Leukocytic Proteases and the Immunologic Release of Lysosomal Enzymes

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LYSOSOMES WERE INTRODUCED into the field of inflammation and connective tissue injury as a result of studies conducted at New York University and at the Strangeways Research Laboratory in Cambridge.[1-4] Shortly after it became clear that vitamin A acted to cause degradation of cartilage matrix both *in vitro* and *in vivo* by the release of proteases from cartilage lysosomes, it was hypothesized that lysosomal enzyme release in various forms of acute tissue injury could account for hydrolysis of connective tissue macromolecules.[5] Further studies in both laboratories have suggested the plausibility of this hypothesis. Thus, lysosomal proteases present in cartilage and in leukocytes have been shown capable of degrading cartilage matrix, isolated proteoglycans and simple chemical substrates.[6-8]

Leukocyte lysosomes of the rabbit contain at least two sorts of proteases capable of cleaving isolated cartilage proteoglycans: an enzyme or enzymes active at near neutral pH, and one enzyme active in the acid pH range.[7,8] When purified, high molecular-weight (10^6) proteoglycan of bovine nasal cartilage (PP-L) was exposed to extracts of leukocyte lysosomes (but not to other subcellular fractions), this substrate was readily cleaved, releasing polyanions of lower molecular weight which resembled those released by trypsin. This uronic acid-containing, readily diffusable material was non-dialyzable and precipitated readily with hexamine cobaltic chloride. Thus, *in vitro* as *in vivo,* when proteoglycans are exposed to excess vitamin A, they could be degraded by lysosomal hydrolases to release anionic polysaccharides; degradation proceeded as readily at neutral as at acidic pH. Since it became clear that proteolytic activity in the acid pH range could be attributed to the cathepsin D in rabbit leukocyte granules, the identification of a neutral protease capable of cleaving extracellular materials became of considerable interest.

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Neutral proteolytic activity in *human* leukocytes has been studied extensively by several investigators and, indeed, the major portion of this sort of activity is usually attributed to the elastin-like enzymes studied extensively by Janoff and his associates.[9] We have investigated the subcellular distribution of a number of enzymes in *rabbit* polymorphonuclear leukocytes, and general results of these data can be seen in Table 1. Rabbit polymorphonuclear leukocytes clearly contain a neutral protease acting on calf thymus histone, using liberation of acid-soluble arginine or tyrosine as assay.[8] The enzyme has relatively little action upon hemoglobin. Its subcellular distribution differs considerably from that of enzymes acting in the neutral pH range on such substrates as acetyl-DL-phenylalanine β-naphthyl esters, on L-leucine-2-naphthyl amide and on N-CBZ-L-alanine *p*-nitrophenol esters.[10] Nor could orcein elastase be demonstrated in rabbit polymorphonuclear leukocytes. These data differ considerably from findings in human granulocytes. The enzyme was clearly shown to be associated with azurophil granules and was readily inhibited by a material present in the cytoplasm of these cells. Moreover, it could be activated by increasing the ionic strength of the medium—*eg*, in 2.5 M KCl, and by polyanions such as heparin and dextran sulfate. The enzyme, conveniently referred to as a *histonase*, was partially separated by Sephadex gel filtration and by isoelectric focusing, until the specific activity of the activated enzyme showed a 300–fold increase over that determined in whole cells. This relatively purified enzyme of rabbit polymorphonuclear leukocytes could be shown preferentially to hydrolyze substrates such as histones or

Table 1—Percentage Distribution of Protein, Histonase, Acid Cathepsin and β-Glucuronidase, in Subcellular Fractions of Rabbit Polymorphonuclear Leukocytes*

	Nuclear debris	Granule	Post granule supernatant
Protein	4.4 ± 0.9 (5)	33.0 ± 5.6 (5)	63.8 ± 5.3 (5)
Histonase (pH 7.2)			
Tyrosine	6.6 ± 5.5 (3)	87.0 ± 14.1 (3)	6.3 ± 11.0 (3)
Arginine	1.8 ± 2.3 (5)	97.2 ± 3.1 (5)	0.8 ± 1.8 (5)
Cathepsin (Hemoglobin pH 3.0)	7.0 ± 2.6 (4)	44.0 ± 17.1 (4)	48.5 ± 18.9 (4)
β-Glucuronidase (pH 4.5)	4.6 ± 3.9 (5)	77.4 ± 9.8 (5)	18.0 ± 6.4 (5)

* Mean ± SE, standard deviation in parentheses. From Davies P, Krakauer K, Rita GA, Weissmann G,[8] in which experimental details may be found.

Table 2—Specific Activity of Acid Hydrolases in Human Leukocyte Subfractions*

	Homogenate	Nuclei and debris	Granules	Postgranule fraction
β-Glucuronidase†	100.5	90.5	1095	12.3
Acid Proteases (pH 3.0)				
Hemoglobin‡	18.0	<1	26.2	6.1
Histone§	8.6	6.6	10.4	8.3
Neutral Proteases (pH 7.4)				
Hemoglobin‡	33.1	53.7	84.4	8.2
Histone§	16.6	17.1	19.3	5.3

* Human leukocytes fractionated in sucrose/heparin and differential centrifugation yielded: nuclei and debris (800g, 5 minutes) granules (15,000g, 10 minutes) and postgranule fractions. Mean of three determinations.

† μg of phenolphthalein/mg protein/hr

‡ μg of tyrosine/mg protein/hr

§ μg of arginine/mg protein/hr, determined in the presence of 2.5 M KCl

proteoglycans, but it displayed only modest activity on hemoglobin and lysozyme.

In direct contrast to this substrate-specificity in *rabbit* polymorphonuclear leukocytes, neutral proteases of *human* leukocytes are much more active upon hemoglobin. From data listed in Table 2, the specific activity of enzymes capable of cleaving both hemoglobin and histone is clearly greater at neutral than in the acid pH range. Moreover, the subcellular distribution of these proteolytic activities differs considerably from the relatively homogeneous distribution of the neutral protease in rabbit polymorphonuclear leukocytes. Thus, considerable proteolytic activity upon both hemoglobin and histone, in the acid pH range, remains in postgranule fractions; significant neutral proteolytic activity sediments with the nuclei. That the subcellular distribution of these enzymes truly reflects a difference from the distribution of a lysosomal (azurophile) marker enzyme may be seen from the subcellular distribution of β-glucuronidase in the same homogenates. These observations are in keeping with the morphologic heterogeneity of leukocyte lysosomes in the human, compared to those of the rabbit. Moreover, protease activity in the neutral and acid range in human leukocytes does not appear to be entirely confined to granules rich in β-glucuronidase.

What is the biologic function of these neutral proteases? We have been intrigued by the possibility that enzymes released from lysosomes within the cell, or perhaps taken up by other cells during inflammation, may provide one stimulus to alterations in transcription or translation.[13,14] In order to test this hypothesis, therefore, we turned to

a system previously studied in relationship to mechanisms of lymphocyte transformation by phytohemagglutinin (PHA). The nucleus of the transformed lymphocyte may be characterized as containing excess of euchromatin over heterochromatin.[15] Moreover, the template activity of nuclei from stimulated lymphocytes for exogenous RNA polymerase exceeds that of unstimulated lymphocytes by severalfold. Indeed, the usual increments in template activity provoked by trypsin, an effect which has been attributed to hydrolysis of repressor materials and/or histone by the enzyme, are less easily demonstrated in nuclei from PHA-stimulated cells. These findings led us to suggest that nuclei of stimulated cells may have been exposed, preemptively, to an endogenous trypsin-like enzyme.[13] Subsequently it was found that, when isolated rabbit liver nuclei were incubated with increasing amounts of crystalline trypsin, their template activity for exogenous, bacterial, RNA polymerase was increasingly enhanced.[14] Were our hypothesis, that neutral proteases present in lysosomes could resemble trypsin in augmenting the template activity of isolated nuclei, correct, then it should be possible to incubate rabbit liver nuclei with fractions rich in neutral protease activity (rabbit leukocyte lysosomal lysates), in order to similarly increase their temperate activity for exogeneous RNA polymerase.

Data presented in Table 3 summarizes experiments in which nuclei from rabbit liver were incubated at 37 C, at neutral pH, with freeze-thawed lysates derived from rabbit polymorphonuclear leukocyte lysosomes, rich in neutral histonase. Template activity of nuclei exposed to histonase-rich fractions rose roughly fivefold when compared to nuclei incubated with buffer alone; exogenous RNA polymerase was added to both groups in order to test template activity. That these increments in RNA synthesis represented true

Table 3—Effect of Lysosomal Granules on Template Activity of Rabbit Liver Nuclei for Bacterial RNA Polymerase

Preparation	cpm*
RNA-Polymerase + nuclei + granules	2934
O + nuclei + granules	170
RNA-Polymerase + nuclei + O	634
O + nuclei + O	146
RNA-Polymerase + nuclei + granules + actinomycin	143
RNA-Polymerase + nuclei + O + actinomycin	154

* Assay: Results expressed as cpm of H_3GTP in RNA in 10 minutes. Assay with RNA-Polymerase = 50 units; Nuclei = 20 μg DNA; Granules = 7.1 μg protein; Actinomycin D = 25 μg; O = Glycerol solvent for RNA polymerase, 0.34 sucrose for granules.

DNA-dependent RNA synthesis was established by the finding that incorporation of H_3GTP into RNA was inhibited by appropriate levels of actinomycin-D. More exhaustive treatment of nuclei with granule preparations at neutral pH, or similar treatment at acid pH resulted in breakdown of DNA, RNA, and polymerase, effects which were attributable to lysosomal nucleases and cathepsins.

These data clearly suggest that neutral proteases derived from leukocyte lysosomes have the capacity to: a) degrade extracellular material such as the proteoglycans of cartilage matrix and b) expose sites in previously intact nuclei otherwise unavailable for transcription by RNA polymerase. Now it is clear that these experiments by no means indicate that such events take place in intact tissues or under pathologic circumstances. However, protease-sensitive steps in transcription have also been identified,[16] and it has recently become possible to suppress many of the inflammatory and proliferative events consequent to leukocyte accumulation in intact animals by administering appropriate protease inhibitors.[17] It will be important to determine whether, in pathologic states, materials extruded from leukocyte lysosomes can gain free access to the cytoplasm and/or the nuclei of, as yet, uninvolved cells.

Mechanisms of Enzyme Release from Phagocytic Cells

Since the description of *cytases* by Metchnikoff, which, he believed, escaped from phagocytic cells when these died, it was not appreciated that alternative means might exist whereby these enzymes might be released from phagocytes. Whereas a series of studies have clearly indicated that extracts of leukocyte lysosomes can provoke acute and chronic inflammation in experimental animals,[18] discrete mechanisms which account for the release of such materials from phagocytes were not clearly identified until recently. In Table 4 are listed four discrete situations under which lysosomal hydrolases and inflammatory substances, ordinarily sequestered in membrane-bounded granules, can gain access to the exterior of cells.

The *first* circumstance is indicated simply as *cell death* and is so readily appreciated that it may appear trivial. When a variety of toxins are added to phagocytic cells, injury to the cell membrane is an early consequence, and all intracellular materials are released *pari passu* from the injuried cell, including those ordinarily sequestered within lysosomes. In this regard we have studied the model amphipath melittin, which is a biologic *detergent* that causes primary lysis of the cell membrane and only subsequently disrupts

Table 4—Mechanisms of Lysosomal Enzyme Release in Tissue Injury

Mechanism	Model	Disease
Cell death	Melittin	Infection, toxins (animal, bacterial, chemical)
Perforation from within	MSU, silica	Gout, silicosis
Regurgitation after feeding	Bulk phase: AG/AB complexes, aggregated AB, zymosan, CPPD	Rheumatoid synovitis, pseudogout, immune complex nephritis
Reverse endocytosis	Surface: AG/AB complexes (? cytochalasin)	Vasculitis, SLE, nephrotoxic nephritis

intracellular lysosomes.[19,20] Similar rupture of the outer membranes of cells can be induced by a number of animal, bacterial and chemical toxins, as well as synthetic detergents such as Triton-X100, freezing and thawing, etc. Under these circumstances, cytoplasmic enzymes, potassium, and cellular constituents, in addition to lysosomal hydrolases, find their way into the surrounding tissues. The cell dies not because lysosomal hydrolyses are released into its cytoplasm but because injury to other parts of the cell, especially the cell membrane, cannot be reversed.

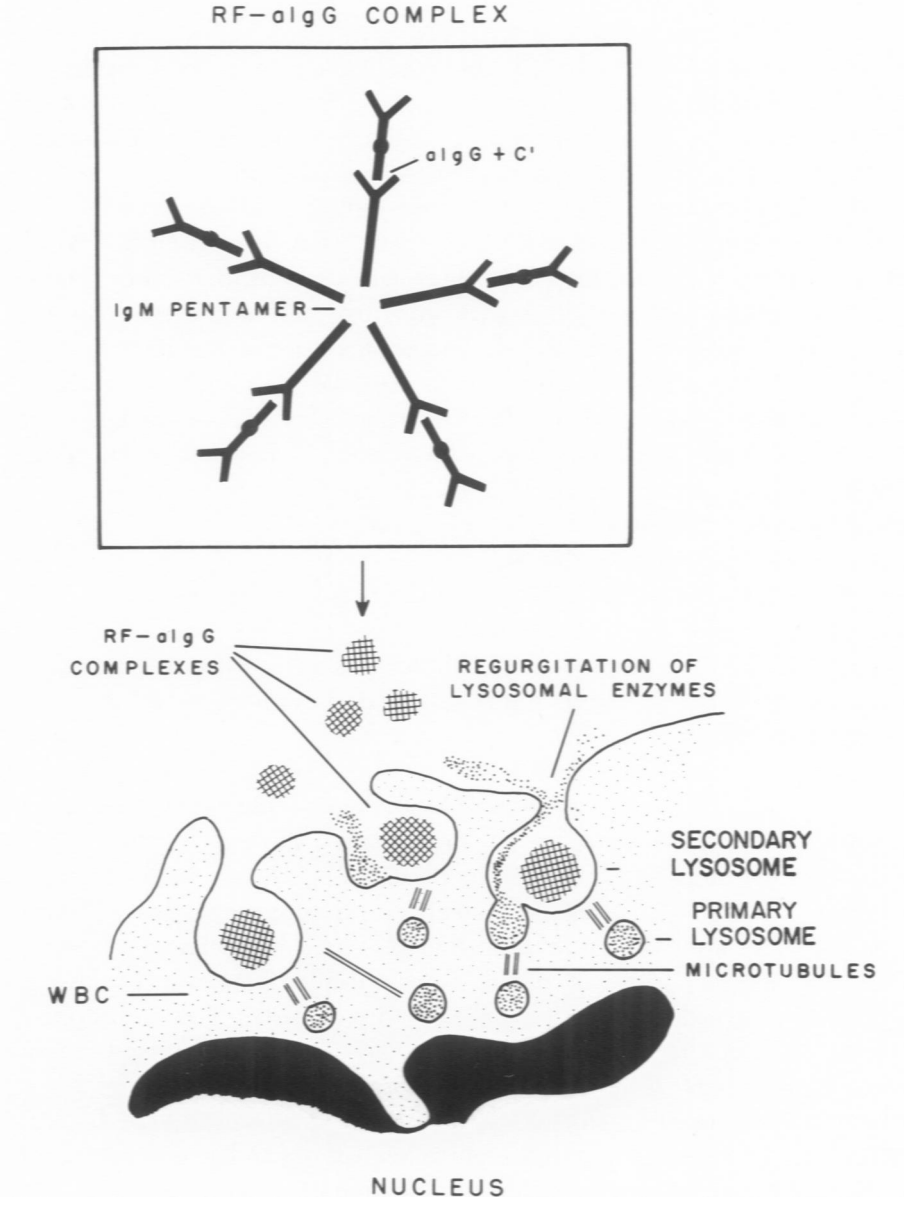
The *second*, albeit rare, circumstance more nearly conforms to the *suicide sac* hypothesis of DeDuve.[21] Under some circumstances, materials gain access to the inside of the lysosomal system wherein they cause membranes of the lysosomes to rupture from within. We have discussed elsewhere [22,23] reasons for believing that crystals of monosodium urate act in the same fashion as silica (elegantly demonstrated in the studies of Allison [24] and his coworkers). Both of these crystal types appear to be membrane-lytic, both for artificial and natural membrane systems, by virtue of their capacity to form hydrogen bonds with appropriately aligned phosphate ester groups, such as those of the internal lysosomal membrane. Damage to the organelle leads to the release of lysosomal enzymes concomitantly with the release of cytoplasmic enzymes and other intracellular contents as the cell dies by a kind of *perforation from within* of the cell's vacuolar system. This mechanism will not be discussed in detail.

The *third* mechanism is perhaps more common in pathology. Whenever cells engage in phagocytosis, they release a portion of their lysosomal hydrolases into the surrounding medium, unaccompanied by seepage of cytoplasmic marker enzymes.[22] This effect, now appreciated

by many authors,[25-30] appears to be due to the extrusion of lysosomal materials from, as yet, incompletely closed phagosomes, open at their external border to tissue spaces but already joined at their internal border by lysosomes actively discharging their acid hydrolases into the vacuole. Pictures consistent with this mechanism have been published by Zucker-Franklin and Hirsch,[31] by Henson [27,29,30] and by several others. This mechanism is diagrammed in Text-figure 1. Under these circumstances, we have demonstrated that lysosomal acid phosphatase and β-glucuronidase are extruded into the surrounding medium without concomitant extrusion of lactate dehydrogenase. Trivial explanations could not account for these findings—*eg*, there was no consumption or degration of any of the enzymes during phagocytosis, nor was there selective absorption of LDH by granules or leukocytes after regurgitation. This sequence of events follows not only the uptake of immune complexes but accompanies ingestion of other inert particles such as zymosan or crystals of calcium pyrophosphate dihydrate.[22] When cells regurgitate their hydrolases after uptake of immune precipitates, they form the sort of inclusions found in the *RA* cells obtained from synovial fluids of patients with rheumatoid arthritis.

Indeed, it is the uptake of antigen-antibody complexes from the bulk phase of the circulation, tissue fluids or joint fluid that may account for the liberation of inflammatory materials from leukocytes in the many forms of tissue injury associated with circulating, large molecular-weight, aggregates.

We have studied the morphologic correlates of hydrolase extrusion, after the ingestion, by polymorphonuclear leukocytes, of inert particles such as zymosan, or of precipitates formed by the interaction of heat-aggregated immunoglobulin G (aIgG) with rheumatoid factor (IgM). In scanning electron photomicrographs (Figure 1), we see the appearance of a normal polymorphonuclear leukocyte, the ruffled leading edge of which is essentially free of cytoplasmic organelles (toward the upper right of the figure). The multilobed nucleus accounts for the snail-like appearance of the raised edge. When such a cell is exposed to zymosan particles (Figure 2) a striking surface rearrangement of the phagocyte is observed. In cell suspensions exposed to zymosan particles, all particles not engulfed by the cells are separated from the phagocyte during fixation and dehydration. The cells themselves undergo a series of internal gymnastics that result in the appearance at their surface of a series of phagocytic *cups* or invaginations presumably forming the residue of appendages designed to engulf the zymosan particles. These re-



TEXT-FIG 1—Lysosomal enzyme release from polymorphs exposed to RF-aIgG complexes.

markable invaginations, or cups, can be seen in over 90% of polymorphonuclear leukocytes from human peripheral blood exposed to zymosan. In direct contrast, when cells are exposed to immune precipitates formed by aggregated IgG and IgM, a different series of morphologic sequelae is observed (Figure 3). The cells become oriented towards one or two poles; at these poles the cells appear to erect a series of phagocytic projections or *tentacles* apparently designed to engulf the masses of immune precipitates still somewhat adherent to the cell. (In contrast to the behavior of zymosan, the immune complexes appear to be far more resistant to dissociation from the phagocyte by the process of fixation and dehydration.) The phagocytic tentacles, which appear almost squid-like in their morphology, do not arise uniformly over the surface of the phagocytic cell, but seem to be concentrated at one or another pole. Previously published results of transmission electron microscopy indicate that, at the base of such phagocytic projections, phagosomes may be seen merging with lysosomes which extrude their electron-opaque material into the still-open phagosome. Indeed, there may be discerned, by transmission microscopy, a phagocytic vacuole joined at its internal border by a lysosome.[22] The open vacuole is ringed by discrete cross-sections of cytoplasmic projections which have the same dimensions as the projections seen clearly in scanning electron photomicrographs (Figure 3).

In most cells, the kinds of intracellular rearrangements described above are accomplished by means of two systems of filamentous proteins: microtubules[32] and microfilaments.[33] The microtubule system, which is sensitive to colchicine,[32,34] has been shown to be responsible for granule movement in many cell types. These include the melanocyte,[35] the pancreatic islet cell,[36] the platelet,[37] the neuron,[38] the salivary gland cell and the basophil.[39] Therefore, it was reasonable to suppose that agents which influenced the state of aggregation of microtubules in such cell types might be used to influence the response of phagocytic cells to the ingestion of inert particles. Moreover, recent studies have suggested that the functional state of microtubules might be regulated by the intracellular level of cyclic AMP.[22,40,41] Consequently, we have exposed cells to a series of agents designed to raise the level of cyclic AMP within the leukocyte. Cells were treated with appropriate concentrations of cyclic AMP, with dibutyryl cyclic AMP and with these nucleotides, to which had been added agents capable of inhibiting the phosphodiesterase which degrades cyclic nucleotides (theophylline, 2-chloroadenosine). Furthermore, adenyl cyclase was stimulated in

leukocytes by prostaglandins of E_1 and F, especially when added with theophylline. In studies described in detail elsewhere,[22] we have demonstrated that these agents have the capacity to retard the release of hydrolase from human peripheral blood polymorphonuclear leukocytes exposed either to particles of zymosan or to immune precipitates. Moreover, these effects could be mimicked by high concentrations of colchicine, a drug known to bind selectively to the tubulin subunits of the final microtubules.

It was therefore of considerable interest to determine the morphologic consequences of the action of these agents upon human white cells exposed to zymosan particles. Figure 4 is a scanning electron photomicrograph of a leukocyte, treated with prostaglandin E_1 and theophylline before challenge by zymosan particles. It may be seen that the phagocytic *cups* are still prominent—cup *formation* has not been inhibited—but within these cups (which indeed have now turned to *saucers*) the outlines of barely ingested zymosan particles may be identified still subjacent to the cell surface. These particles, which by virtue of their intercellular location, have not been washed from the cell during fixation and dehydration, can be seen only in outline. These data are compatible with the hypothesis that prostaglandin E_1 and theophylline inhibited the translocation of zymosan particles from loci near the periphery of the cell (the granule-poor, ruffled edge) to more central locations. It may, therefore, be inferred that lysosomal hydrolases still packaged within lysosomes concentrated at the granule-rich pole of the cell might have trouble gaining access to the peripheral phagocytic vacuoles. Indeed, recent studies in our laboratory have quantitatively documented the failure of leukocyte lysosomes to degranulate into phagocytic vacuoles of cells treated with prostaglandin E_1, mimicking, in this regard, the action of colchicine. These data would clearly suggest that at least one consequence of raising the level within cells of cyclic AMP is to impede the traffic of lysosomes to the phagocytic vacuoles. Consequently, extrusion of acid hydrolases into the surrounding medium by the *regurgitative* pathway would be significantly retarded.

Unfortunately for this simple scheme, detailed studies of the action of cyclic nucleotides (or agents which raise the levels within cells of cyclic AMP) have indicated that, at least for some particles, there is a significant retardation of uptake by phagocytic cells.[42] Thus it was found that concentrations of cyclic AMP above 10^{-4} M with theophylline, or prostaglandin and theophylline, have the ca-

pacity to retard phagocytosis of ^{125}I-labeled, heat-aggregated BSA by mouse peritoneal macrophages. Contrarywise, and perhaps more interestingly, very low concentrations of cyclic AMP itself (10^{-10} M) have the capacity to stimulate uptake of these particles. Therefore, it was difficult to decide whether inhibition of hydrolase extrusion in phagocytic cells was due to an intracellular event (interference with microtubule function) or due to inhibition of phagocytosis *per se*. Consequently, together with Dr. Peter Dukor, now of Basle, we studied the degradation of previously ingested ^{125}I-labeled BSA by mouse peritoneal macrophages.[42] In keeping with our previous results, we observed both effects. At low concentrations of cyclic AMP—$<10^{-8}$ M—translocation of previously ingested BSA to lysosomes was enhanced (judged by its subsequent degradation into TCA-soluble fragments). Contrarywise, at high concentrations of cyclic AMP—above 10^{-4} M, especially with theophylline—a colchicine-like effect was noted. Retardation of the breakdown of previously ingested aggregated ^{125}I-labeled BSA by the macrophage was observed. These data were entirely compatible with the hypothesis that cyclic nucleotides exert a biphasic effect intracellularly: acceleration of granule translocation by low concentrations and, retardation at high concentrations, the latter effect mimicking the effect of colchicine or incubation at 4 C. But data shown in Table 5 clearly indicate that when the metabolic response of human peripheral blood polymorphonuclear leukocytes to challenge by immune precipitates was measured, levels of prostaglandin and theophylline sufficient to inhibit hydrolase extrusion were also sufficient to inhibit the normal

Table 5—Glucose Oxidation in Human Polymorphs During Particle Uptake*

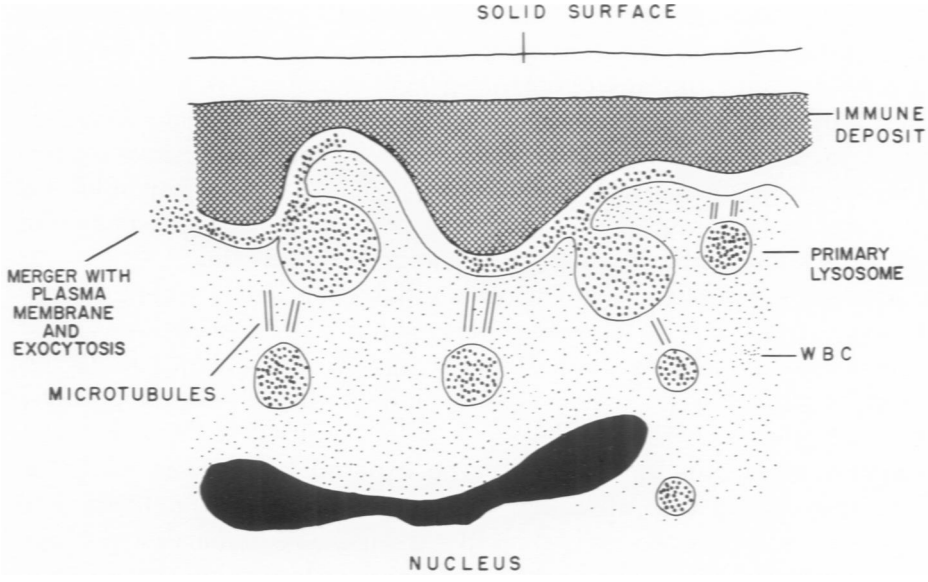
	Exposure of cells to RF-aIgG complex		
Compound added	Concentration (mM)	Difference resting vs phagocytic (cpm × 10^{-3})	% Inhibition
None	(Control)	96	00.0
PGE_1	0.28	48	50.0
Theophylline	1.0	61	36.5
PGE_1 + theophylline	0.28	24	75.0
	1.0		
Colchicine	1.0	28	70.6
	0.001	100	00.0

* C^1 oxidation of glucose, expressed in differences between resting and phagocytic cells.

metabolic accompaniment of phagocytosis, namely C-1 oxidation. Similarly, concentrations of colchicine sufficient to retard hydrolase release (1 mM) also inhibited C-1 oxidation of glucose. Consequently, in the human leukocyte system, it remained entirely possible that instead of interfering with the intracellular event we wished to study, we were interfering with the first part of the sequence of hydrolase extrusion, namely phagocytosis *per se*. Since in human leukocytes it was difficult to dissociate effects of phagocytosis *per se* from intracellular effects, another system was sought for.

Thus, we encountered the *fourth* mechanism of hydrolase release. Peter Henson [27,29,30] has been studying the effect of coating various solid surfaces with immune complexes and exposing these to polymorphonuclear leukocytes. When polymorphonuclear leukocytes make contact with such surfaces they discharge their hydrolases onto the surface by a mechanism that we have termed *reversed endocytosis* and Henson has called *frustrated phagocytosis*.[27] The former term may be preferable because superficially the process resembles protein extrusion from the salivary gland or from the pancreas and indeed, this process is basically one in which material previously stored within organelles is exported to the external milieu. Although in the leukocyte, this milieu is usually the phagocytic vacuole, under the special circumstances described above, stimulated by IgG in its antigen-reactive form, the granules appear to flow to the surface where their contents are directly discharged onto the filter.

In reverse endocytosis, phagocytosis *per se* does not take place. Therefore, it was of considerable interest to see whether agents which inhibited the export of acid hydrolases during uptake of immune precipitates from the bulk phase would have an effect upon extrusion of acid hydrolases onto the filters coated with immune complex. The scheme for such experiments is shown in Text-figure 2; from this diagram it will be clear that we postulate normal functioning of microtubules in order for granule extrusion to proceed. Data shown in Table 6 indicate that in this system, as well as in the previous system (regurgitation during feeding), release of acid hydrolases is significantly retarded by agents which elevate the level within the cells of cyclic AMP, as well as by colchicine. These data would indicate that the effect we have studied truly represents an *intracellular* effect of high levels of cyclic AMP rather than an indirect effect upon phagocytosis *per se*, since, in reverse endocytosis phagocytosis cannot proceed. Indeed, control experiments, in our laboratories and in that of Henson, have clearly shown that insufficient immunoglobulins are leeched off the filter paper during



TEXT-FIG 2—Reverse endocytosis of lysosomal enzymes from white blood cells encountering immune deposits on a surface.

incubation (less than 10% of applied material) to cause enzyme extrusion were this small amount of immunoglobulin alone to be present in the bulk phase.

Not all immunoglobulins or precipitates *per se* will cause hydrolase extrusion. Thus, coating of the surface with IgM alone or of nonaggregated IgG does not cause extrusion of acid hydrolases.

Table 6—Inhibition of Enzyme Release from Human Leucocytes Exposed to Immune Complex on Nonphagocytosable Surface

Compound	Percent of control enzyme release	
	β-Glucuronidase	LDH
None (control)	100.0*	100.0*
Dibutyryl cAMP (10^{-3} M)	75.7	99.4
PGE_1 (2.8×10^{-4} M)	81.3	97.8
Theophylline (10^{-3} M)	83.1	101.4
2-Chloroadenosine (10^{-3} M)	73.0	97.8
PGE_1 + theophylline	55.9	96.6
PGE_1 + 2-chloroadenosine	62.9	99.0
Colchicine (10^{-6} M)	73.3	102.5

* Human WBC's exposed to aIgG + IgM (200 μg) on millipore filter released 16.5 ± 3.2% of total β-glucuronidase and 9.5 ± 3.7% of total LDH as opposed to resting cells, which released 3.2 ± 0.6% of total β-glucuronidase and 8.0 ± 4.4% of total LDH (N = 7). Drug-treated samples: N = mean of 4 determinations.

Nor is this process complement-dependent. Extrusion is unassociated with damage to the cells (as indicated by release of lactate dehydrogenase or other indexes of impaired cell viability). Nor is it likely that inhibition of enzyme release is due to some extraneous influence of cyclic AMP in combination with theophylline alone, since exhibition of prostaglandin E_1 and 2-chloroadenosine (another inhibitor of phosphodiesterase) is as effective as admixture of prostaglandin with the known inhibitor phosphodiesterase, theophylline.

Further support for this hypothesis has come from recent experiments in which cells have been pretreated with cytochalasin B, at concentrations ranging from 3 to 10 μg/ml. This agent, as Davies *et al*[43] and Malawista[44] have shown, inhibits phagocytosis of particles by polymorphonuclear leukocytes. After pretreatment with cytochalasin B, human leukocytes can be exposed to zymosan particles. The cells do not take up the particles, which aggregate onto the cell surface and cause the cells directly to extrude lysosomal hydrolases from organelles which discharge immediately subjacent to the adherent zymosan particles. What this experimental system accomplishes in effect, is to turn the regurgitation-during-feeding model into the reverse-endocytosis model. Biochemical studies of this event, to be published in detail elsewhere, indicate that when cells are treated with cytochalasin B and subsequently exposed to zymosan, hydrolase release is markedly enhanced compared to control cells fed zymosan alone. This explanation was appreciated from morphologic studies in which granules could be seen to merge with the cell membrane subjacent to the adherent zymosan particles.

Consequently, as background, we have studied the enhanced release of enzymes from cytochalasin B-treated cells exposed to zymosan. Such *relaxed* or *paralyzed* cells constitute another test for the hypothesis that agents which elevate the level of cyclic AMP within cells act via the microtubule system rather than by means of the microfilaments. While it is appreciated that cytochalasin B interferes with the function with microfilaments, indirect effects on microtubules have also been postulated. Consequently, we treated such cells with dibutyryl cyclic AMP, cyclic AMP and theophylline, prostaglandin and theophylline and with colchicine. When leukocytes were treated with each of these agents, inhibition of enhanced release was readily demonstrable. Such experiments tend to confirm the hypothesis that agents which raised the levels of cyclic AMP within cells acted at a site also sensitive to colchicine, probably the microtubules.

If cells relaxed by treatment with cytochalasin B could still respond with enhanced extrusion of lysosomal enzymes into the surrounding medium after contacting zymosan, and if translocation of granules to the cell surface was unimpaired (except when disrupted as above, by cAMP, etc), microtubules would be expected to be quite prominent in cytochalasin B-treated cells exposed to zymosan. Indeed, that is exactly what was found: cells treated in this fashion displayed an abundance of microtubules as regularly aligned as in control cells. Moreover, such cells treated with prostaglandin and theophylline also displayed intact microtubules. But, if anything, the microtubules were even longer and randomly arrayed throughout the cell. The possible significance of this observation will be discussed below.

A General Mechanism for Immunologically Induced Endocytosis

Since the observations of Lay and Nussenzweig,[45] it has been appreciated that peripheral blood leucocytes contain receptors for immunoglobulin G (but not immunoglobulin M). These receptors, which are not sensitive to trypsin, are discrete from the complement receptors present on the same cells, which are sensitive to trypsin. Moreover, Henson[27] has found that endocytosis and subsequent release of lysosomal hydrolases from polymorphonuclear leucotyes could be readily induced by aggregates of immunoglobulin G and of immunoglobulin A but not of immunoglobulin M or other classes. Immunoglobulins of the subgroups IgG_1 and IgG_3 were most effective in inducing release when ingested in the aggregated state, whereas native immunoglobulins induced little or no enzyme release. Therefore, it appears that immunoglobulins G (and perhaps A) in the antigen-reactive configuration have the property of interacting with nonprotein sites of the leukocyte membrane in order to provoke endocytosis (and hydrolase release). Recent studies in our laboratory by Mrs. Ann Brand have indeed shown that aggregated human IgG, but not native IgG, interacts with artificial lipid structures in the smectic mesophase (bilayer configuration). These structures (liposomes) undergo modest perturbation in contact with immunoglobulins in the antigen-reactive configuration. Studies of the association between immunoglobulins and liposomes by means of Sepharose chromatography on Sepharose 4B show both anionic (dicetyl phosphate) and cationic (stearylamine) liposomes capable of interacting with immunoglobulin G in the heat-aggregated stage. Moreover, the enhanced diffusion was demonstrated of previously sequestered

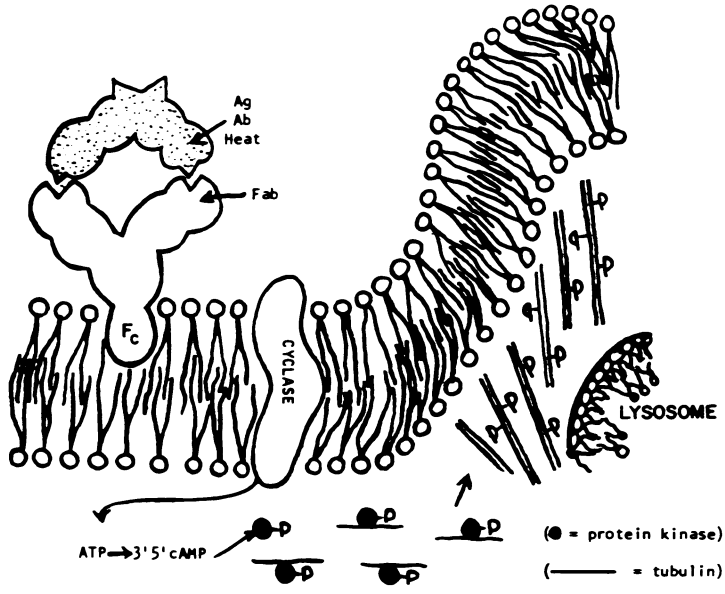
marker anions from liposomes after their contact with these immunoglobulins.

Therefore, it is reasonable to suppose that the first stimulus to endocytosis of aggregated immune complexes from the bulk phase, or to extrusion of enzymes onto immunoglobulins in the antigen-reactive configuration when present on a filter paper, is the interaction of the Fc fragment of immunoglobulins with the lipid bilayers at the surface of leucocytes. This interaction (see above) would occur when IgG molecules become fixed in the antigen-reactive configuration by virtue of union of Fab with divalent antigen, antibody to Fab determinants, or by heat aggregation. Each of these can promote lattice formation of IgG monomers and would render available regularly arrayed, now hydrophobic, Fc ends for interaction with lipid bilayers (Text-figure 3).

What would be the consequences of this interaction between immunoglobulins and surface lipids? Membrane-perturbing agents such as digitonin, etc are capable of altering the reactivity of adenyl cyclase.[46] It would, therefore, be reasonable to suppose that it is membrane perturbation by Fc regions of immunoglobulins which stimulates leucocyte membrane adenyl cyclase to increase the level of cyclic AMP within cells.* Furthermore, in order for a phagocyte to respond to membrane perturbation by endocytosis and movement of lysosomes to phagocytic vacuoles or to the cell exterior (depending upon whether its task is to ingest from the bulk phase or to extrude onto a solid surface), the microtubule system must remain intact and under appropriate controls.

Within cells, microtubules are in a constant state of assembly and, disassembly. Monomers of tubulin are in equilibrium with the aggregated state in which intact microtubules exert their influence on cell form.[32,40,41] Goodman *et al*[41] have postulated that, in nervous tissue at least, microtubule protein can function as an appropriate substrate for phosphorylation by cyclic AMP-dependent protein kinases. Were this situation to hold in the case of the leukocyte, we would expect that cyclic AMP would activate a specific protein kinase in the leucocyte cytosol to phosphorylate microtubule subunits. It would therefore be expected that phosphorylation of tubulin subunits might promote

* Recent data[47] suggest that increments of cAMP in leukocytes after phagocytosis are found, not in polymorphs, but in mononuclear cells. This observation came from experiments using the cyclic AMP-binding assay. Since the protein kinase of human polymorphs has been found (by Dr. P-K. Tsung in our laboratory) to be stimulated equally well by *cyclic IMP*, assays of other cyclic nucleotides are required in phagocytic cells.



TEXT-FIG 3—Relationship between immunoglobulins and endocytic rearrangement.

aggregation of microtubules and thus regulate their function. Indeed, Gillespie [40] has presented evidence (albeit in tubule-rich pancreatic strips), which suggests that cyclic AMP exerts a biphasic effect on microtubule protein, as judged by the affinity of the tubulin units for colchicine. Low concentrations of cAMP induced *aggregation* of microtubules (decreased colchicine-binding) whereas higher concentrations induced *disaggregation* (enhanced colchicine-binding by tubulin monomers).

For this hypothesis to have any validity in the leukocyte, it would be necessary to demonstrate that human polymorphonuclear leukocytes do indeed have a protein kinase which is activated by cyclic AMP. Recent experiments in our laboratory by Dr. Pi-Kwang Tsung have indeed identified such a protein kinase in purified preparations of human polymorphonuclear leucocytes. The protein kinase is maximally stimulated by 5×10^{-6} M cyclic AMP and demonstrates the usual requirements for absence of calcium and presence of magnesium. The protein kinase, which, as in other systems, consists of a regulatory and a catalytic subunit, could be purified approximately 400-fold over its content in the crude homogenate. On DEAE cellulose chromatography the protein kinase in its catalytic form could be shown to be specifically precipitable by vinblastine together with microtubule protein. Thus, the first step for suggesting that cyclic

AMP-dependent protein kinases can act to regulate tubule structure has been fulfilled, but it remains to be determined whether the tubulin subunits of human polymorphonuclear leukocytes can indeed serve as substrates for the protein kinase.

Were this hypothesis to be true, how can one explain the *inhibition* of granule extrusion by increasing the level of cyclic AMP within cells? This appears to be a paradox, since we would expect that cyclic AMP would *stimulate* endocytosis by promoting tubule function. We have previously shown (see above) that low levels of cyclic AMP *do* enhance translocation of lysosomes to phagosomes or the cell periphery (experiments with mouse macrophages done with Peter Dukor) and that high concentrations inhibit.[42] But high concentrations of cyclic AMP may have another interesting effect. We have noted before the polarity of endocytosis and phagocytosis. When cells are stimulated to endocytose foreign material they appear to rearrange their surfaces at specific sites rather than over the general cell periphery (Figure 3). This would imply that directed traffic of lysosomes to these locally perturbed areas is required and that a gradient of cAMP exists, which is highest in the perturbed region. Were cyclic AMP to be elevated uniformly throughout the cell, we would expect random aggregation of microtubules, thereby preemptively causing aggregation of tubulin into microtubules at areas not necessarily directed toward the cell surface or toward phagocytic vacuoles. A paraphrase of this process would be "if all tracks are open, train traffic cannot be directed where needed."

This series of events, it will be easily appreciated, is at the moment highly speculative. The actual data we have obtained indicate only that there may be an interaction between immunoglobulins and cell surfaces in general, specifically those of the polymorphonuclear leucocyte. Moreover, we have shown that agents which elevate the levels within cells of cyclic AMP have the capacity to interfere with the flow of lysosomes to the phagocytic vacuole or to the cell periphery. This effect is biphasic, as many effects of cyclic AMP are within cells (low concentrations appear to enhance and high concentrations appear to inhibit the flow and shuttle of granules). We have obtained evidence for the presence of a protein kinase (the catalytic unit of which is associated with microtubule protein) within human white cells. Much more experimentation will have to be done in order to verify the hypothesis presented above. It may well be, however, that phagocytic cells such as the polymorphonuclear leucocyte fall into a class of cells, the activities of which can be

regulated by cyclic AMP, acting, as in other cells, via protein kinases to regulate the fully differentiated function of the cell.

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[*Illustrations follow*]

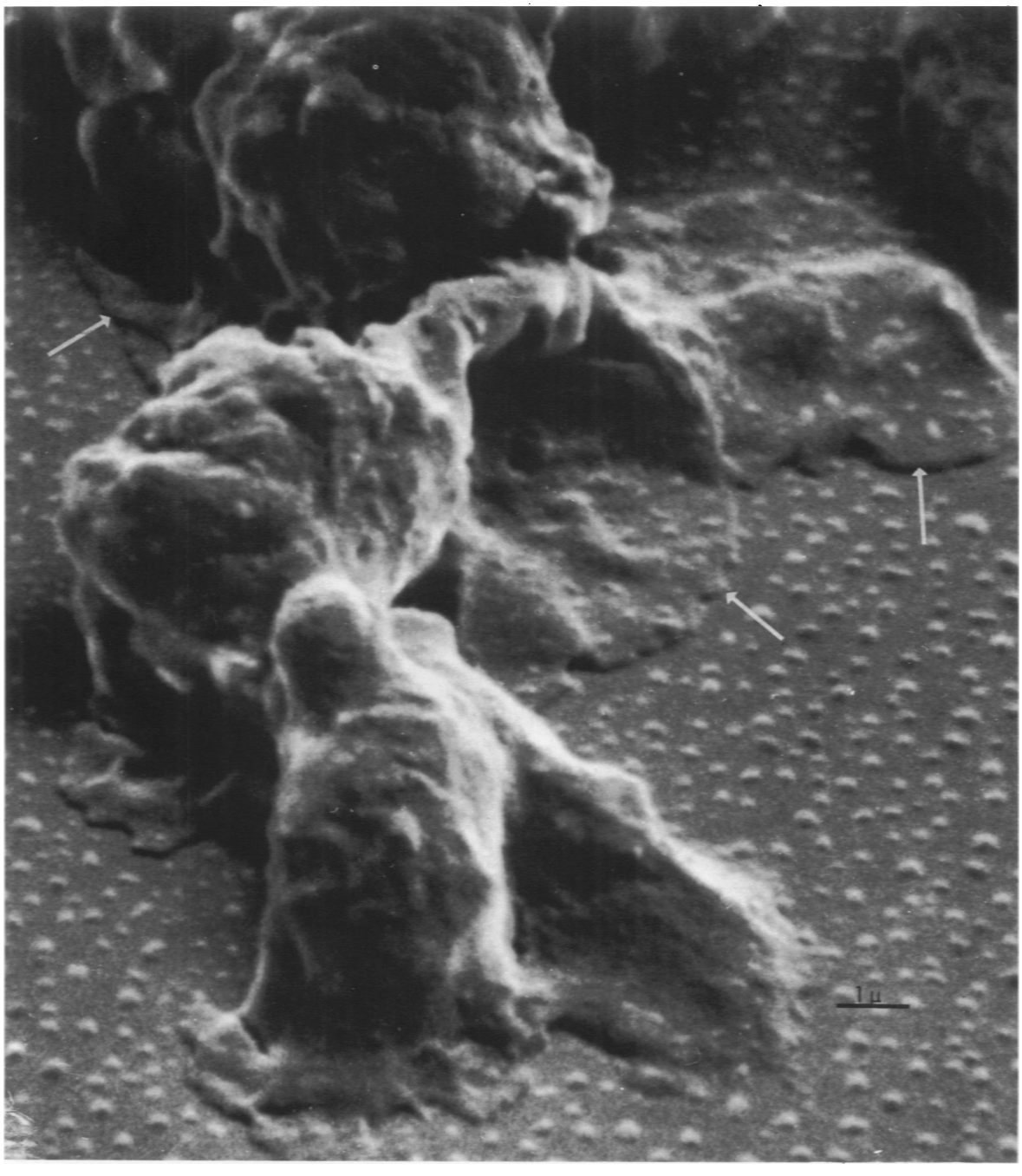


Fig 1—Human peripheral blood polymorphonuclear leucocytes, incubated for 1 hour in phosphate-buffered saline. The cells have flat ruffled extensions (*arrow*), less than 0.1 μ thick at the edge. The bulk of the cytoplasm is massed around the nuclear lobes.

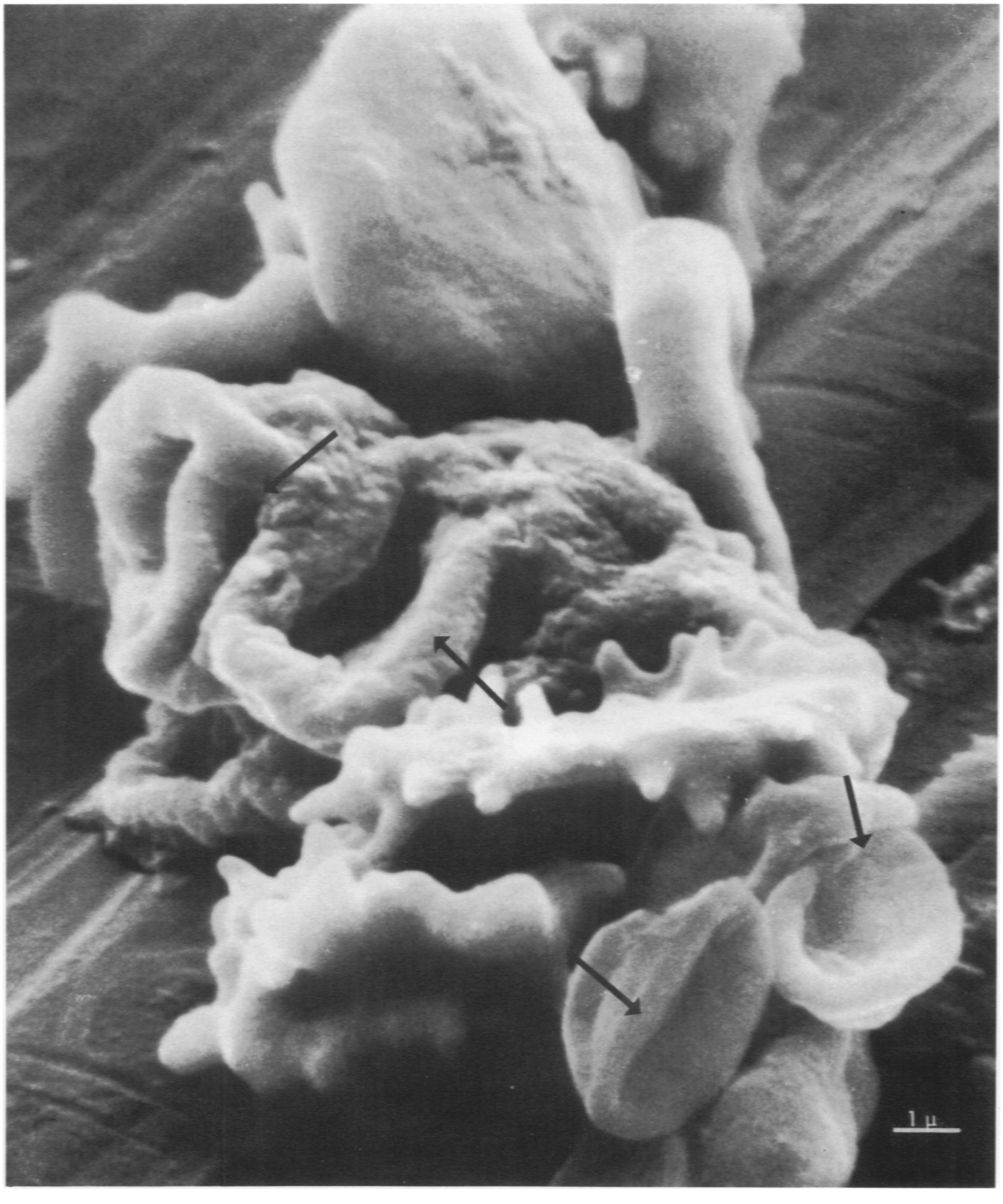
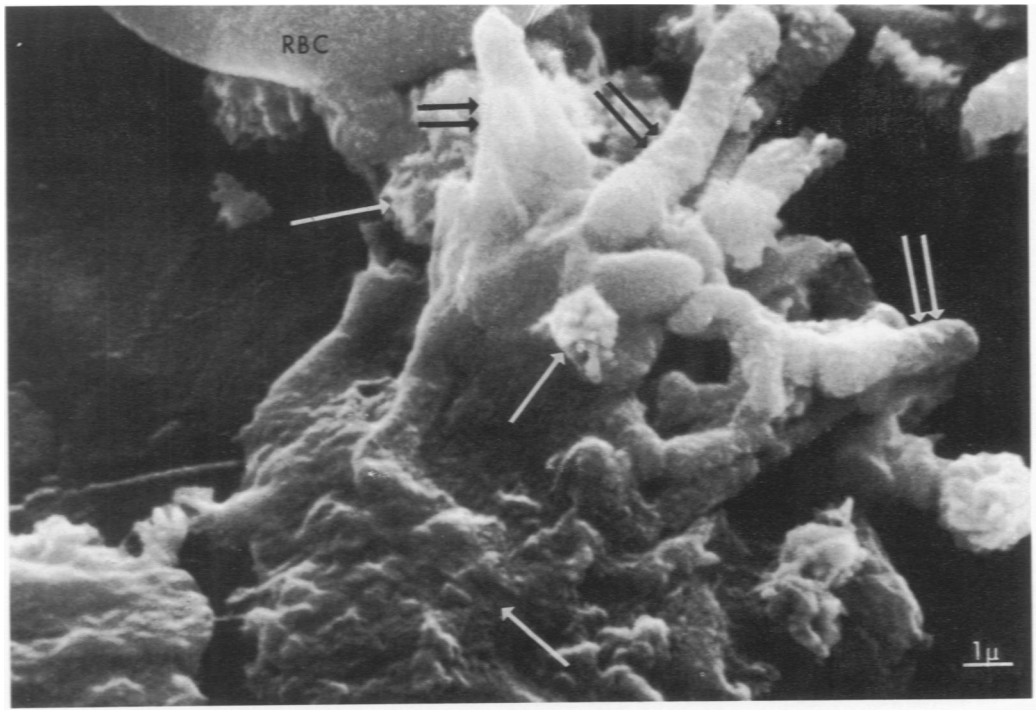


Fig 2—Human peripheral blood polymorphonuclear leucocyte, incubated for 1 hour with zymosan. *Arrows* indicate phagocytic cups. These cups have an inside diameter of approximately 2 μ and a wall thickness of 0.4 to 0.6 μ; each cell has several.



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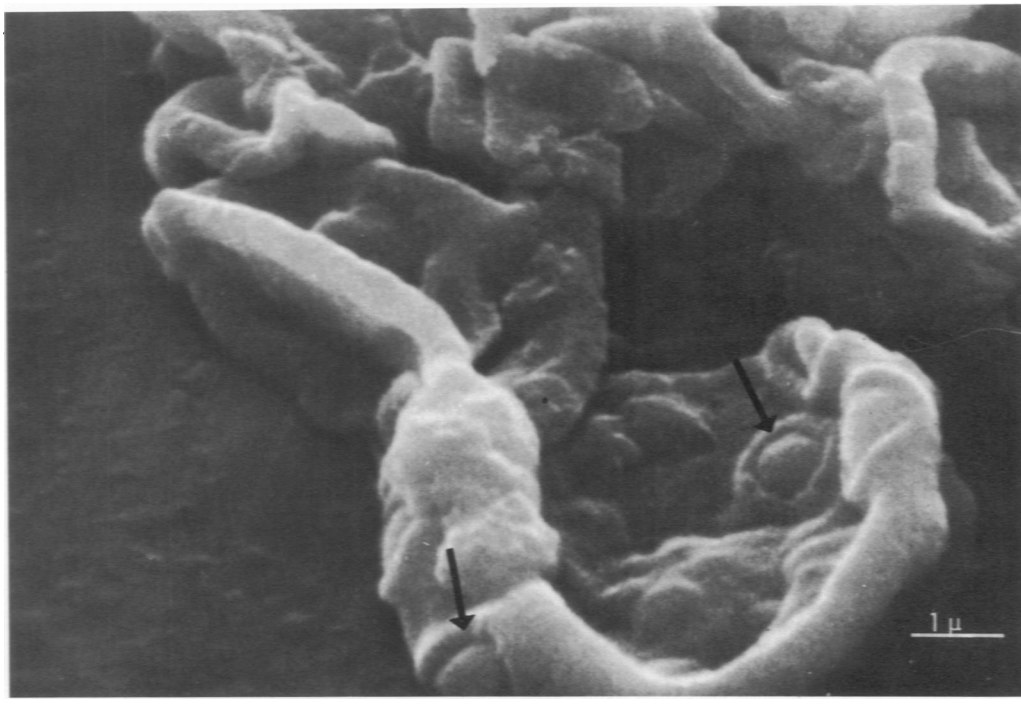

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Fig 3—Human peripheral blood polymorphonuclear leucocyte, incubated for 1 hour with immune precipitates of IgG with rheumatoid factor. *Double arrows* indicate the multiple finger-like projections elaborated by these cells. The projections are approximately 1 μ in diameter with lengths of 2 to 5 μ. *Single arrows* indicate the amorphous precipitate of immune complex. **Fig 4**—Human peripheral blood polymorphonuclear leucocyte, incubated for 60 minutes with 2.8×10^{-4} M prostaglandin E_1 before exposure to zymosan (1 hour). The cup is transformed into a saucer and rounded projections (approximately 1 μ diameter) within these saucers indicate the presence of particles subjacent to the cell surface.

[*End of Article*]