performed in conditioned medium containing 10% fetal calf serum, uptake of EPO was decreased and fewer cells exposed to EPO appeared damaged. EPO uptake also appeared reduced, although to a lesser extent, when medium containing 0.8% fetal calf serum was used.

EPO-positive tubules and vesicles fused with nega-

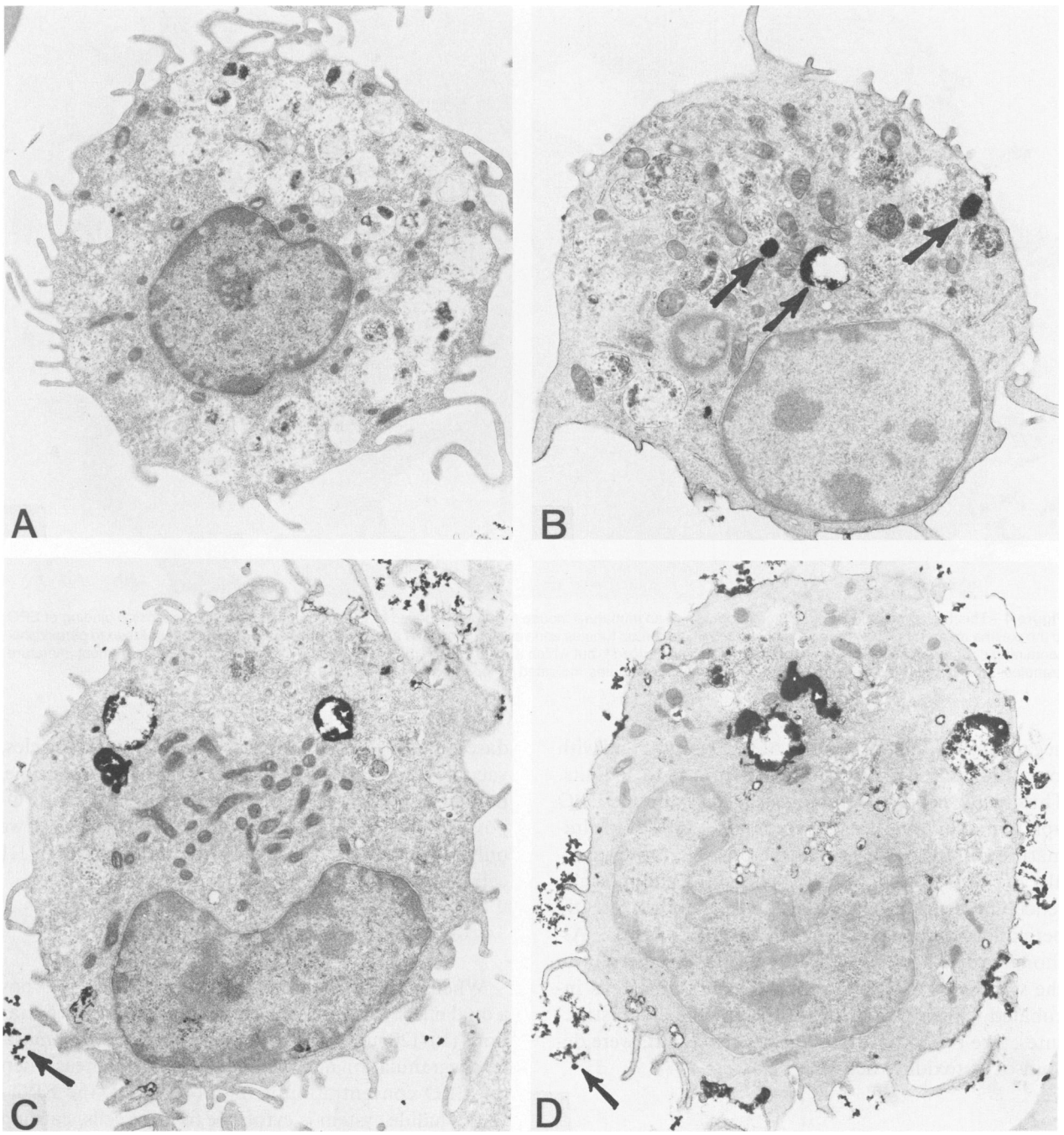


Figure 3—Immature mouse mast cells prepared to show endogenous peroxidase (**A**) or reacted for peroxidase activity following exposure to EPO for 5 minutes (**B**), 15 minutes (**C**), or 30 minutes (**D**). The cytoplasm of the cell in **A** contains numerous immature granules, all of which are peroxidase-negative. In **B**, three granules (*arrows*) contain EPO, which appears markedly dense. Cells studied after 15 (**C**) or 30 (**D**) minutes' exposure to EPO show several EPO-positive granules and vacuoles, large numbers of positive cytoplasmic vesicles, and (in **D**) large amounts of EPO bound to the cell surface. Extracellular aggregates of peroxidase positive material are also evident (*arrows* in **C** and **D**). (**A**, ×8500; **B**, ×8500; **C**, ×9000; **D**, ×9000)

tive immature granules and multivesicular bodies, and some of these structures became EPO-positive (Figures 4 and 5). The contents of immature granules are distinctive[23,24]; they include numerous vesicles and some granular material which does not display sufficient inherent density to be confused with EPO reaction product. An admixture of positive and negative vesicles was seen within multivesicular bodies and immature granules (Figure 6). Some immature granules contained EPO in their matrix, surrounded by peroxidase-negative vesicles. However, in contrast to guinea pig basophil granules, the majority of immature granules remained EPO-

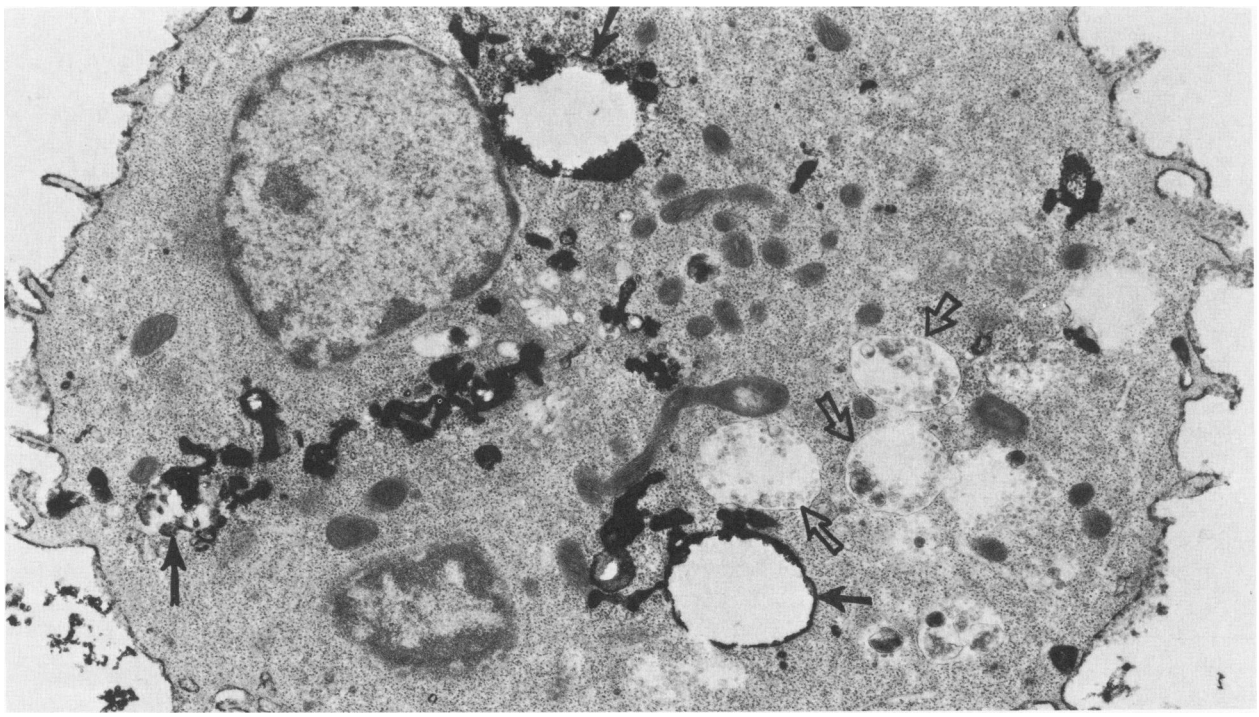


Figure 4—This higher magnification photomicrograph of an immature mouse mast cell exposed to EPO for 5 minutes shows extensive binding of EPO to the plasma membrane and internalization of EPO in numerous tubules and vesicles. Note that a number of positive vesicles appear fused to peroxidase-containing structures the size of immature granules (*solid arrows*), but which appear to lack the granular and vesicular material seen in most immature granules, for example, the peroxidase-negative immature granules indicated by *open arrows*. (×16,000)

negative, even after 60 minutes of EPO uptake and with exposure to doses of EPO ranging from 18 to 36 units. Thus, while nearly all viable mast cells exhibited EPO uptake and in some cells approximately 10% of the immature granules were peroxidase-positive, more generally only 2–3% were positive. The rough endoplasmic reticulum of cells exposed to EPO remained consistently peroxidase-negative during cellular EPO uptake. Although a few positive vesicles/tubules were present in the vicinity of the Golgi apparatus of some cells incubated with EPO, for the most part the Golgi structures, like the rough endoplasmic reticulum, were devoid of peroxidase activity.

Mouse Granulated Lymphoid Cells

When J11 cells were examined for endogenous peroxidase activity, we found occasional positive cytoplasmic lipid bodies; whereas the secretory apparatus and the mature granules were all peroxidase-negative. The cells contained inherently dense virus particles, but these were present only in the cisternae of rough endoplasmic reticulum. EPO uptake experiments done for 60 minutes and with doses of EPO up to 36 units revealed vesicular transport of EPO in cells which did not appear to be damaged (Figure 7). As in mast cells, peroxidase-positive coated vesicles, larger smooth vesicles, vacuoles, and elongated tubules were seen. With time, numerous multivesicular bodies also became EPO-positive (Figures 7C and 8). In contrast to mast cells, we only saw a few EPO-positive immature granules in J11 cells.

Discussion

When combined with H_2O_2 and a halide, EPO has a dual effect on mast cells; at relatively low concentrations (16–120 mU*), EPO provokes noncytotoxic mast-cell degranulation and mediator release; whereas when the EPO concentration is high (400 mU), the EPO–H_2O_2–halide system is cytotoxic to mast cells, causing cell lysis and release of all organelles.[39] A third effect of EPO on mast cells and basophils is shown here: its uptake and incorporation into cytoplasmic granules by vesicular transport. All granule-containing leukocytes tested, including freshly isolated guinea pig basophils, neutrophils, and eosinophils, cloned mouse mast cells, and cloned mouse granulated lymphoid cells, internal-

* The unit values given in Henderson et al,[39] in error, were one-fourth the actual values shown here.

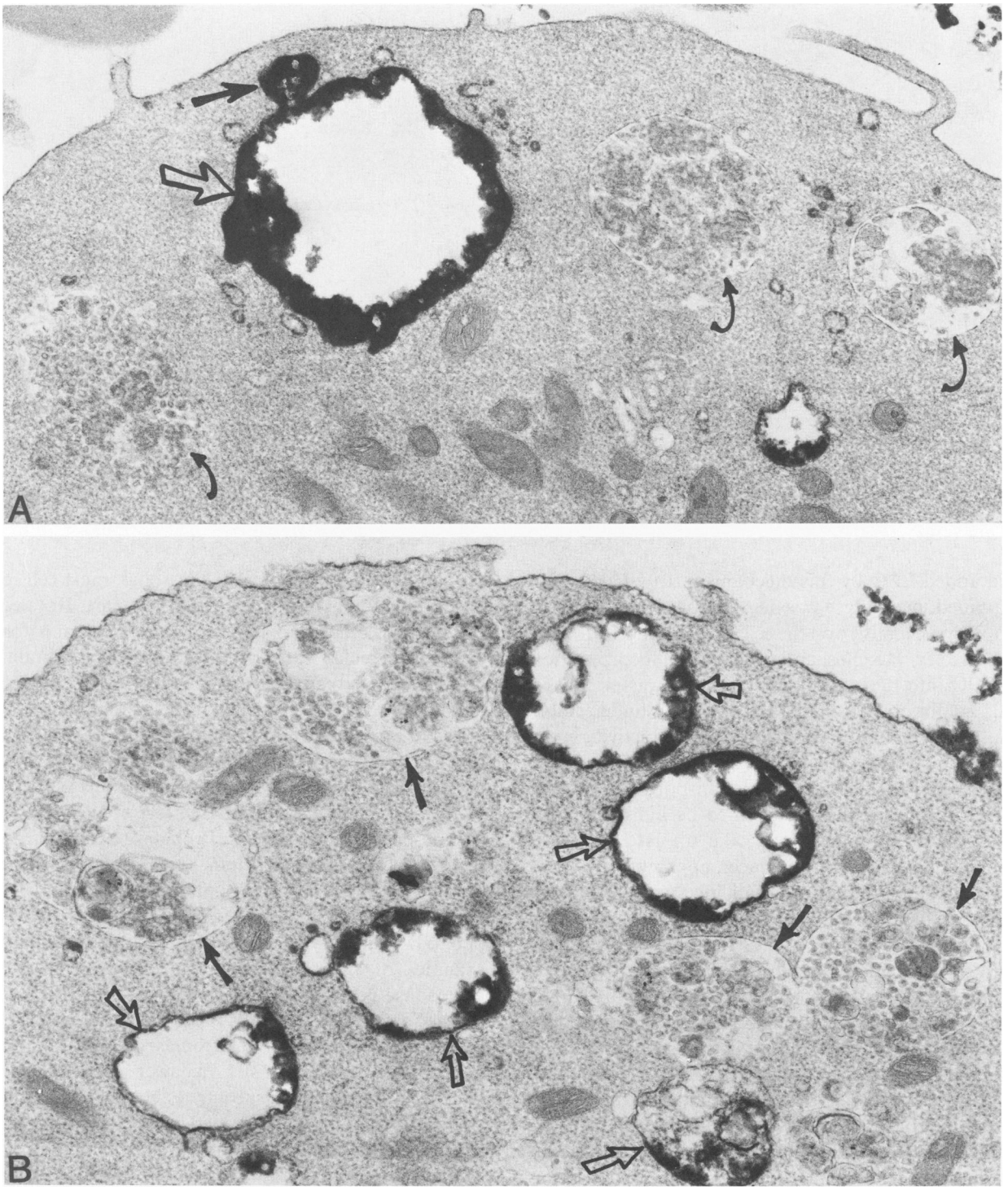


Figure 5—Mouse mast cells after 30-minute uptake of EPO. **A** shows one large peroxidase-positive structure (*open arrow*). While this probably represents an immature granule, native particulate and vesicular granule components like those present in EPO-negative granules (*curved arrows*) are not apparent. There are several EPO-positive vesicles in the immediate vicinity of the EPO-positive structure, which exhibits one appendage (*solid arrow*) that probably represents the result of fusion with an EPO-positive vesicle/vacuole. **B** shows several EPO positive (*open arrows*) and several EPO-negative (*solid arrows*) immature granules. In this cell, EPO surrounds but does not enter some vesicular structures within the granules. (**A**, ×30,500; **B**, ×33,500)

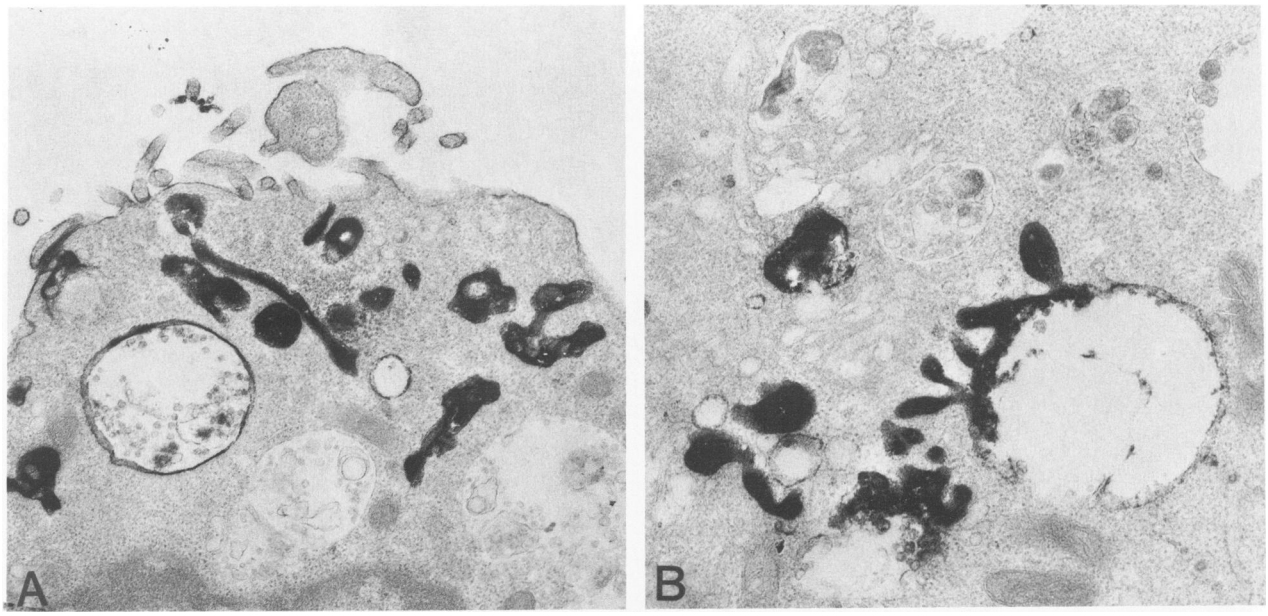


Figure 6—Mouse mast cells after 30-minute exposure to EPO. The cell in **A** has extensively labeled tubules and vesicles in the peripheral cytoplasm. One of the three immature granules has EPO bound to its inner membrane. **B** shows EPO-positive tubular structures in continuity with a granule. This granule also contains EPO, particularly along its inner membrane in the region of continuity with EPO-positive tubules. (**A**, ×17,500; **B**, ×23,500)

ized added EPO by this mechanism. In all cell types examined, peroxidase was detected by electron-microscopic cytochemistry on the cell surface and in cytoplasmic vesicles, vacuoles, and tubules; and incorporation of EPO into the cytoplasmic granules was evident in cells exhibiting minimal (basophils) or undetectable (mast cells) granule-associated peroxidase activity in the absence of EPO.

In earlier studies, vesicular transport of HRP by guinea pig basophils appeared to be bidirectional.[19] Thus, basophils allowed to take up HRP released significant amounts of this tracer, apparently by vesicular transport, during a 1-hour incubation period in HRP-free medium. By contrast, basophils that had internalized EPO did not appear to discharge the incorporated peroxidase under the same conditions of washout. EPO is a strongly basic protein that binds firmly to the negatively charged mast-cell granule matrix,[10] and this may account for its retention by basophil granules. The vesicular transport of EPO occurred with no morphologic evidence of cell damage; however, rare guinea pig basophils that had incorporated EPO exhibited fusion of granule membranes identical to that accompanying degranulation provoked by specific antigen or Con A.[36,37]

The ability of basophils and mast cells to take up EPO *in vitro*, together with the variable peroxidase positivity of mast cell or basophil granules reported in other studies[11-15] and the frequent association of mast cells or basophils with eosinophils in tissues,[17,21,22] suggests that the peroxidase present in some mast-cell or basophil granules is derived, at least in part, from eosinophils (or other peroxidase-containing inflammatory cells) by vesicular transport. Neither we nor Murata[40] detected any peroxidase activity in the secretory apparatus of guinea pig basophils, which suggests that these cells make no endogenous peroxidase. However, in other species, basophil granule-associated peroxidase activity may be derived in part from endogenous synthesis of the enzyme. For example, Bentfeld et al[11] reported the presence of endogenous peroxidase activity in the rough endoplasmic reticulum, Golgi apparatus, and granules of immature rat basophils. Interestingly, while all the granules of immature basophils exhibited definite peroxidase activity of variable intensity, some of the granules of mature rat basophils appeared to be peroxidase-negative.[11]

The basophil or mast-cell granule acquisition of EPO by vesicular transport may be representative of a more general phenomenon. Thus, Padawer reported that a variety of extracellular substances could be taken up by mast cells and incorporated into the cytoplasmic granules.[41] He suggested that the granule matrix acquired and stored these substances by acting as an ion exchange resin, and proposed that this may represent a mechanism for removing toxic substances from the interstitial fluid. In the case of EPO, the removal of extracellular peroxidase from tissues experiencing eosinophil degranulation may limit EPO toxicity or may modulate other effects of this enzyme on adjacent cells

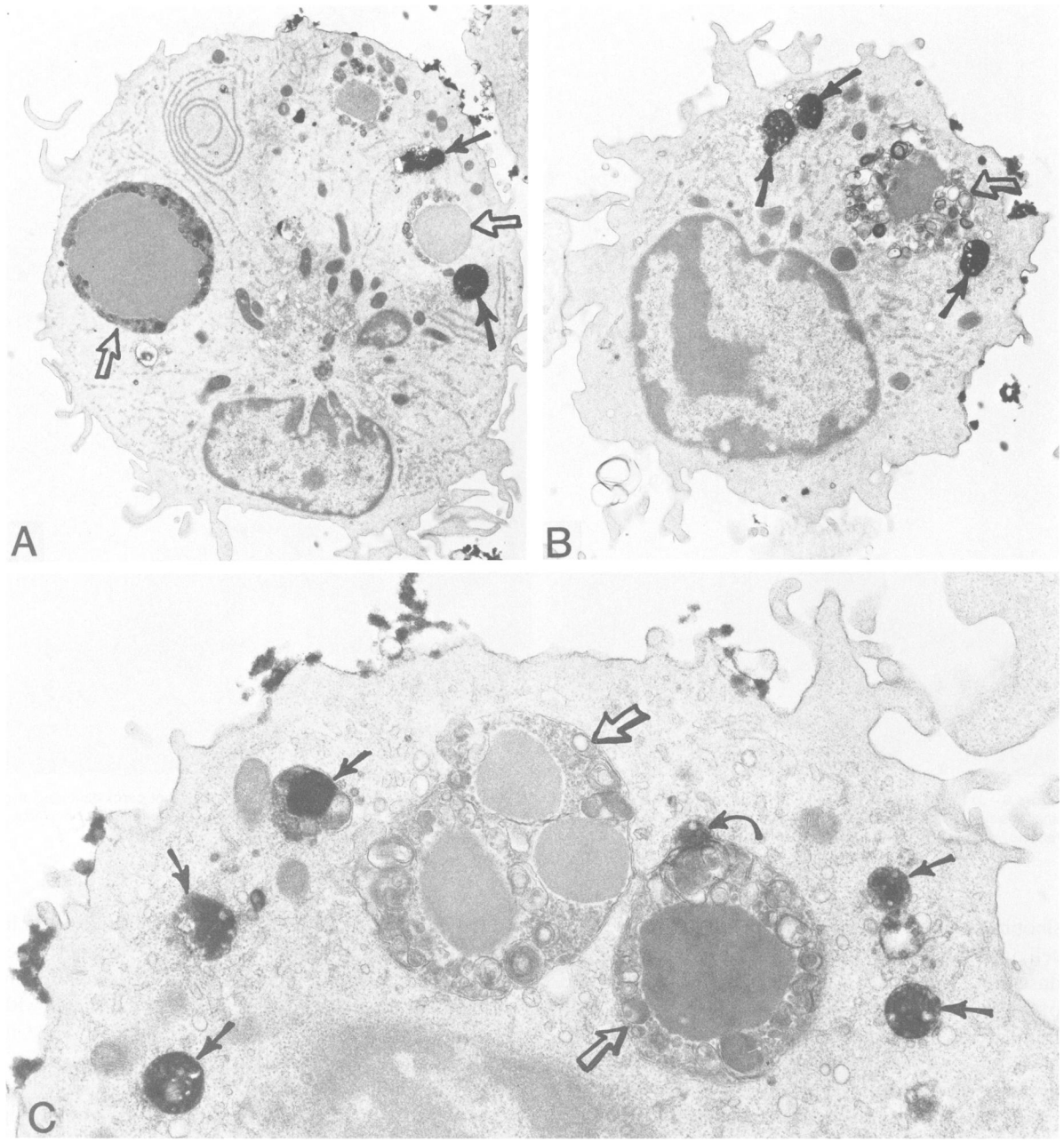


Figure 7—Cloned mouse J11 cells exposed to EPO for 60 minutes. EPO is focally bound to the cell membrane and is also present within the cell in membrane-bound structures (*solid arrows*). Note that the large granules (*open arrows*) generally do not contain peroxidase activity, although there is one apparent point of fusion between a peroxidase-positive structure and an otherwise peroxidase-negative granule (*curved arrow*). (**A**, × 6000; **B**, × 8000; **C**, × 18,500)

or soluble mediators.[42] Furthermore, eosinophil major basic protein has recently been detected within basophils,[43] and it is tempting to speculate that this finding also reflects basophil uptake and sequestration of potentially toxic basic proteins derived from eosinophils.

The uptake of EPO (or other basic proteins) and their incorporation into mast cell or basophil granules could theoretically have additional effects on the inflammatory reaction. Once EPO is stored within basophil or mast-cell granules, extracellular expression of the biochemical properties of this enzyme would be regulated by basophil or mast-cell secretagogues. Because signals controlling the degranulation of basophils and mast cells may differ significantly from those affecting eo-

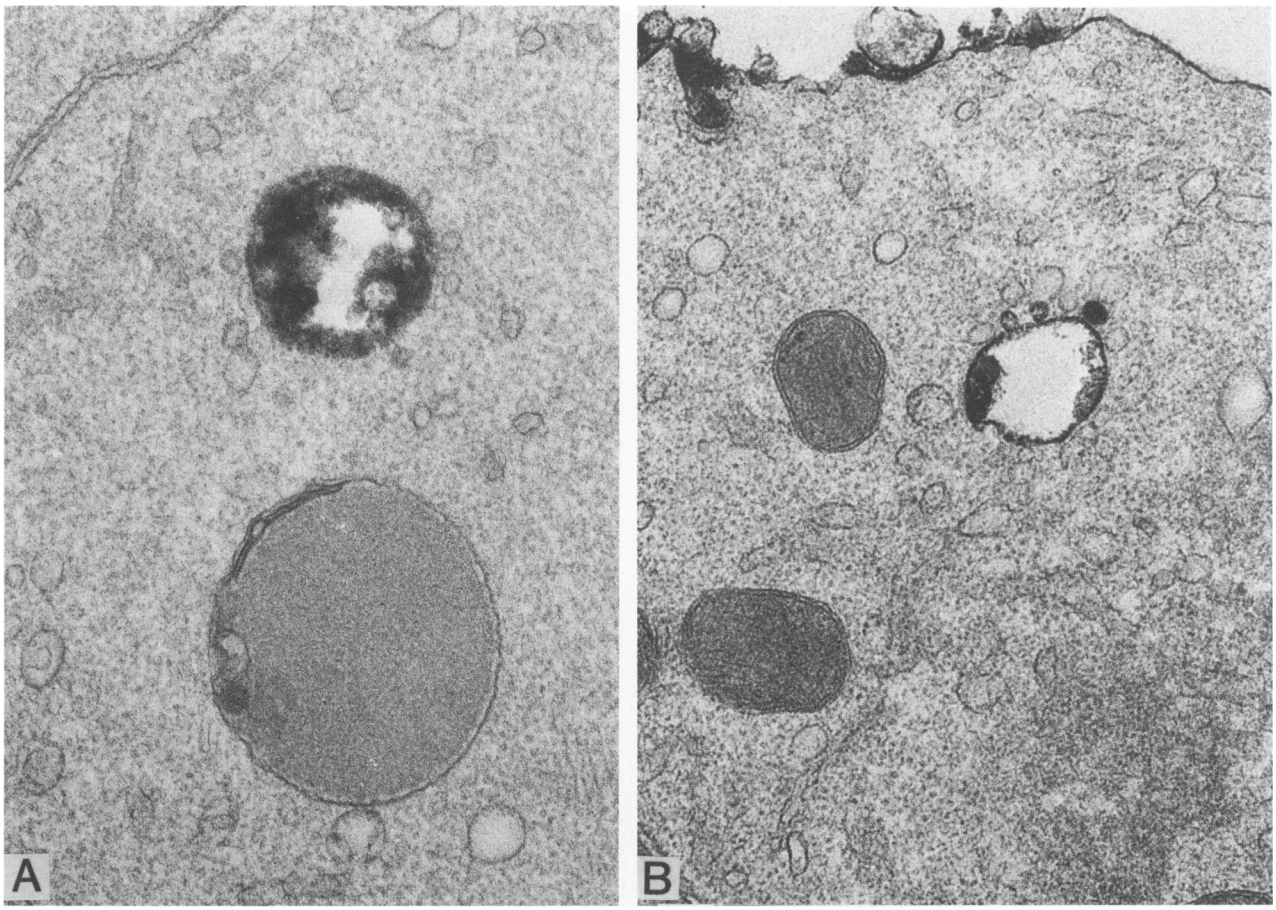


Figure 8—J11 cells exposed to EPO for 15 minutes. **A** shows an EPO-positive large vacuole with several immediately adjacent peroxidase-negative small vesicles. There is also a granule without peroxidase activity. In **B**, EPO is present in the plasma membrane and on the inner membrane of a cytoplasmic vacuole with several immediately adjacent EPO-positive vesicles and EPO-negative vesicles. (**A**, ×51,000; **B**, ×36,000)

sinophil activity, the release of peroxidase-positive mast-cell or basophil granules could reintroduce the peroxidase in a form and at a time more suited to the needs of the inflammatory response. Thus, Henderson et al[44] found that rat peritoneal mast cells combined with H_2O_2 and iodide were toxic to tumor cells *in vitro*, and degranulation of the mast cells by H_2O_2 followed by the interaction of the endogenous peroxidase of the released mast-cell granules with H_2O_2 and iodide to form a cytotoxic system was proposed as the mechanism.[44] The cytotoxicity of this system was enhanced significantly by the addition of EPO.[44] EPO bound to mast-cell granules retained its peroxidatic activity, and the mast-cell-granule–EPO complex was toxic to tumor cells[44] when combined with H_2O_2 and a halide. Such mast-cell-granule–EPO complexes were also toxic to microorganisms.[10] Indeed, when standardized to the same guaicol units of enzyme activity, EPO bound to mast-cell granules appeared to be more effectively cytotoxic than free EPO.[10,44]

Others have reported that mast cells have a role in the limitation of tumor growth *in vivo*[45] and in the destruction of certain tumor cells *in vitro*.[46] Basophils may also be present in leukocytic infiltrations associated with the immune destruction of certain tumors,[22] and in some of these reactions basophils can be seen in intimate anatomic association with dead or damaged tumor cells.[47] In addition, preparations of guinea pig peripheral-blood leukocytes highly enriched in basophils, when stimulated with specific antigen to induce basophil degranulation, appeared to damage these same tumor cells *in vitro*.[37] Although the extent to which peroxidase contributed to the cytotoxicity observed in these models was not investigated, the studies with guinea pig basophils employed leukocytes isolated by techniques known to increase the "background" peroxidase activity of basophils.[19,20] Mast cells and basophils have also been implicated in the expression of cytotoxicity to parasites. Their numbers in some lesions greatly exceed those found in the vicinity of

tumors[22,48]; furthermore, *in vivo* studies have provided strong evidence that mast cells[49] or basophils[50] are essential to the immune rejection of certain parasites, possibly working in conjunction with eosinophils. Several mechanisms by which mast-cell or basophil products can augment eosinophil-mediated cytotoxicity to parasites have been proposed.[51-54] The uptake of EPO by basophil or mast-cell granules and the release of the granule–EPO complexes provide yet another potential tactic of host defense, whereby eosinophil products can augment cytotoxicity of basophils and mast cells.

In conclusion, the experiments reported here indicate that basophils, mast cells, and perhaps other types of granulated leukocytes can take up EPO from the extracellular fluid, transport it in cytoplasmic vesicles, and concentrate it within cytoplasmic granules. This mechanism may have a role in the sequestration of potentially toxic EPO or in the storage of this material for future use. But it is important to emphasize that the latter ideas simply represent working hypotheses. First, all of our studies to date have employed relatively high concentrations of EPO (1800–3600 mU). Secondly, although electron-microscopic cytochemistry can identify the uptake of enzymatic activities such as EPO, it does not permit direct quantitation of the amount of enzyme incorporated. Finally, it may be difficult to estimate the concentration of EPO experienced by basophils and mast cells during inflammatory reactions *in vivo*. For these reasons, considerable additional work may be required before the biologic significance of the uptake of EPO by basophils and mast cells is understood.

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