THE IN VITRO DIFFERENTIATION OF MONONUCLEAR PHAGOCYTES

V. The Formation of Macrophage Lysosomes*

By ZANVIL A. COHN, M.D., MARTHA E. FEDORKO, M.D., and
JAMES G. HIRSCH, M.D.

(*From The Rockefeller University*)

Certain factors involved in the formation of the dense, acid phosphatase–positive granules of cultivated macrophages have been discussed in previous publications (1–5). From these studies it appeared that the granules arose from pinocytic vacuoles which accumulated in the juxtanuclear region. In this location the phase-lucent vacuoles became increasingly dense and following this transition were shown to contain both extracellular components of their environment as well as new hydrolytic enzymes. Additional experiments documented the close association between the rate of pinocytosis and the production of both granules and hydrolases.

Some of the major questions which arose during these studies were related to the detailed mechanism of granule formation and the method by which acid hydrolases were transferred to the pinocytic vacuole. This report will deal with these problems by following: (*a*) the life history of pinocytic vesicles labeled with colloidal gold; (*b*) the intracellular flow of newly synthesized protein by electron microscopic autoradiography; and (*c*) the localization of acid phosphatase at the ultrastructural level.

Material and Methods

The techniques for cultivating mouse peritoneal phagocytes and the preparation of cells for electron microscopy have been detailed in a previous publication (5). For the purpose of this study cells were maintained in T flasks in the presence of 50% newborn calf serum in No. 199 medium.

The Labeling of Cells with Colloidal Gold.—Colloidal gold, nonradioactive, was obtained from Abbott Laboratories, North Chicago, Illinois. Before use in cell cultures it was dialyzed against large volumes of phosphate-saline (pH 7.5) at 4°C for 48 hr to remove the preservative and other small molecules.

Mass cultures in T flasks were incubated for 36 to 48 hr in the 50% serum medium. At this

time the medium was removed and 8.0 ml of fresh, warm 50% serum medium containing 10 μg/ml of colloidal gold was added to the flasks. The cells were then incubated in the presence of colloidal gold for periods of 30 to 120 min. The medium was aspirated thoroughly and the cell sheet washed once with 10 ml of warm No. 199. The cells were then fixed for 15 min with 1% buffered OsO_4. After fixation, the cell sheet was rinsed with 10 ml of physiological saline and the macrophages were scraped off the glass surface. The cells were collected by low speed centrifugation and processed as described previously (5). Thin sections of Epon-embedded material were stained with both uranyl and lead ions (6) and examined in a Seimens Elmiskop I. In most experiments the contents of two T flasks were pooled.

Electron Microscopic Autoradiography of Cultivated Macrophages Pulse Labeled with Leucine-H^3.—

(a) *Labeling of macrophages:* Macrophages were cultured in T flasks for 42 to 44 hr in 50% newborn calf serum medium. At this time the cells were highly polarized (5) with maximum development of the Golgi region. DL-leucine-4,5-H^3 with an activity of 5.4 c/mmole was purchased from New England Nuclear Corporation, Boston, as a sterile solution and employed as the label in all experiments. Preliminary studies were performed to investigate the incorporation of the isotope and the degree of labeling of cell organelles when examined by autoradiography. The following method gave sufficient labeling so that an exposure of thin sections to the emulsion gave adequate numbers of grains in 2 wk. The general plan of these experiments was patterned after that followed by Caro and Palade (7).

The following procedures were performed in an incubator room at 37°C and all solutions were equilibrated to this temperature before being employed. At the time of an experiment, the culture medium was removed and replaced with fresh 20% newborn calf serum, No. 199, containing 30 μc/ml of tritiated leucine. The cultures were immediately gassed with 5% CO_2 air, stoppered, and incubated for 15 min at 37°C. The isotope-containing medium was then aspirated with a fine tipped pipette and the cell sheet washed thoroughly with two 15 ml volumes of 50% newborn calf serum, No. 199, in order to remove the vast majority of isotope in the medium. Following this 15 min pulse, the medium was replaced with fresh 50% newborn calf serum, No. 199, containing 2 mg/ml of nonradioactive L-leucine. Both the high concentration of serum and the added "cold" amino acid precluded any further utilization of the small amount of labeled amino acid present in the system. Immediately after the pulse of tritiated leucine and at 30, 60, and 180 min thereafter, sets of two flasks were harvested.

(b) *Processing of macrophage cultures:* At the time of harvest, the medium was aspirated and 8.0 ml of ice cold 1.25% buffered glutaraldehyde was added to the flask. Fixation was allowed to continue for 15 min at 4°C and the cell sheet was subsequently rinsed with three 12 ml volumes of physiological saline. Postfixation was accomplished with 1% OsO_4 for 60 min at 4°C. Following another series of saline rinses, the cells were scraped from the glass surface and collected by low speed centrifugation. The cell pellets were then imbedded in agar and processed for electron microscopy. Cells from similarly treated preparations were also assayed for isotope by liquid scintillation spectroscopy. The distribution of isotope in the trichloracetic acid (10%) precipitable and soluble fractions were determined at time periods up to 3 hr after the pulse.

(c) *Autoradiography:* Thin sections were cut on a Porter-Blum microtome and prepared by the general procedure of Caro and van Tubergen (8). A detailed description of this technique has recently been outlined by Harford and Hamlin (9). Ilford L-4 emulsion was applied by means of a large wire loop and the coated grids stored with a dessicant at room temperature for 2 to 6 wk. Coated grids were removed and examined at weekly intervals up to 6 wk. They were developed with Microdol (Eastman Kodak Co., Rochester, New York) and emulsion was removed by the method of Revel and Hay (10) with the alkaline lead reagent. A comparative study revealed that no significant redistribution of label occurred during removal of the excess emulsion. Additional staining was accomplished with uranyl acetate.

(d) *Examination of sections and counting of grains:* For the purposes of grain counts, multiple grids, obtained from 2 to 3 blocks were examined for each time point. Grids were routinely observed in the Seimens electron microscope at a magnification of either 10 or 20,000. Counts were performed on intact cells which had been cut longitudinally and demonstrated all segments of cytoplasmic organization. A total of at least 500 gr were counted for each time point. Approximately 10% of the total grains at each time point could not be accurately localized gecause of vagaries in fixation. These grains were omitted from the calculations.

Electron Microscopic Localization of Acid Phosphatase.—T flask cultures maintained in 50% serum medium for periods of 24 to 48 hr were examined for the presence of acid phosphatase. Most experiments were performed with cells cultivated for 40 to 44 hr. At the time of harvest, the growth medium was removed and 5.0 ml of ice cold 1.25% glutaraldehyde (cacodylate buffer pH 7.4) was added to the cell sheet. The cells were allowed to fix for 15 min at 4°C, the glutaraldehyde removed, and the cells rinsed briefly with 10 ml of saline. 7 ml of 0.88 M sucrose was then added to the flasks and they were allowed to stand at 4°C for 30 min. The sucrose step was found to be essential for good staining of the dense granules. The sucrose solution was then replaced with 8 to 10 ml of prewarmed Gomori substrate pH 5.0, which had been freshly prepared with β-glycerophosphate (Eastman Kodak). Incubation in the presence of substrate took place for exactly 15 min at 37°C. The substrate was aspirated, the cell sheet rinsed twice with cold saline and the cultures were then postfixed with 1% OsO₄ for 30 min at 4°C. After this step the cells were scraped from the surface of the flask and processed for electron microscopy. The contents of two flasks were routinely pooled. Cells scraped from the surface of T flasks were also observed with both bright field and phase microscopy in thin cover slip preparations.

RESULTS

The Intracellular Distribution of Colloidal Gold.—Prior studies at the light microscope level revealed that colloidal gold was taken up by macrophages in vitro and was subsequently segregated into the granules of the centrosphere region (4). Once in this locus, a given pulse of gold was quantitatively retained in the cell for at least 24 hr. These data suggested that gold was taken up by a process analagous to pinocytosis and would therefore be an excellent marker for the intracellular membrane systems of pinocytic origin.

Fig. 1 illustrates a cell after exposure to colloidal gold for 120 min. Occasional particles were seen attached to the limiting membrane although no striking surface accumulation has been observed in these experiments. The uptake of the colloid occurred by means of small vesicles which were indistinguishable from the usual pinocytic vesicles. These small vesicles moved toward the centrosphere and fused to form the larger, electron-lucent pinocytic vacuoles. The gold was homogeneously distributed in the pinocytic vacuole and this structure was often surrounded by small vesicles which appeared to be fusing with it. At no time was there evidence of particles of gold in the cisternae of the endoplasmic reticulum or in Golgi saccules and associated vesicles.

The Fusion of Membranous Structures of Pinocytic Origin.—The fusion of pinocytic vesicles with the larger pinocytic vacuoles was a common event which could be observed by direct observations of living cells. This fusion also occurred with micropinocytic vesicles which were too small to be resolved with

the light microscope. An example of this process is presented in Fig. 2 *a*. Three micropinocytic vesicles containing colloidal gold are seen fusing with a larger, electron-lucent pinocytic vacuole containing gold. This picture strongly suggested that the contents of micropinocytic vesicles were emptied into the larger pinosome whereas their membranes fused with and became part of the wall of the pinocytic vacuole.

When cells were exposed to colloidal gold for various periods of time it was apparent that pinocytic vesicles also fused with preformed dense granules. In many instances, e.g. Fig. 2 *b*, the gold particles were localized at the periphery of the dense granule, in contrast to their homogeneous distribution in the pinocytic vacuole. As the exposure period was prolonged, the colloidal gold often became more homogeneously distributed in the dense bodies. Occasionally, however, the multivesiculated dense granules contained gold localized to certain of the internal vesicles and not to others. This type of result would be expected if vesicles of pinocytic origin actually penetrated the wall of the granule without a fusion of their external membranes.

The Relationship Between the Golgi Apparatus and the Pinocytic Vacuole.— It was mentioned previously that small vesicles of the size and configuration of Golgi vesicles accumulated about the electron-lucent pinocytic vacuoles. Because of the similarity in size between the Golgi and micropinocytic vesicles it was difficult to be sure of the identification of these structures. Studies were therefore performed to examine this interaction in cells which were heavily exposed to the colloid. The examination of thin sections from such samples showed that many of the vesicles which accumulated about and were fusing with the pinocytic vacuole did not contain gold. An example of this process is shown in Fig. 2 *c*, in which a gold-labeled pinocytic vacuole in the juxtanuclear region, is surrounded by nongold-containing vesicles. In this particular cell, more than 90% of the peripherally located vesicles, outside the juxtanuclear zone, were labeled whereas none of the vesicles in close proximity to the Golgi lamellae contained colloidal gold. Although this type of evidence is by no means conclusive it suggests the fusion of Golgi vesicles with structures of pinocytic origin.

The Intracellular Flow of Newly Synthesized Protein as Ascertained by Electron Microscopic Autoradiography.—Studies were next done employing electron microscopic autoradiography in an effort to obtain evidence on the role of the Golgi complex in the formation of lysosomes in maturing macrophages.

Text-fig. 1 shows the composite results obtained by localizing grains at the electron microscopic level after a 15 min pulse of tritiated leucine. Immediately after the pulse, 80% of the grains were located over the rough surfaced endoplasmic reticulum and were primarily found on the side of the nucleus opposite to the centrosphere region and in the neighborhood of lipid droplets. Less than

5% of the grains were associated with the Golgi apparatus and dense granules. The remainder were distributed over the nucleus, pinocytic vacuoles, and mitochondria.

30 min after the pulse there was a marked redistribution of grains. At this
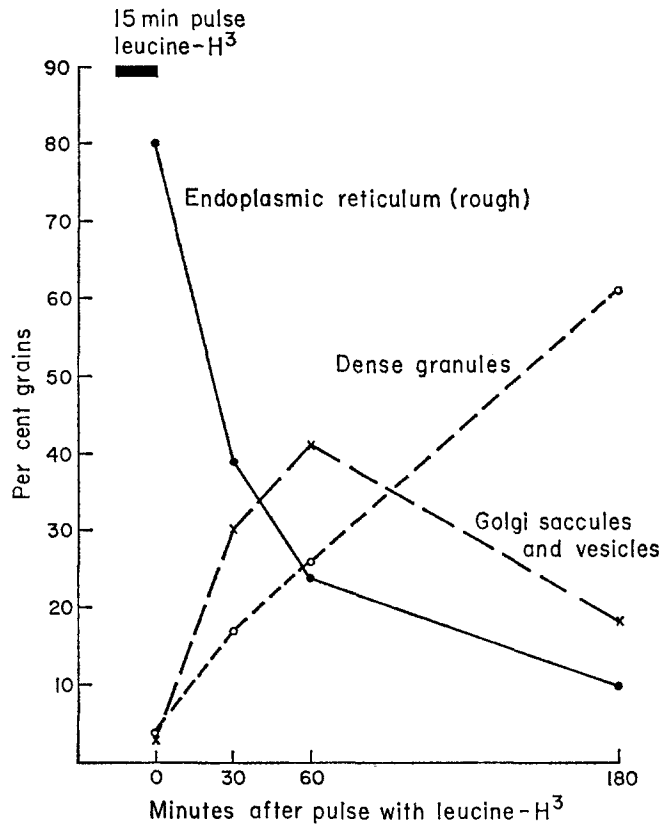
TEXT-FIG. 1. The intracytoplasmic redistribution of incorporated leucine-H³.

time, the percentage of grains over the rough endoplasmic reticulum had fallen to 40% whereas there was a substantial increase in the activity over the Golgi apparatus. Grains over the Golgi zone were found in association with both the saccules and vesicles. A small percentage of grains was now associated with dense granules. The appearance of a cell at this time period is shown in Fig. 3.

60 min after the pulse, the activity of the endoplasmic reticulum had fallen still further and the Golgi apparatus had increased to over 40% of the total grains. Granule labeling at this time had increased to approximately 25%.

After 180 min in the cold medium, both the endoplasmic reticulum and the Golgi apparatus had fallen to low levels. In contrast, the dense granules were now highly labeled and accounted for over 60% of the total grains.

The percentage of grains distributed over other cellular structures is shown in Table I and was taken from the same experiments as presented in Text-fig. 1. Only a small percentage of grains were associated with these organelles and no apparent rearrangement of isotope occurred during the experiment. A comparison of counts performed at weekly exposure intervals did not demonstrate any significant difference in localization between the 2nd and 6th wk, even though the number of grains had increased.

Experiments were also performed to quantitate the amount of isotope present

TABLE I

Distribution of Grains after a 15 min Pulse of Leucine-H³

Structure	Minutes after pulse			
	0	30	60	180
	%	%	%	%
Nucleus	3	5	4	3
Pinocytic vacuoles	5	6	7	6
Mitochondria	2	2	1	2

in cells after exposure to tritiated leucine. Direct isotopic measurements of washed cells, harvested immediately after the pulse and at 180 min of residence in cold medium revealed that over 90% of the isotope was trichloracetic acid precipitable and was quantitatively retained for this period of time. This is in keeping with the grain counts performed at the light microscope level (2). It suggested that newly formed protein remains within the cell for 3 hr and that the grain counts reflect an intracellular redistribution of protein.

The Localization of Acid Phosphatase Activity.—The previous evidence suggested that the Golgi vesicle might play a role in the transfer of hydrolases to the pinocytic vacuole and dense bodies. From the isotope experiments, however, no evidence concerning the nature of the newly formed protein could be ascertained. It was therefore of importance to localize one of the hydrolases at the electron microscope level and study its intracellular distribution.

Fig. 4 a illustrates a typical cell stained for acid phosphatase after 42 hr of cultivation in the 50% serum medium. At this time, the rate of accumulation of this enzyme is maximal as ascertained biochemically (3). The lead sulfide reaction product was uniformly present in the large dense granules. In this location it was distributed throughout the granule without localization to the granule membrane. Within the matrix of the granule it often exhibited a particu-

late appearance and seemed to be sharply localized, whereas other portions of the granule matrix were negative. Electron-lucent pinocytic vesicles and the larger vacuoles were without reaction product. On occasion, reaction product was present about the periphery of electron-lucent pinocytic vacuoles which were in the juxtanuclear region. The endoplasmic reticulum did not exhibit large amounts of reaction product. Some lead sulfide may have been deposited over the rough endoplasmic reticulum (ER) but this was difficult to ascertain in view of the small amounts of nonspecific product in the cytoplasm. Lipid droplets and nuclei were uniformly negative.

Of particular interest was the consistent presence of lead sulfide within the Golgi complex. The reaction product was present within the saccules, outlining these structures, as well as in what appeared to be small vesicles. An example of this localization is seen in Fig. 4 b. Examination of 200 appropriately sectioned cells indicated that at least 80% contained clearly demonstrable reaction product in the Golgi apparatus. Because of the poor preservation of membranes it was not possible to state whether or not all Golgi saccules were positively stained. Intact cells from the same preparation were also examined in cover slip preparations with phase-contrast microscopy. These illustrated strong staining of granules as presented previously (1) but no discernible reaction product in the Golgi region.

DISCUSSION

The evidence presented in this and other articles in this series indicates that the dense granules which accumulate in the juxtanuclear region originate from pinocytic vacuoles. During phases of active pinocytosis there is a constant flow of both newly internalized membrane and medium constituents into the cytoplasm of the cell. The pinocytic vesicles, which vary markedly in size, demonstrate directional movement and accumulate in the centrosphere region. This flow of vesicles through the peripheral cytoplasm is accompanied by fusion of many of the smaller pinosomes so that larger pinocytic vacuoles are eventually formed. It is these structures which clearly demonstrate the transition in density and are the eventual site of hydrolytic enzyme deposition. Although the fusion of both small and large pinosomes is the rule, there are instances in which there appears to be actual penetration without membrane fusion. This type of interaction has been demonstrated previously in time lapse cinematographic studies (3) and may account for the complex multivesiculated granules observed in similar preparations (see Fig. 4 b) (5).

In addition to the fusion of pinosomes with each other and with preformed dense granules there is strong evidence for the interaction of the pinosome with the Golgi vesicle. Many small, smooth surfaced vesicles become preferentially aligned about large, electron-lucent pinocytic vacuoles and presumably fuse with these structures. The difficulty of differentiating between Golgi vesicles

and micropinocytic vesicles in any given instance may be overcome to a considerable degree by the use of colloidal gold as a pinosome marker. Morphological evidence leading to similar conclusions on the role of the Golgi vesicle has been presented previously by Novikoff and his colleagues (11), and more recently by others (12).

Although the macrophage system does not permit a direct isolation and enzymatic analysis of Golgi vesicles it is ideally suited for the type of combined morphology and autoradiography employed by Caro and Palade to examine the role of the Golgi complex in the formation of zymogen granules in the pancreas (7). Following a pulse of leucine-H^3 in the macrophages cultured in vitro a flow of protein apparently occurs from its site of synthesis in the endoplasmic reticulum through the Golgi apparatus and finally to the dense granule. From this type of evidence it seems likely that the majority of newly synthesized protein is initially packaged in the Golgi vesicle. The Golgi vesicles then fuse with pinocytic vacuoles which subsequently develop into the dense granules. The dense granule can then be considered the final repository of both endogenous proteins and a variety of exogenous macromolecules. Whether or not the preformed dense bodies also receive newly synthesized protein by this mechanism remains uncertain. However, in view of the constant membrane fusion which occurs between "new" and "old" granules, protein and presumably hydrolytic enzyme might well be redistributed.

It is still unclear as to what types of protein are being synthesized by the macrophage. Some of this is thought to be hydrolytic enzyme. This reasoning is reinforced by the presence of acid phosphatase in the Golgi apparatus and its subsequent appearance in the dense granules. No evidence for the direct passage of enzyme from either the endoplasmic reticulum or cytoplasmic membrane has been found in this system. It is therefore suggested that the Golgi vesicle represents the "primary lysosome" in the macrophage which then interacts with the pinosome to form the "secondary lysosome". The Golgi vesicle would then be analogous to the polymorphonuclear leukocyte granule (13) in that it represents a membrane-bound package of enzymes prior to its interaction with exogenous substrate. This role of the Golgi vesicle is also in keeping with the enlargement of the Golgi complex in cells which are forming large amounts of hydrolytic enzyme (5). Whether this type of mechanism functions in other cell types is unknown.

SUMMARY

A combined morphological, autoradiographic, and cytochemical study at the electron microscope level has been directed towards the formation of electron-opaque granules of cultured macrophages.

Labeling of the membrane-bound vesicular structures of pinocytic origin was accomplished with colloidal gold. The initial uptake of gold occurred within

micropinocytic vesicles. These electron-lucent vesicles subsequently fused with and discharged their contents into larger pinocytic vacuoles. Colloidal gold was homogeneously distributed in the large pinosomes. In contrast, gold was initially deposited in the periphery of preformed dense granules indicating that these structures were also in constant interaction with the external environment.

Colloidal gold was not observed within the cisternae of the endoplasmic reticulum nor within the saccules or vesicles of the Golgi apparatus. There were, however, many small, gold-free vesicles, indistinguishable from Golgi vesicles, which were preferentially aligned about and appeared to fuse with the large pinosomes.

The intracellular flow of leucine-H^3-labeled protein was followed by electron microscopic autoradiography. After a 15 min pulse of labeled amino acid there was initial labeling of the rough endoplasmic reticulum. Subsequently, much of the label appeared in the Golgi complex. At still later time periods the cytoplasmic dense granules contained the majority of the isotope.

Acid phosphatase activity was localized to the dense granules and in the majority of cells to the Golgi apparatus.

It is suggested that hydrolytic enzymes are initially synthesized in the endoplasmic reticulum and are then transferred to the Golgi apparatus. Here they are packaged into small Golgi vesicles which represent the primary lysosome of macrophages. The Golgi vesicles subsequently fuse with pinosomes, thereby discharging their hydrolases and forming digestive granules or secondary lysosomes.

BIBLIOGRAPHY

1. Cohn, Z. A., and Benson, B., The Differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry, *J. Exp. Med.*, 1965, **121**, 153.
2. Cohn, Z. A., and Benson, B., The in vitro differentiation of mononuclear phagocytes. I. The influence of inhibitors and the results of autoradiography, *J. Exp. Med.*, 1965, **121**, 279.
3. Cohn, Z. A., and Benson, B., The in vitro differentiation of mononuclear phagocytes. II. The influence of serum on granule formation, hydrolase production, and pinocytosis, *J. Exp. Med.*, 1965, **121**, 835.
4. Cohn, Z. A., and Benson, B., The in vitro differentiation of mononuclear phagocytes. III. The reversibility of granule and hydrolytic enzyme formation and the turnover of granule constituents, *J. Exp. Med.*, 1965, **122**, 455.
5. Cohn, Z. A., Hirsch, J. G., and Fedorko, M. E., The in vitro differentiation of mononuclear phagocytes. IV. The ultrastructure of macrophage differentiation within the peritoneal cavity and in culture, *J. Exp. Med.*, 1966, 123, 747.
6. Karnovsky, M. J., Simple methods for "staining with lead" at high pH in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 729.
7. Caro, L. G., and Palade, G. E., Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study, *J. Cell Biol.*, 1964, **20**, 473.

8. Caro, L. G., and van Tubergen, R. P., High resolution autoradiography. I. Methods, *J. Cell Biol.*, 1962, **15**, 173.

9. Harford, C. G., and Hamlin, A., Electron microscopic radioautography of Hela cells infected with adenovirus, *J. Bact.*, 1965, **89**, 1540.

10. Revel, J. P., and Hay, E. D., Autoradiographic localization of DNA synthesis in a specific ultrastructural component of the interphase nucleus, *Exp. Cell Research*, 1961, **25**, 474.

11. Novikoff, A. B., Essner, E., and Quintana, N., Golgi apparatus and lysosomes, *Fed. Proc.*, 1964, **23**, 1010.

12. Gordon, G. B., Miller, L. R., and Bensch, K. G., Studies on the intracellular digestive process in mammalian tissue culture cells, *J. Cell Biol.*, 1965, **25**, 41.

13. Hirsch, J. G., and Cohn, Z. A., Digestive and autolytic functions of lysosomes in phagocytic cells, *Fed. Proc.*, 1964, **23**, 1023.

EXPLANATION OF PLATES

PLATE 76

FIG. 1. Cell cultivated in 50% serum for 42 hr and exposed to 10 μg/ml colloidal gold for 2 hr before fixation. × 30,000.

Particles of gold are present in all structures of pinocytic origin. On the upper left, a string of pinocytic vesicles (*Ves*) contains gold and one gold particle is present on the cell surface. Small pinosomes scattered throughout the cytoplasm, including some in the Golgi zone (*Go*), are seen to contain the colloid. Two large, electron-lucent pinocytic vacuoles (*Vac*) contain gold which is homogeneously distributed. These are surrounded by small vesicles, some of which are labeled. The larger dense granules (*GR*) are also labeled. In contrast, the Golgi saccules and associated vesicles, as well as cisternae of the rough endoplasmic reticulum are free of gold.
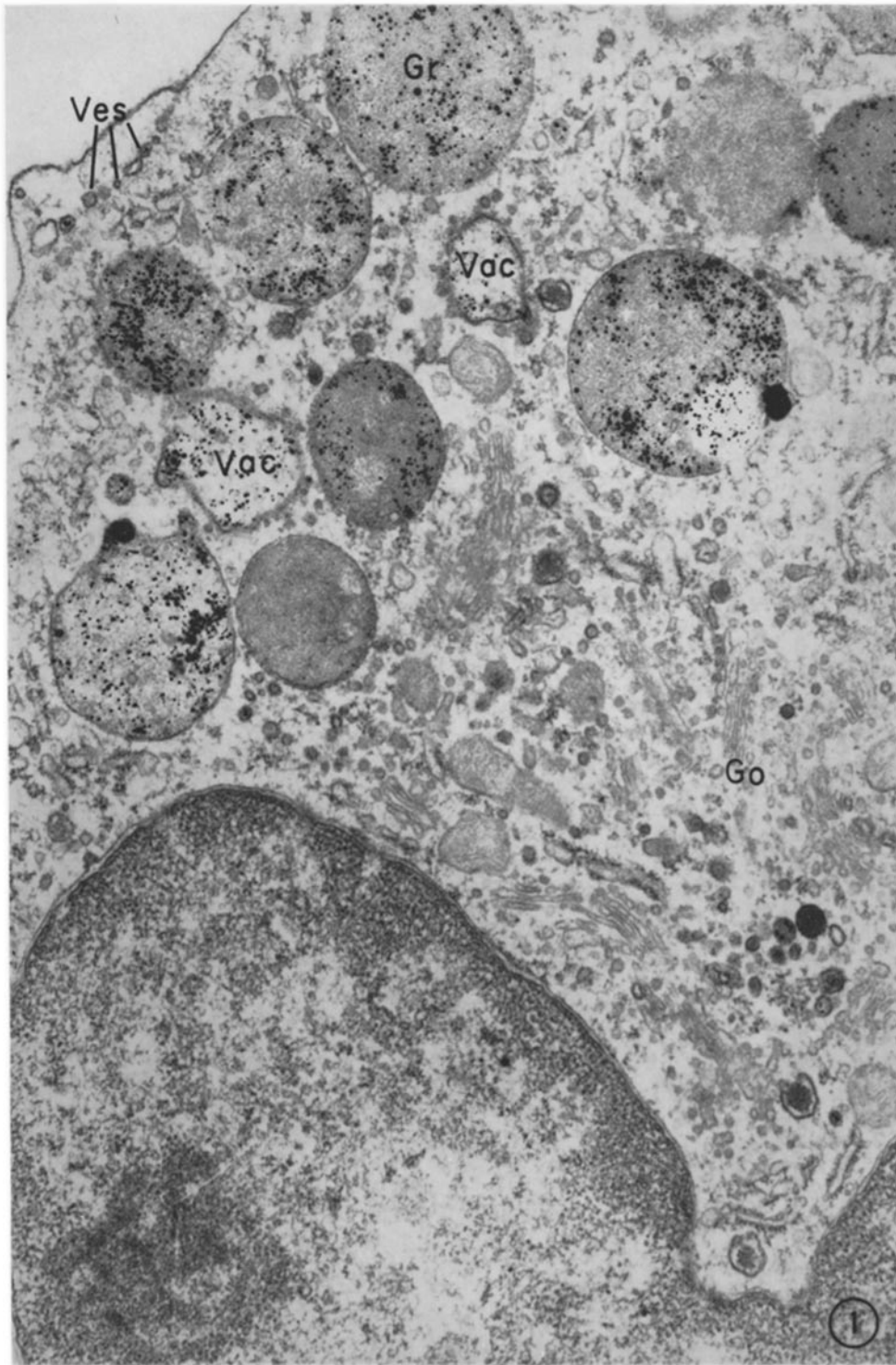
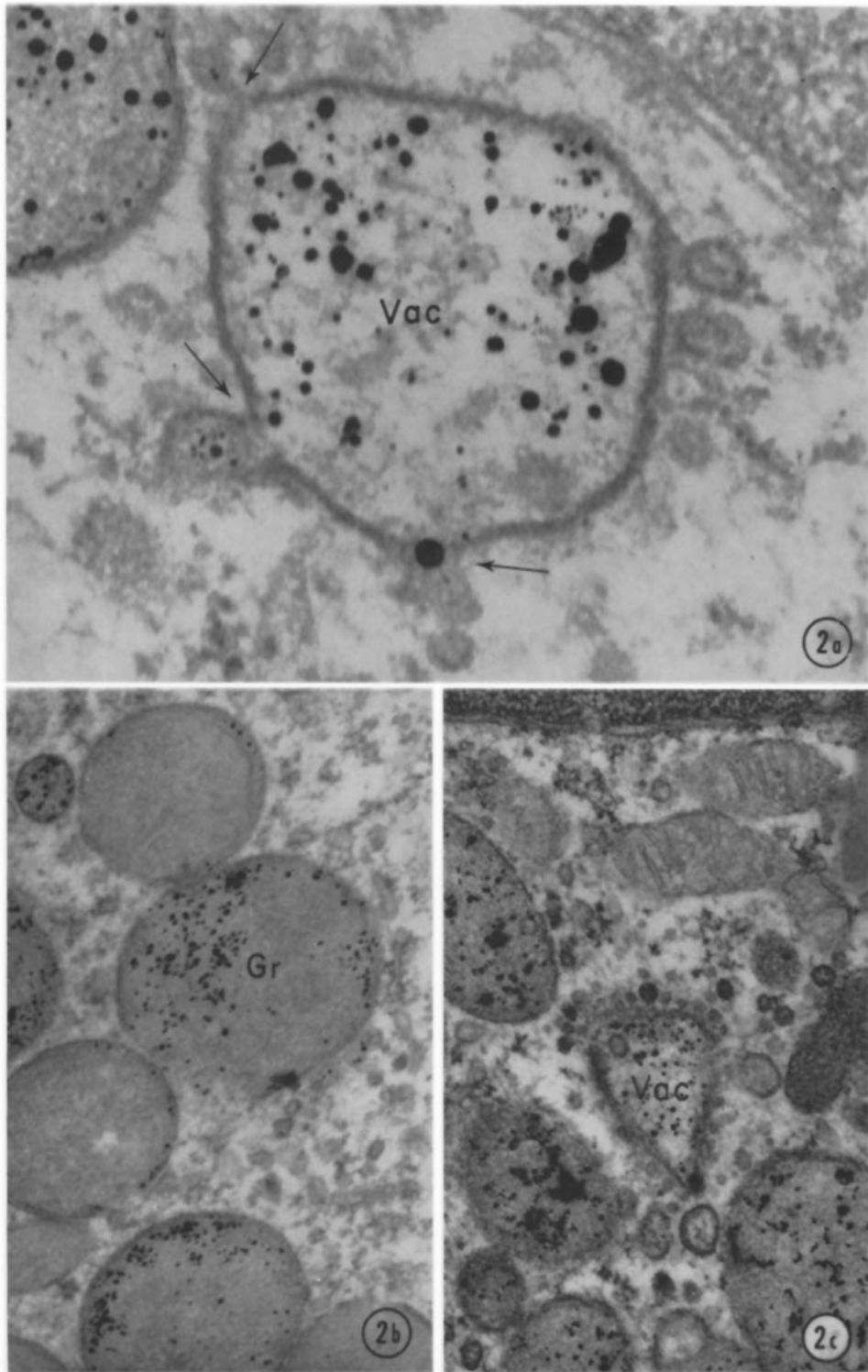(Cohn et al.: Macrophage differentiation)

FIGS. 2 *a* to 2 *c*. Cells exposed to colloidal gold for various periods of time.

FIG. 2 *a*. A section of a cell exposed to 10 µg/ml colloidal gold for 1 hr, showing the fusion (*arrows*) of three gold containing pinocytic vesicles with a larger, electron-lucent, pinocytic vacuole (*Vac*). × 132,000.

FIG. 2 *b*. A portion of a cell exposed to 10 µg/ml colloidal gold for 1 hr, demonstrating a nonhomogeneous distribution of gold in the electron-opaque granules (*GR*). In most cases the gold has a peripheral distribution and in two instances it is apparently segregated by a membrane from the homogeneous matrix of the granule. A few, small, gold containing pinosomes are in close proximity to the central large granule. × 37,000.

FIG. 2 *c*. A cell exposed to 10 µg/ml colloidal gold for 2 hr before fixation showing in the juxtanuclear region an electron-lucent, gold-containing pinocytic vacuole (*Vac*) surrounded by clusters of small vesicles which are free of the label. The majority of these vesicles are indistinguishable from Golgi vesicles. Micropinocytic vesicles present in the peripheral cytoplasm of this cell were heavily labeled with the colloid. This is thought to represent the fusion of Golgi vesicles with the larger pinosome. × 40,000.

FIG. 3. An electron microscopic autoradiogram of a cell fixed 60 min after a 15 min pulse of tritiated leucine. Grains are present over the Golgi zone (*Go*), pinocytic vacuole (*Vac*), nucleus (*N*), and dense granule (*GR*). × 28,000.
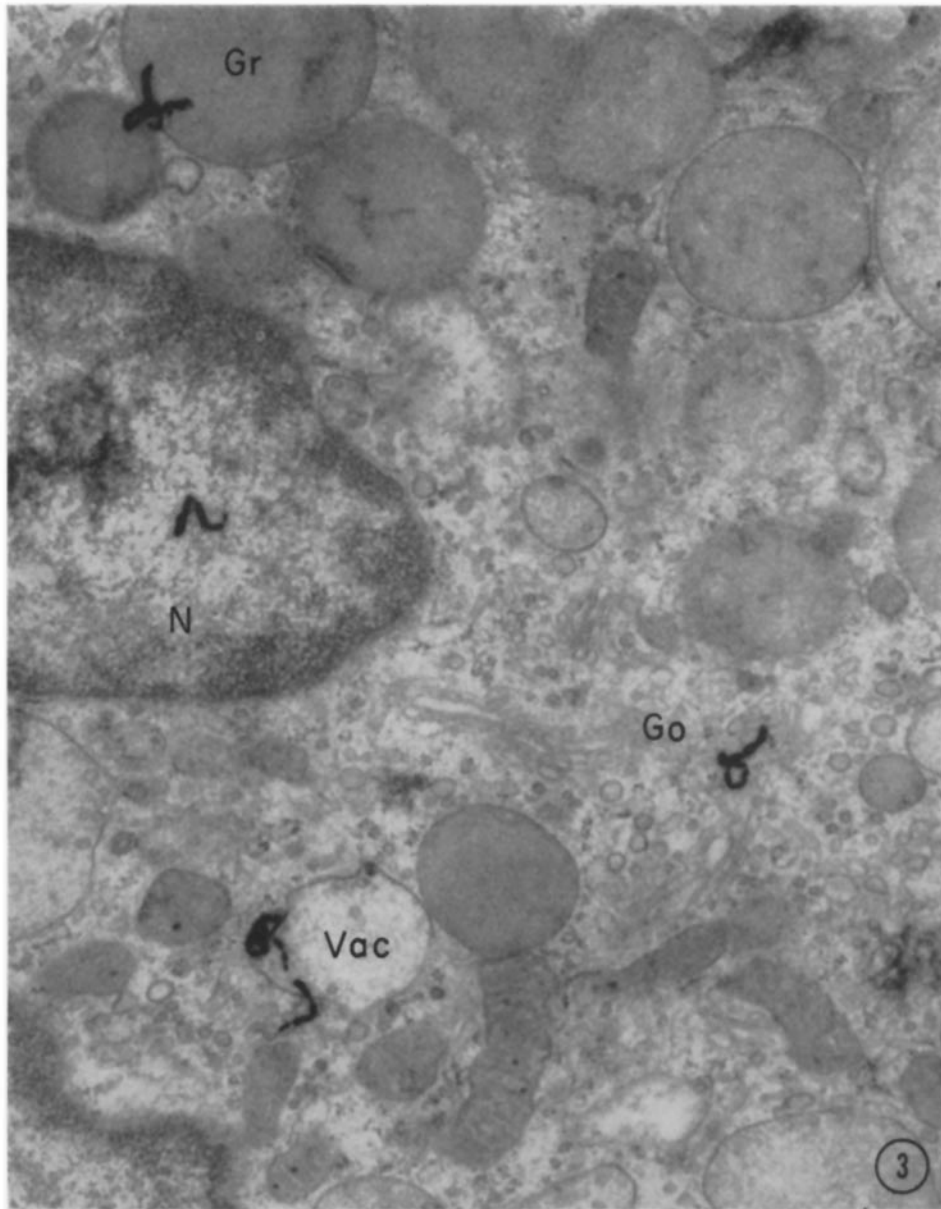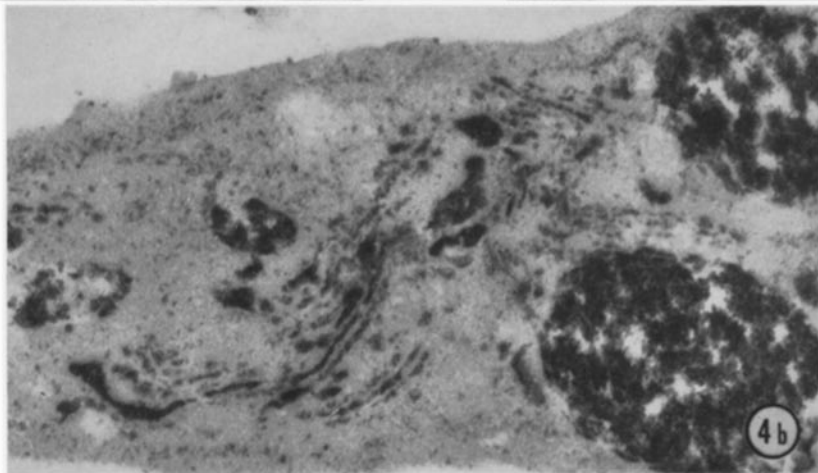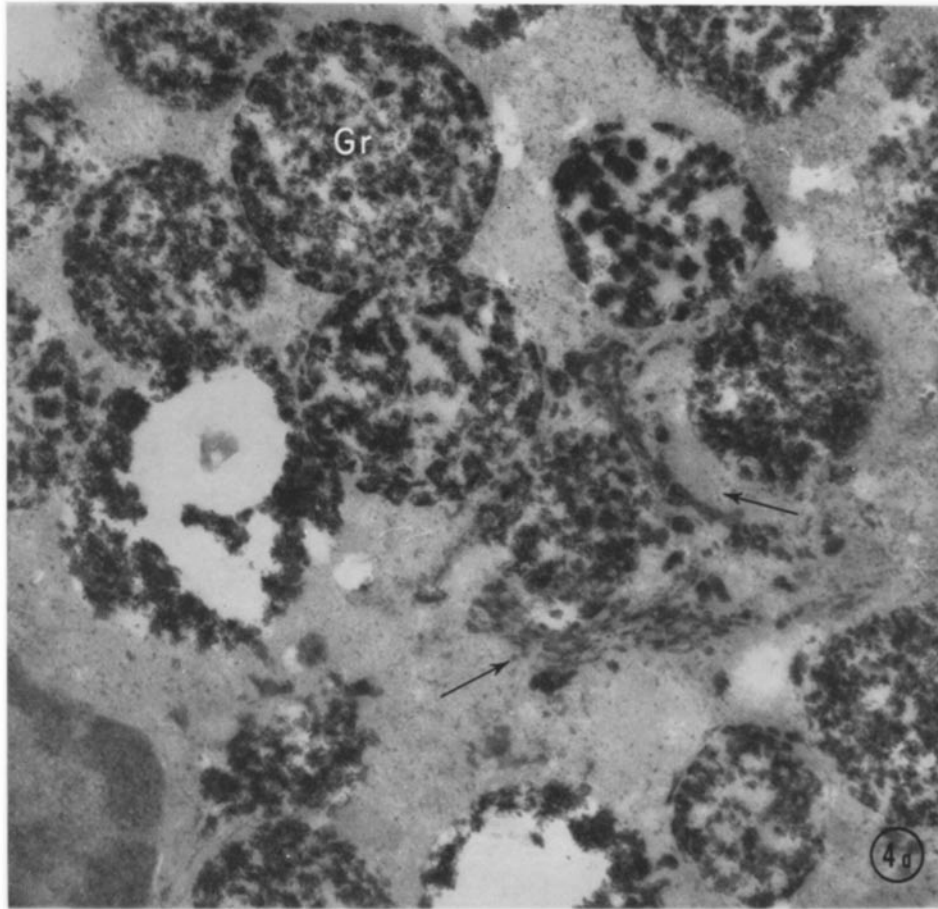
(Cohn et al.: Macrophage differentiation)

FIGS. 4 *a* and 4 *b*. Acid phosphatase stains of cells cultivated for 42 hr in vitro.

FIG. 4 *a*. The juxtanuclear region of a cell. The lead sulfide reaction product is localized to the dense granules (*GR*). In the central region of the cell, reaction product is seen (*arrows*) outlining the cisternae of the Golgi saccules and is also present in small granules which may represent Golgi vesicles. × 27,000.

FIG. 4 *b*. Another example of acid phosphatase reaction product outlining Golgi saccules and associated small vesicles. × 41,000.

(Cohn et al.: Macrophage differentiation)