AUTOPHAGIC VACUOLES PRODUCED IN VITRO

I. Studies on Cultured Macrophages Exposed to Chloroquine

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ABSTRACT

Mouse macrophages exposed to 30 μg/ml of chloroquine in vitro develop autophagic vacuoles containing various cytoplasmic components and acid phosphatase. The early toxic vacuoles appear in the perinuclear region within 15 min; on electron microscopy, they show irregular shape, amorphous moderately dense content, apparent double membranes, and in some instances curved thin tubular extensions with a central, dark linear element. Cytoplasmic structures are probably transported into the vacuoles by invagination of the vacuolar membrane. After exposure to chloroquine for 1–4 hr, macrophages display large vacuoles containing degraded cytoplasmic structures, membranous whorls, and amorphous material. When chloroquine is removed by changing the culture medium after 4 hr, the cells survive and 24 hr later they exhibit no abnormality except for large cytoplasmic dense bodies packed with membrane lamellae. During recovery chloroquine disappears from the cells. 24 hr after exposure to chloroquine the macrophages have accumulated less hydrolases than control cells.

INTRODUCTION

Autophagy is a mechanism whereby cells sequester within vacuoles and digest portions of their own cytoplasm. Numerous recent studies on autophagy (discussed in references 1–3) have established that the process occurs in various types of cells under physiologic as well as pathologic conditions. Most of these studies have been made on whole animals or on perfused organs.

The antimalarial drug chloroquine produces vacuoles possibly of an autophagic nature in leukocytes of man (4), in pancreatic cells of rats (5), and in cultured fibroblasts (6). We report in this paper and in the accompanying paper (7) observations of the effects in vitro of chloroquine on cultures of mouse macrophages and L cells. Low concentrations of this drug rapidly induce autophagic vacuole formation in these cells. The tissue-culture systems have proved particularly useful for studies on formation and subsequent development of the toxic vacuoles,[1] and on cell recovery following drug withdrawal.

MATERIALS AND METHODS

Cell Cultures

Macrophages were obtained from 25-g male mice by washing the peritoneal cavity with heparinized phosphate buffered saline as described previously (8). The cells were deposited by centrifugation at 400 *g* and were resuspended at a concentration

[1] The adjectives toxic and autophagic will both be used in this and the following paper to describe the abnormal vacuoles produced by chloroquine. The term toxic seems more appropriate for the early vacuoles in which no cytoplasmic components are visible.

of 2 million per milliliter in medium 199 (Microbiological Associates, Inc., Bethesda, Md.) containing 20% newborn calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 200 U/ml of penicillin. Cells were cultured in Leighton tubes on flying cover slips for light microscopic observations, or in T flasks for the electron microscopy. Cultures were gassed with 5% CO_2 in air. After incubation at 37°C for 1 hr, all cultures were washed twice with medium 199 for removal of cells not adherent to the glass; adherent cells remaining in the culture were nearly all macrophages.

Chloroquine Preparations

Most of the studies were performed with chloroquine [7-chloro-4-[(4-diethylamino)-1-methylbutylamino) quinoline] phosphate (Winthrop Laboratories, New York) freshly dissolved in culture medium. The hydrochloride form of chloroquine (Aralen, Winthrop Laboratories) was used in a few experiments. Radioactive drug, labeled with ^{14}C in the ring-3 carbon (New England Nuclear Corp., Boston, Mass.), was utilized for studies on the fate of chloroquine in recovering cells. Cells were washed five times in medium 199 to remove extracellular label, then suspended in distilled water, and frozen and thawed six times to remove all cells from the glass. An aliquot of the suspension was added to Brays fluid for scintillation counting. Protein determinations were done on other aliquots by the modified Folin procedure (9).

Light Microscopy of Fixed Cells

The flying cover slips were fixed for 10 min at 4°C in 2.5% glutaraldehyde (Fischer Scientific Company, Fair Lawn, N. J.) in 0.1 M phosphate buffer at pH 7.4. The cover slips were then mounted by inverting them over a drop of water, sealed with a paraffin-vaseline mixture, and observed with oil-immersion positive phase-contrast optics with a Zeiss ultraphot.

Histochemical Study for Acid Phosphatase

Macrophages on flying cover slips were fixed for 30 min in 2.25% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 in the cold. The specimens were kept in cold 0.88 M sucrose for 15 min before incubation for 30 min at 38°C in warm, freshly filtered Gomori medium containing beta glycerophosphate at pH 5.0 (10). The Gomori reagent was prepared 12–18 hr prior to use. The cover slips were rinsed in distilled water before exposure first to 1% acetic acid and then to dilute ammonium sulfide. Specimens were inverted over a drop of water and observed with oil-immersion bright-field and phase-contrast microscopy. For control purposes cells were processed in the same fashion except that substrate was omitted.

Analysis of Hydrolase Content of Macrophages

Cultures on glass in T flasks were frozen and thawed six times for insured release of intracellular enzymes. Protein, acid phosphatase, beta glucuronidase, and cathepsin were measured in the extracts by methods described previously (8).

Electron Microscopy

Medium in the T flasks was removed by aspiration and the cell sheet (still warm) was fixed by flooding it with a cold, freshly prepared mixture of one part 2.5% glutaraldehyde and two parts 1% osmium tetroxide, both in 0.1 M cacodylate buffer pH 7.4 (11). After standing on ice for 2 min, the cells were scraped from the glass with a plastic policeman, transferred to 3-ml conical centrifuge tubes, and spun at 300 *g* for 1 min. Supernatant fixative solution was removed thoroughly with a Pasteur pipette, and the cell pellet was chilled on ice, resuspended in 2 ml of fresh mixed fixative, and kept at 0°C for 15–30 min. Washing with saline, postfixation in uranyl acetate, and embedding in agar were as described elsewhere (11). Dehydration in alcohol and propylene oxide and infiltration with Epon were according to Luft (12). Thin sections (silver to gold) were cut on a Porter-Blum microtome with diamond knives, were picked up on 200-mesh grids lightly coated with Formvar and carbon, and were stained with uranyl and lead solutions (13). Specimens were examined and photographed in a Siemens Elmiskop I with 80 kv and a 50 μ objective aperture.

RESULTS

Phase-Contrast Appearance of Control Macrophages

In most experiments the cultures used for assessment of chloroquine cytotoxicity were mouse macrophages which had been maintained for 48 hr in a 20% serum medium. The morphology, under phase contrast, of these macrophages has been described previously (8) and is illustrated in Fig. 1. The cells were typically well spread with radiating pseudopods containing elongated mitochondria and tiny (approximately 0.2 μ) clear pinocytic vesicles. The body of the cell had a large oval or horseshoe-shaped nucleus, often with one to three nucleoli. In the perinuclear hof area were a few large (0.5–1.0 μ), lucent pinocytic vacuoles and numerous dense granules ranging from 0.2 to 0.4 μ in size. Some cells showed clusters of refractile lipid droplets, usually located at the junction of the pseudopods and the body of the cell or

along the margin of the nucleus nearest the cell membrane.

Phase-Contrast Appearance of Macrophages Exposed to Chloroquine

Addition to macrophages in culture of 30 μg/ml of chloroquine resulted in visible toxic effects on more than 90% of the cells within an hour. As is shown in Fig. 2, the cells appeared somewhat contracted, and they had developed numerous lucent vacuoles, 0.2–0.8 μ in diameter, in their perinuclear cytoplasm. On continued exposure to chloroquine, the clear vacuoles grew larger, and some showed dense amorphous material or refractile lipid droplets within them. Cytoplasmic granules were reduced in number in the severely vacuolated cells. After exposure to chloroquine for 3 hr (Fig. 3) some of the vacuoles had become very large, and many of them contained dense or refractile inclusions. Chloroquine at this concentration produced little or no cell detachment or death during a 3 hr period, but after expsoure to 30 μg/ml for 18 hr, the number of cells was markedly reduced. Some macrophage cultures were exposed to 30 μg/ml of chloroquine for 2 hr and then, after being washed, they were placed in medium without drug and allowed to recover. 3 hr later such recovering cells (Fig. 4) commonly showed in the perinuclear cytoplasm large clear or mottled vacuoles, and also large (up to 1 μ), irregularly shaped dense structures, some of which contained lipid inclusions. After recovery from chloroquine for 24 hr, the macrophages were essentially normal in appearance except for dense cytoplasmic granules larger than those seen in control cells. Cytotoxic effects of chloroquine were detectable at concentrations as low as 8 μg/ml. The toxic action was the more rapid and severe the higher the concentration of chloroquine. At 20 μg/ml most of the cells survived for at least 18 hr; at this time they showed dense cytoplasmic granules two to four times normal size. Chloroquine at 50 μg/ml produced rapid and severe toxic effects, most of the macrophages being dead or detached in 2 hr. Chloroquine hydrochloride exhibited cytotoxicity essentially identical with that of the phosphate. The age of the macrophage culture was of little importance in terms of susceptibility of the cells to chloroquine, but cultures older or younger than 48 hr did, of course, show somewhat different morphology initially and after exposure to drug.

Acid Phosphatase Histochemistry on Control and Chloroquine-Treated Cells

Histochemistry for acid phosphatase on 48-hr-old control cultrues of macrophages showed reactivity localized to small structures near the perinuclear hof (Fig. 5). The size and distribution of reaction product corresponded to that of cytoplasmic granules. Similar macrophages exposed to chloroquine for 3 hr before fixation and histochemistry showed reactivity in large round structures (Fig. 6). Phase-contrast microscopy established that these positive structures were the large toxic vacuoles produced by chloroquine.

Ultrastructure of Control Macrophages

Electron microscopic features of the perinuclear region in control macrophages are illustrated in Fig. 7. The Golgi complex was prominent, consisting of multicentric stacks of cisternae and associated vesicular profiles. Cytoplasmic vesicles were very numerous and widely distributed. At least three classes of vesicles could be discerned: (*a*) a small number, 50–100 mμ in size, showing surface spikes typical of coated vesicles, possibly arising from cell surface membrane, Golgi elements, or endoplasmic reticulum (ER) elements (14, 15); (*b*) moderate numbers of vesicles or vacuoles, often irregular in shape, with clear content, and representing pinocytic vesicles and various stages of their fusion with one another to form large (0.5–1.0 μ) pinosomes (16–18); and (*c*) a very large number of small (20–100 mμ) vesicles with smooth membrane and an internal density similar to that of cytoplasmic matrix, probably derived from the Golgi apparatus. In some areas the vesicles seemed to be aligned in linear or circular patterns. Cytoplasmic dense bodies or granules[2] were commonly present at the periphery of the Golgi zone; these granules ranged from 150 to 400 mμ in size and showed a moderately dense, structureless matrix and a thin band of lesser density immediately beneath the limiting membrane. Mitochondria exhibited a moderately electron-opaque matrix and a well-ordered pattern of cristae. The ER was limited to a few short strips of dilated partly rough, partly smooth elements. Peripheral cytoplasm of the macrophages (not shown in Fig. 7) contained smaller numbers

[2] Granule and dense body are used interchangeably in discussing macrophages in this and the following paper.

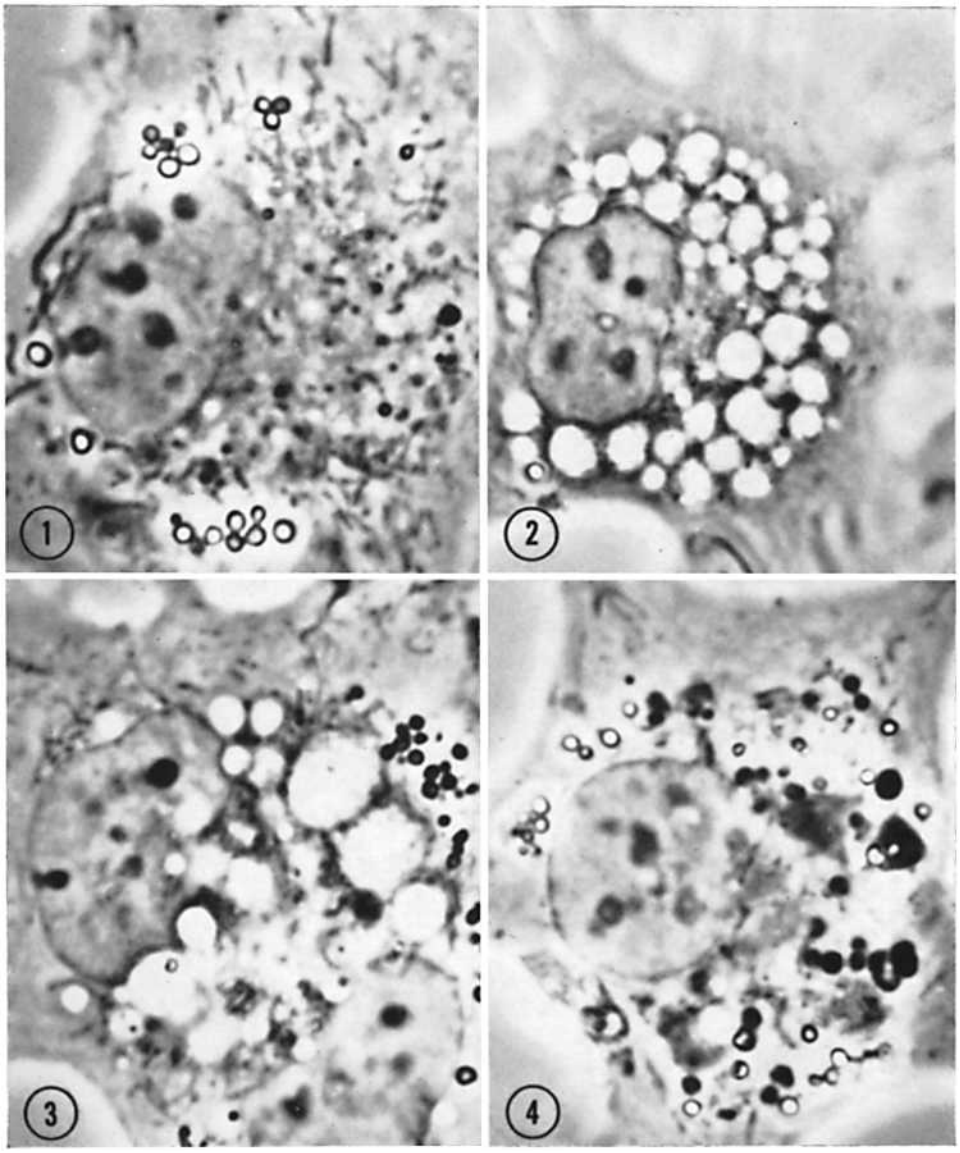

FIGURE 1 Phase-contrast appearance of the perinuclear area in a mouse peritoneal macrophage maintained in vitro for 48 hr in a 20% serum medium. The kidney-shaped nucleus is seen on the left. Cytoplasmic organelles in the nuclear hof region are clear pinocytic vesicles and vacuoles, and small dense granules. Refractile lipid droplets are located about the nucleus and at the periphery of the granule area. The peripheral cytoplasm contains elongated mitochondria and tiny, lucent pinocytic vesicles. × 2,500.

FIGURE 2 A macrophage maintained in vitro for 48 hr and then exposed for 1 hr to 30 μg/ml of chloroquine phosphate. The cell is somewhat contracted. Large clear vacuoles are seen in the perinuclear region. Pinocytic vesicles and dense bodies in the centrosomal region appear to be reduced in number. × 2,500.

FIGURE 3 This macrophage had been exposed to chloroquine for 3 hr. Perinuclear vacuoles are very large, and some of them contain dense amorphous material or refractile lipid droplets. Organelles in the peripheral cytoplasm appear to be unaffected. × 2,500.

FIGURE 4 Phase-contrast appearance of a macrophage exposed to chloroquine for 2 hr and then allowed to recover for 3 hr in a drug-free medium. The marked vacuolization is no longer evident. Large dense structures of irregular shape are seen in the perinuclear zone. × 2,500.

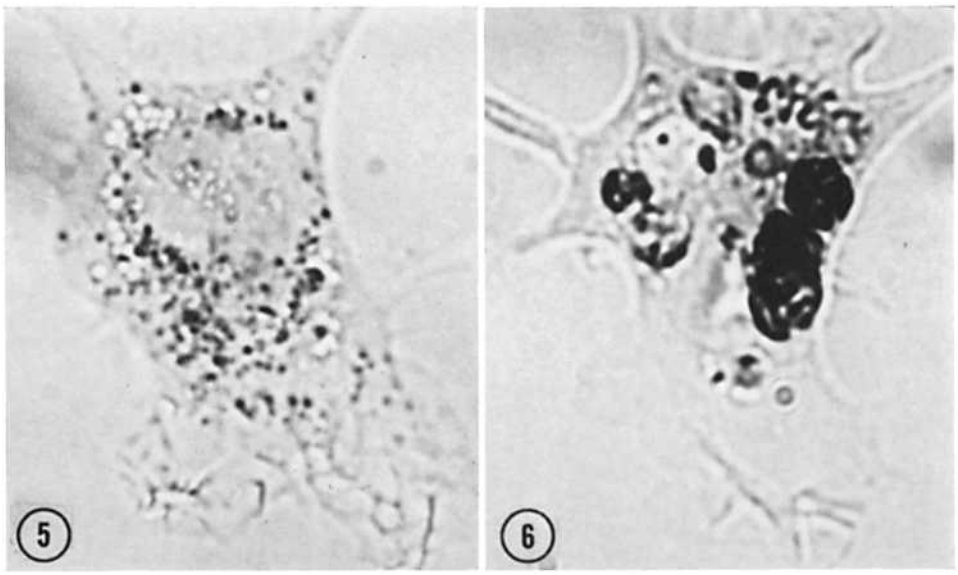

FIGURE 5 The Gomori acid-phosphatase reaction in a control macrophage. Reaction sites are of the same dimensions and distribution as the granules normally present in this cell. × 1,800.

FIGURE 6 The acid phosphatase histochemical response in a macrophage exposed to 30 μg/ml of chloroquine for 3 hr. Reaction product is located in large round structures which were identified as the toxic vacuoles when viewed under phase-contrast optics. The total reaction product in this cell appears to be greater than that of the macrophage in Fig. 5, but this probably reflects cell to cell variation rather than any increase in reactivity following chloroquine exposure. × 1,800.

of vesicular and granular structures, many mitochondria, some lipid deposits, and scattered ER. Typically the lipid droplets were approximately 1 μ in size with irregular outline and no limiting membrane. Strips of rough ER often were located around the lipid.

Ultrastructural Changes in Macrophages Exposed to Chloroquine

Abnormalities were detected in marcophages within 15 min after exposure to 30 μg/ml of chloroquine. These early changes (Figs. 8 and 9) consisted of the appearance at the periphery of the Golgi region of irregularly shaped vacuoles up to 1 μ in diameter with an amorphous moderately dense content. In some areas (Fig. 8, V_1) the vacuoles showed a thin clear zone and an electron-opaque layer immediately beneath the limiting membrane; this gave the impression of a double membrane. Only in rare sections, however, did the inner dense band show at higher magnification trilaminar membrane structure. Some early toxic vacuoles had curved tubular extensions with a centrally placed, prominent, dense element (Fig. 8, V_2; Fig. 9, V_2). Many vacuole profiles showed prominent invaginations of the limiting membrane (Fig. 9, V_1); the appearance of these images and the frequency of their occurrence suggested invagination as a likely mechanism for transfer of cytoplasmic components into the vacuoles.

After exposure of macrophages to chloroquine for longer periods of time (2–5 hr), the toxic vacuoles were very large, ranging up to 3 μ in diameter, and contained cytoplasmic components or their digestion products. The vacuoles were commonly located in a rosette pattern about the Golgi region (Fig. 10). The double-membrane image was still present in some of these late toxic vacuoles (Figs. 11 and 12) but more often a single limiting membrane was seen. The late toxic vacuoles were irregular in shape, and contained large, clear, electron-transparent areas, aggregated amorphous material of moderate density, lipid droplets, membrane whorls resembling myelin figures, and cytoplasmic components apparently in various stages of digestion (Figs. 10–12). A clear image of a

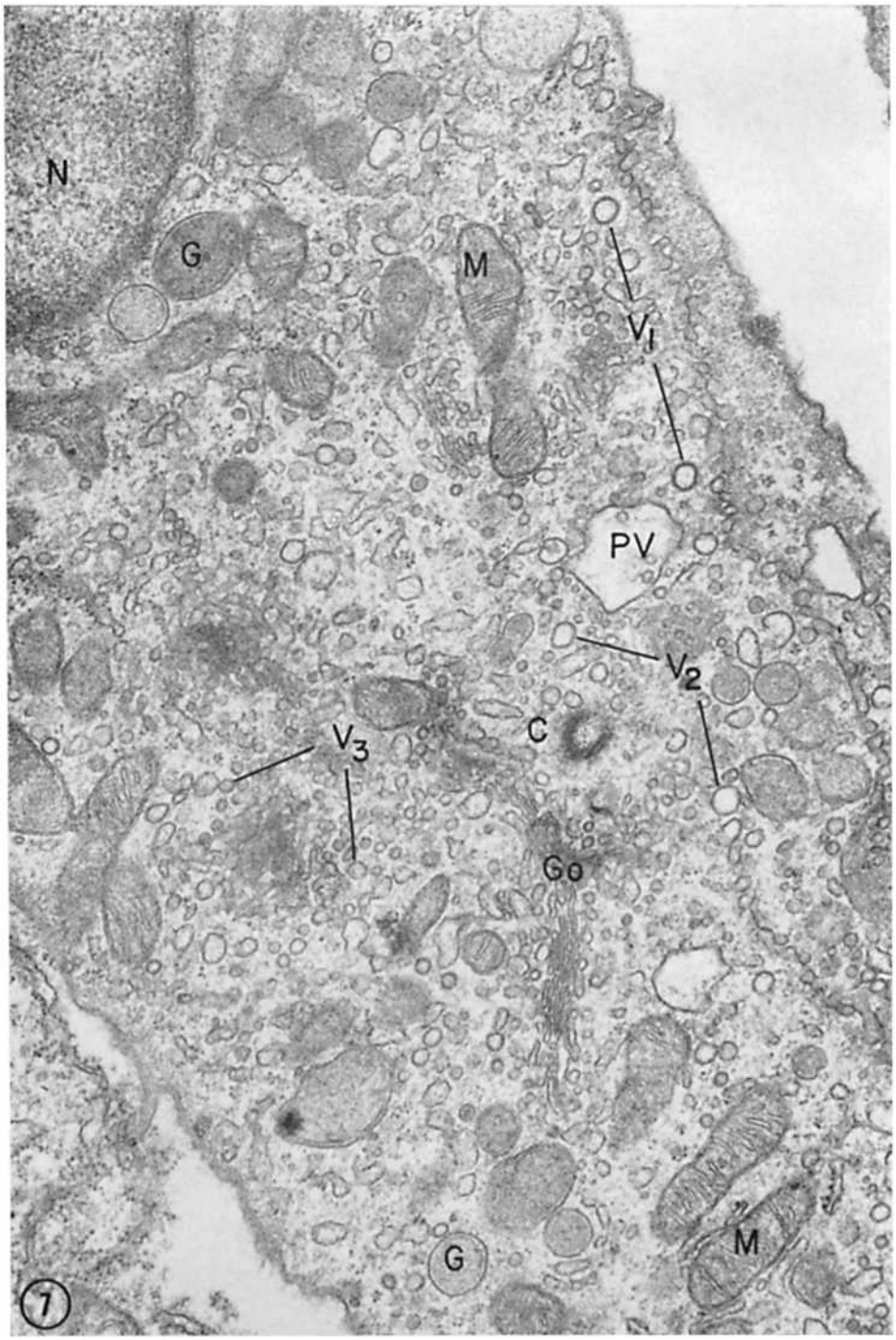

FIGURE 7 The ultrastructure of the perinuclear area in a control macrophage maintained for 48 hr in vitro. The nucleus (*N*) is at upper left. The centriole (*C*) is surrounded by Golgi (*Go*) complex composed of cisternal and vesicular elements. A pinocytic vacuole (*PV*) shows irregular shape and clear content except for internal vesicles. The centrosomal region presents a very large number of small round profiles. These vesicles are of three general types: a few (V_1) which show surface spikes typical of coated vesicles; some large vesicles (V_2) with smooth surface and clear content, perhaps of pinocytic origin; numerous vesicles (V_3) of varying sizes and showing a smooth rather indistinct membrane and a slightly electron-opaque matrix, probably representing elements of the Golgi complex. Granules (*G*) are located at the periphery of the Golgi vesicular region. These granules show an amorphous, moderately dense content and a clearly defined limiting membrane; often there is a thin clear zone immediately beneath the membrane. Many mitochondria (*M*) and a few short strips of partly rough, partly smooth dilated ER are also seen. Peripheral macrophage cytoplasm (not shown here) typically contains lipid deposits not bounded by membranes, scattered rough ER, and mitochondria. × 30,000.

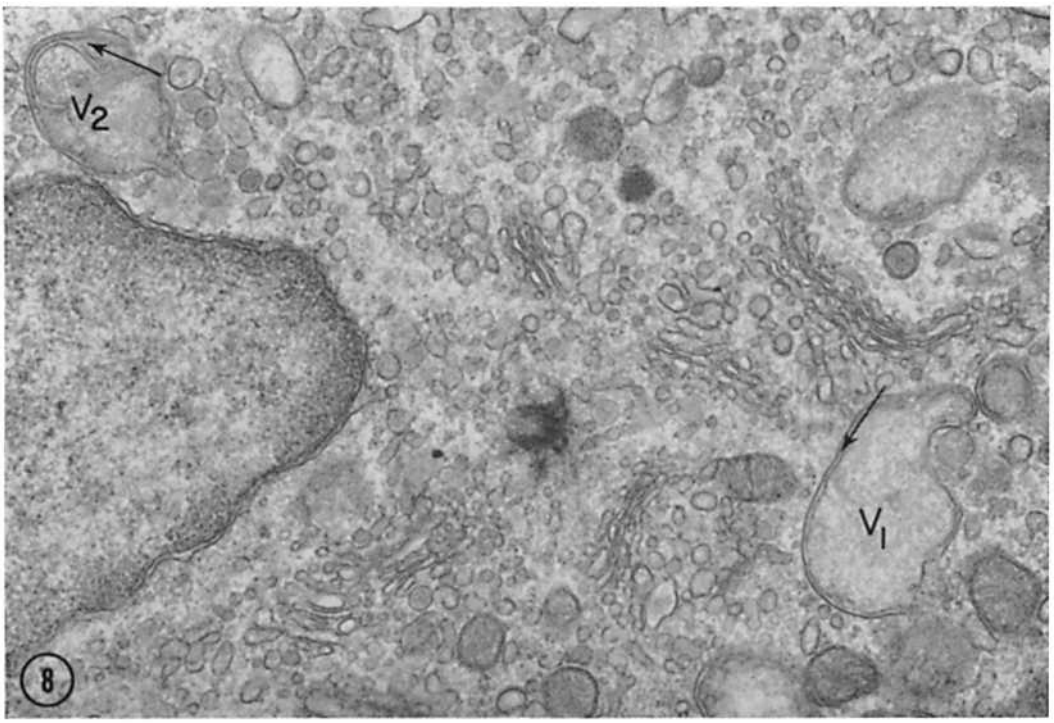

FIGURE 8 The perinuclear area of a macrophage fixed 15 min after exposure to chloroquine. The nucleus, centriole, and Golgi complex appear as in the control cells. Two early toxic vacuoles are present. V_1 shows an irregular outline, a moderately electron-transparent content, and an apparent double membrane (arrow). V_2 has a curved tubular extension or connection with a prominent, central, dense, linear shadow (arrow) which appears to be continuous with the inner membranous elements of the vacuole. × 27,000.

mitochondrion within a chloroquine-induced autophagic vacuole is presented in the accompanying paper (Fig. 7 in reference 7).

Many other macrophage components appeared to be unaffected by chloroquine. Nuclear morphology was not altered. Mitochondria and rough ER in the peripheral cytoplasm appeared normal in cells showing severe vacuolization after 5 hr exposure to chloroquine. Examination of many of these severely affected cells established that there had been a marked reduction in numbers of small cytoplasmic vesicles, pinosomes, and granules; this indicates incorporation of these elements into the toxic vacuoles or interference with formation and replacement of these structures by the cell. The Golgi apparatus in some of the severely damaged cells appeared normal, but in others exposed to drug for 5 hr the Golgi cisternae were markedly dilated.

Recovery of Macrophages from Chloroquine Toxocity

When macrophage cultures were exposed to 30 μg/ml of chloroquine for 2 hr and, after examination under phase contrast as a confirmation of the presence of toxic vacuoles, were washed and placed in fresh medium free of chloroquine, the cells recovered, and 18–24 hr later they appeared normal by light microscopy except for unusually large, dense cytoplasmic granules. Electron microscopic study of these recovered cells (Fig. 13) revealed as the only abnormality large (0.5–1.0 μ), perinuclear residual bodies containing membrane aggregates, electron-opaque amorphous material, and lucent areas.

Macrophages which had been exposed to chloroquine for 2 hr and allowed to recover thus displayed morphologic changes in some ways similar to the changes which occur when pinocytosis is

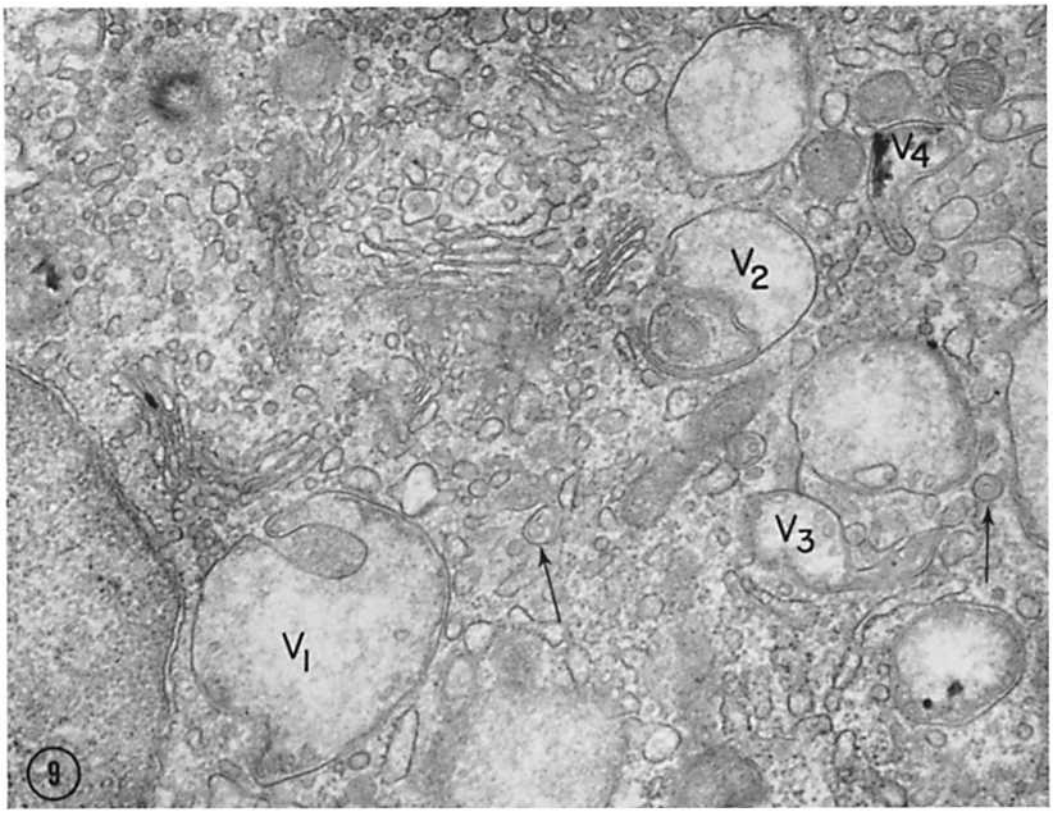

FIGURE 9 Shows early toxic vacuoles at the periphery of the Golgi zone in another macrophage exposed to chloroquine for 15 min. V_1 exhibits an invagination apparently about to pinch off and thus transport cytoplasm into the vacuole. V_2 has a curved tubular process with central electron dense element. V_3 and V_4 also display extensions. Cross-sections of these narrow extensions appear as target or multilaminate vesicles (arrows). The early toxic vacuoles have an amorphous, generally transparent interior. No normal dense bodies are seen. × 30,000.

stimulated in these cells (16). Increased pinocytosis leads to vacuolization which is followed by a marked increase in hydrolase content, and it was therefore of interest to determine whether a similar increased synthesis of digestive enzymes would be associated with toxic vacuolization due to chloroquine. As is shown in Fig. 14, the cells exposed to chloroquine accumulated less acid phosphatase, beta glucuronidase, and cathepsin during the recovery period than did the control "unstimulated" cells. Chloroquine exposure of macrophages resulted initially in stimulation of pinocytosis, but within 30 min this activity returned to control values and thereafter was suppressed (see Fig. 1 in the accompanying paper, reference 7). Pinsome counts 24 hr after chloroquine removal were still suppressed to approximately one-half control levels.

The fate of cell-associated chloroquine in the recovering cells was determined by employing ^{14}C-labeled drug and following radioactivity of the washed cells. Intracellular chloroquine or its derivative was lost from the macrophages, since only 10–15% of the initial cell-bound radioactivity remained after 24 hr. Total protein content of the cell sheet at the end of this 24 hr recovery had increased somewhat, and thus it was established that the loss of bound radioactivity was not merely a reflection of cell detachment or death.

DISCUSSION

In 1962 Ashford and Porter described "lysosomal" membrane-bounded bodies containing cytoplasmic structures in rat liver cells following perfusion of the organ with glucagon (19). In the short time since this report, the phenomenon of segregation

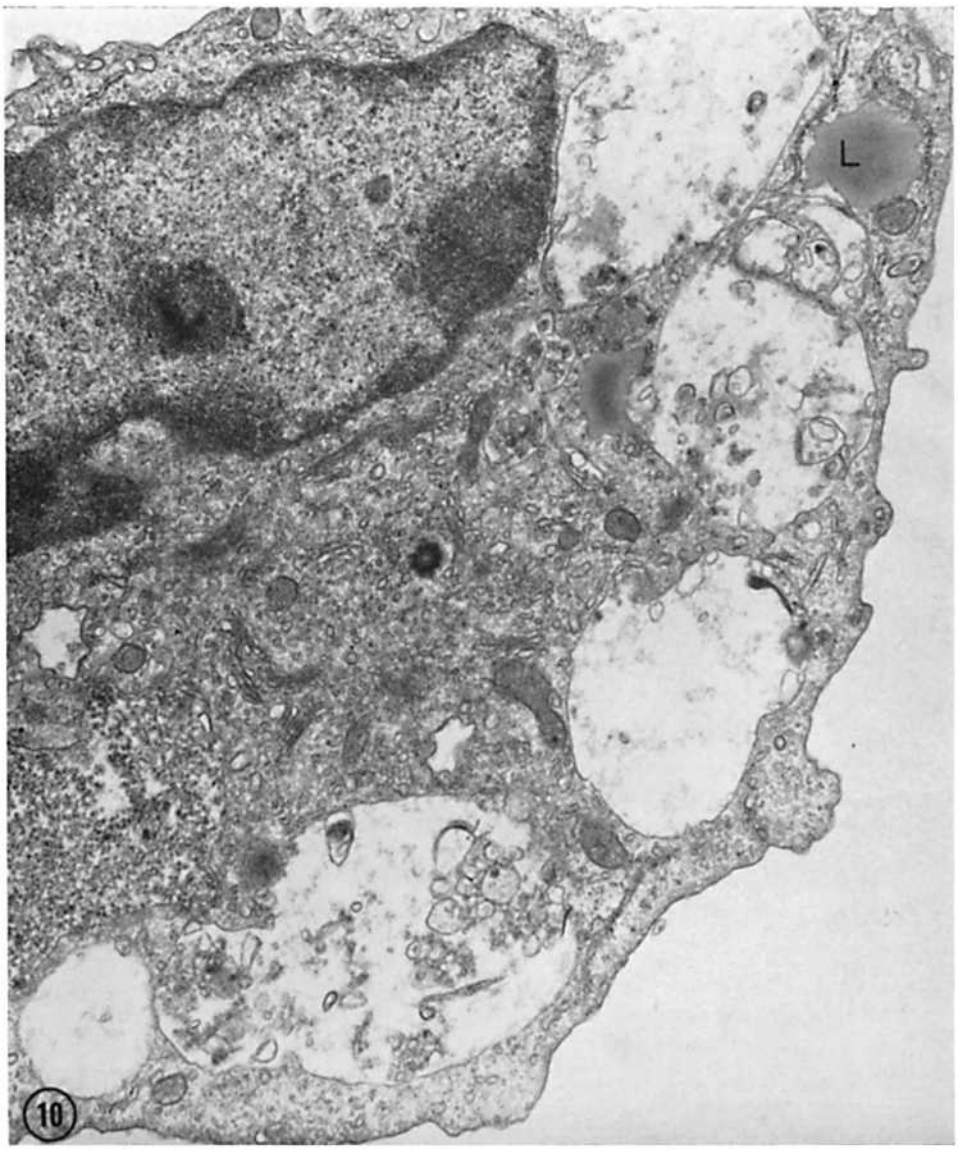

FIGURE 10 A low power view of a macrophage exposed to 30 μg of chloroquine per milliliter for 30 min. The huge vacuoles are located about the Golgi zone and occupy most of the space in the peripheral cytoplasm. The vacuoles have a well-defined limiting membrane (single in most places), and contain amorphous material and membranous structures in an electron-transparent medium. Nucleus, Golgi complex, mitochondria, and lipid (*L*) appear normal. × 17,500.

and digestion of parts of cells within cytoplasmic vacuoles has been investigated extensively, and is generally now termed autophagy. Autophagy occurs in many different cell types under physiologic and pathologic conditions (reviewed in references 1–3, 20, 21). Most observations of autophagy have been made on whole animals or on organ systems, although the phenomenon also has been seen in tissue culture (22). Some aspects of the autophagic process remain unclear despite the numerous studies. For example, the origin of the membrane bounding the autophagic vacuole

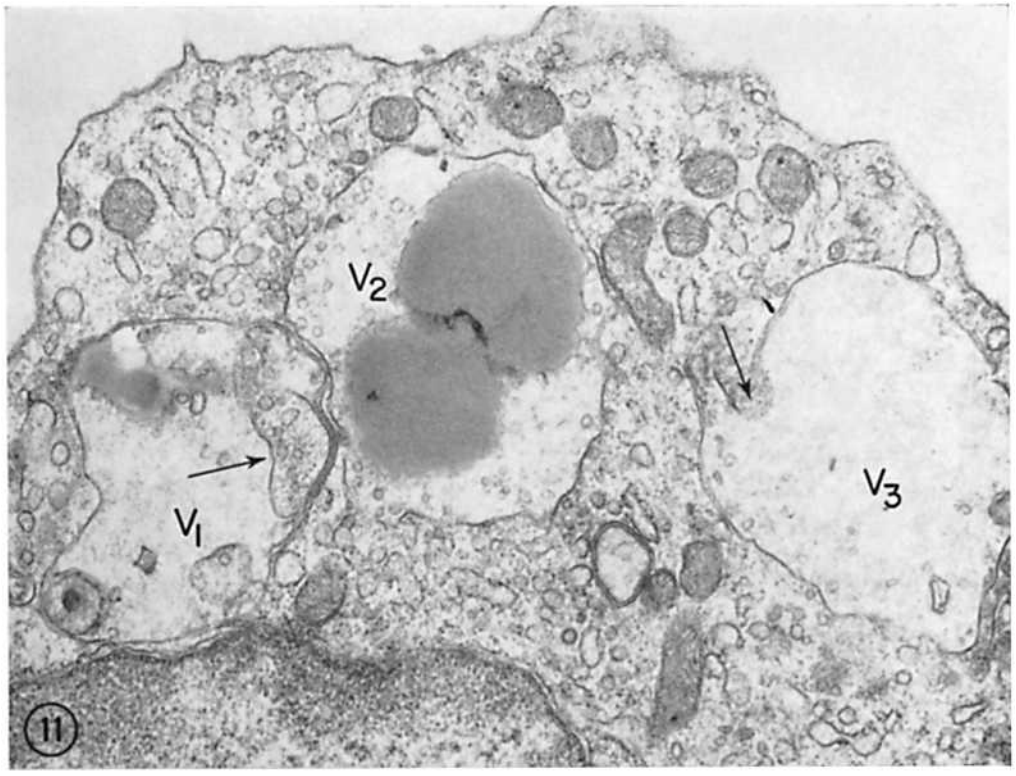

FIGURE 11 Shows three large toxic vacuoles in a cell exposed to chloroquine for 2 hr. V_1 contains a lipid deposit and an apparent membrane-bounded piece of cytoplasm (arrow). Between this cytoplasmic inclusion and the vacuolar membrane is a thin electron-opaque element; this gives a three-layered appearance similar to that of the tubular extensions in Figs. 8 and 9. V_2 contains two large lipid droplets and numerous very small vesicles. V_3 shows an amorphous content and, at the arrow, possible early invagination. × 28,000.

is not established, at least in some instances, and there are differences of opinion as to the means by which cytoplasmic elements are transferred into the vacuole.

The macrophage-chloroquine system has proved to be a particularly convenient model for studies on autophagy. Macrophages are readily available and are easily maintained in culture, and they do not display autophagy normally under these conditions except perhaps for autophagic contributions to pinocytic vacuoles (23). Addition of small concentrations of chloroquine to the culture medium results in rapid development of autophagic vacuoles; the timing and the extent of the vacuolization can be varied by altering the dose of drug. Macrophages spread thinly on glass can be observed and photographed in the living state by phase-contrast microscopy, and thus the formation of autophagic vacuoles can be followed continuously in living cells exposed to chloroquine (see the following report, reference 7). The toxic action of chloroquine appears to be directed against certain cytomembranes and is reversible, at least at low concentrations, so that recovery and fate of the autophagic vacuoles can be studied.

The studies reported here also were facilitated by the newly developed technique of simultaneous fixation with glutaraldehyde and osmium tetroxide and postfixation with uranyl acetate (11). This fixation procedure is much more satisfactory than previous fixative combinations for preservation and definition of macrophage organelles in general, and of vesicular and vacuolar structures of the Golgi region in particular.

The vacuoles which appear in macrophages or in L cells (7) after exposure to chloroquine are properly classed as autophagic, since they contain cytoplasmic fragments or organelles (see Figs. 11

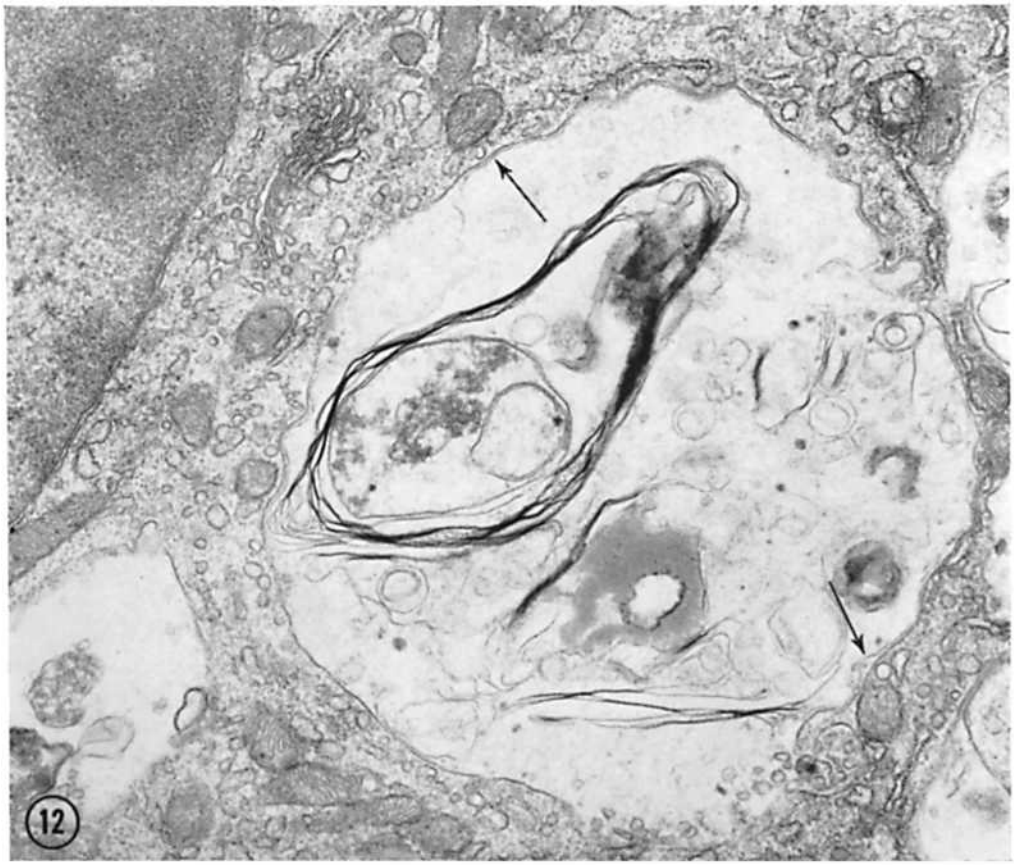

FIGURE 12 A very large (4 μ in diameter), perinuclear autophagic vacuole in a macrophage exposed to chloroquine. The vacuole presents a double membrane image at several places (arrows). It contains amorphous material, lipid, membranous layers, and small vesicles. Nucleus, Golgi cisternae, mitochondria, and ER appear essentially normal. × 25,000.

and 12, or see especially Fig. 7 in the following report). These vacuoles also give positive histochemical reactions for acid phosphatase. The development of autophagic vacuoles may be discussed in relation to two distinct phases of the process: (*a*) the nature of the initial damage and the source of the membrane for the vacuole and (*b*) the mechanism for trapping or transporting cytoplasmic components into the vacuoles and for activating or delivering digestive enzymes. Discussion of the first phase, i.e. the phase of vacuole formation, will be deferred to the following report (7) which presents experimental findings pertinent to this topic.

Proposals made by others to account for incorporation of cytoplasmic structures into the autophagic vacuole include the following: (*a*) envelopment of a focal area of cytoplasm by cisternae of smooth ER and fusion of these cisternae to form a vacuole (2, 14, 24), (*b*) trapping of cytoplasmic elements between two or more preformed vacuoles which then fuse (25), or (*c*) invagination of the membrane of a preformed vacuole with pinching off from the invaginated pouch, a process analogous to phagocytosis at the cell surface (3, 26). In many studies, including the present one, autophagic vacuoles have exhibited in the early stages an apparent double membrane (2, 26–31). A double membrane could arise from encirclement by ER cisternae, or from the fusion, or invagination of preformed vacuoles if the internalized portion is sufficiently large or is so located that its membrane lies adjacent to the limiting vacuolar membrane. Usually late states of autophagic vacuoles show

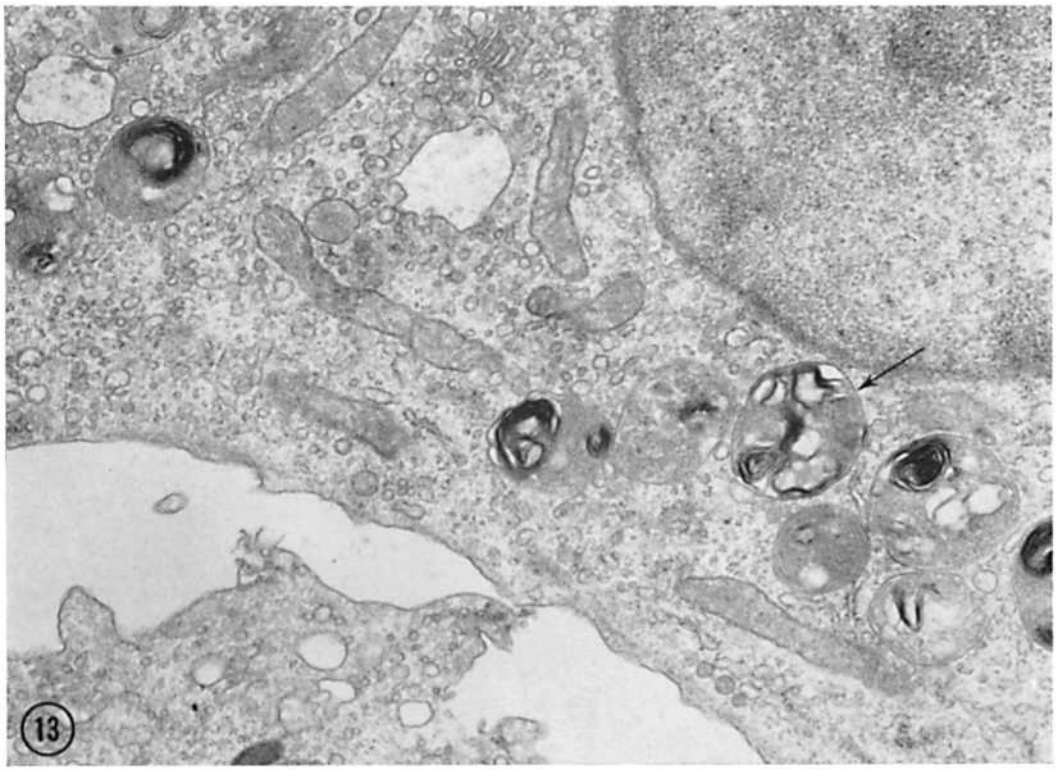

FIGURE 13 The perinuclear cytoplasm of a macrophage exposed to chloroquine for 2 hr to produce marked vacuolization, and then maintained in drug-free medium for 24 hr to allow recovery. There are prominent, large (1–1.5 μ) residual bodies (arrow) containing membrane whorls, dense amorphous material, and clear areas. The cell shows normal nucleus, Golgi complex, pinocytic vacuoles, small vesicles, and mitochondria. Numerous polyribosomes are evident. × 21,000.

only a single limiting membrane; this suggests that the inner membrane has been degraded or relocated. In the present observations on autophagic vacuole formation in macrophages, it seems unlikely that these structures arise by fusion of cisternae of ER, for cisternae of this type are rare in the cytoplasm of this cell in general and are essentially absent from the cytoplasmic region in which the autophagic vacuoles arise. Early vacuoles seen after exposure to chloroquine do not, as a rule, show discernible cytoplasmic organelles. The findings thus indicate first a formation of vacuoles, and then entrapment of cytoplasmic components in these vacuoles by fusion or by invagination. The configuration of many of the early toxic vacuoles (see Figs. 9 and 11) in macrophages exposed to chloroquine strongly suggests invagination and pinching off as the likely mechanism for this entrapment. The autophagic process in other cells under other conditions obviously may be quite different so that no general conclusions about mechanisms are justified.

Many of the electron micrographs in cells exposed to chloroquine show early toxic vacuoles with long, curved tubular extensions, or in some instances apparent tubular connections between vacuoles. In many instances these tubular structures have a prominent electron-opaque, linear central element. Interpretation of the origin and significance of these peculiar structures is uncertain; the central dense structure may represent fusion of the inner of the double membrane leaflets in a portion of a vacuole stretched very thin, or it may result from condensation of nonmembranous material of an undetermined nature.

As previously discussed elsewhere (3), autophagic vacuoles may be of two different types in terms of their origin and development. Speaking

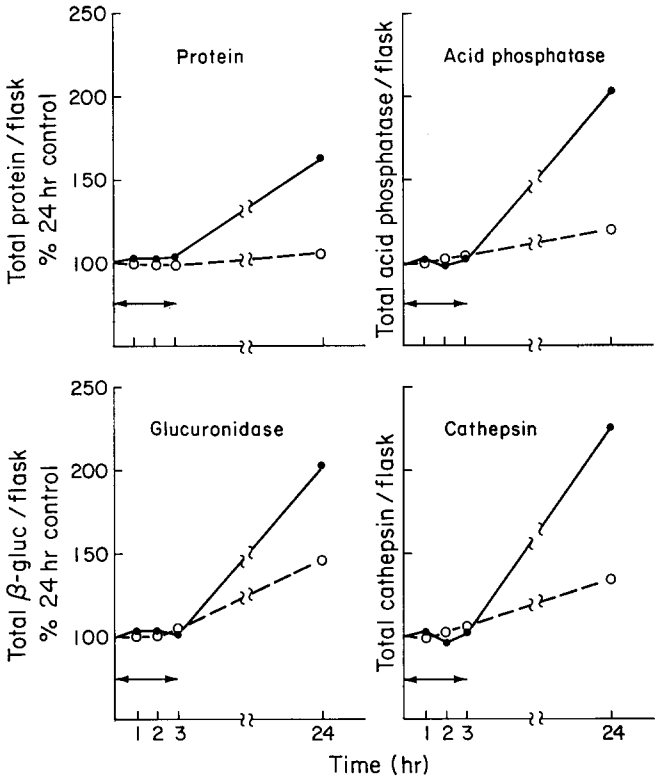

FIGURE 14 Hydrolase accumulation in macrophages recovering from chloroquine. Closed circles, control cells (not exposed to chloroquine); open circles, cells exposed to chloroquine; arrows, period of chloroquine exposure.

teleologically, in one of these types, which might be termed "purposeful," the intial event is focal cytoplasmic damage or degeneration, and the cell responds by incorporating the damaged material in a digestive vacuole to permit its removal and the possible reutilization of some of the degradation products. The other type of autophagy, which might be called "accidental," would involve initially alteration in cellular membrane systems so as somehow to produce vacuoles whose membranes display an abnormal tendency to fuse with themselves and with other organelles. Movement of these vacuoles in the cytoplasm might then result in "ingestion" of certain cytoplasmic structures which the vacuoles happen to encounter, the ingestion being accomplished by entrapment between vacuoles or by invagination as discussed above. Our studies on chloroquine toxicity in macrophages give no evidence of focal cytoplasmic destruction or autolysis as the initial event; rather the picture seems to indicate an initial drug action on membrane systems leading to abnormal vacuole formation and subsequent accidental incorporation of cytoplasmic elements such as lipid and mitochondria into these vacuoles.

As noted above, acid phosphatase activity in macrophages treated with chloroquine is found in large vacuoles rather than in small dense granules (see Fig. 6). This relocation of hydrolase is not accompanied by any change in total hydrolase content of the cells or any detectable release of hydrolases into the medium. At no time in the course of chloroquine effect is focal cytoplasmic degeneration seen. Thus the evidence suggests transfer, probably by fusion, of hydrolases from Golgi vesicles and dense bodies into the autophagic vacuoles without significant release into the cytoplasm and associated cell damage or death.

Recovery of macrophages from chloroquine toxicity was studied simply by removing the drug and observing the evolution of the autophagic vacuoles into residual bodies (telolysosomes) on continued cultivation. The ultrastructure of these residual bodies is similar to that often seen as a result of autophagic or heterophagic digestion, showing as a principal component lamellae of membrane-like elements which probably represent indigestible or slowly digestible lipid materials. Not yet determined is the eventual long term fate of these residual bodies: progressive digestion and

absorption, excretion to the outside in bulk, or persistence unchanged in the cells.

When pinocytosis is stimulated in macrophages in vitro by, for example, increasing the content of serum in the medium, the cells develop large pinocytic vacuoles in the perinuclear region. Golgi vesicles, which likely are the primary lysosomes of this cell, then apparently fuse with the pinosomes to convert them into digestive vacuoles (18, 23). This sequence of events is soon followed by synthesis of large amounts of hydrolases by the cells, so that their digestive enzyme content 24 hr later is much greater than control unstimulated cells (16). It seems reasonable to speculate, as others have done (3), that some step in this sequence, perhaps the discharge and thus depletion of the reserve supply of primary lysosomes, somehow creates a message which directs the cell to manufacture new hydrolases. The macrophages exposed to chloroquine present many morphologic features similar to those seen in cells upon stimulation of pinocytosis, including the development of large perinuclear vacuoles and depletion of the Golgi vesicle primary lysosomes. Yet the chloroquine treated cells do not make large amounts of new enzymes; in fact their total hydrolase content 24 hr later, after apparent recovery from chloroquine toxicity, is less than that of parallel control macrophages. Perhaps the message for new enzyme synthesis arises from some subtle intracellular event which occurs in the case of stimulated pinocytosis but not in the case of chloroquine toxicity, or perhaps the message created in the macrophages exposed to chloroquine is not delivered or acted on because of residual damage due to the drug.

Chloroquine is a stabilizer of isolated liver lysosomes in vitro (32), but in intact macrophages, and probably also in man or animals treated with this drug, it clearly does not act in this manner. The present results emphasize the uncertainties of conclusions about the properties of organelles, based on observations made on isolated cell fractions in an in vitro environment.

Human monocytes in tissue culture (7), human leukocytes in patients receiving chloroquine (4), and pancreatic acinar cells in rats given this drug (5) all show evidence of probable autophagic vacuole formation, and thus it is likely that effects similar to those seen in our macrophage cultures occur in the whole antimal. These effects may conceivably be the basis, at least in part, for some of the toxic complications, e.g. retinal degeneration, of long term chloroquine treatment in man. The mechanisms of antimalarial action of chloroquine, or of its apparent anti-inflammatory action in various chronic diseases of man are unknown, and it is possible that the cytotoxic effects reported here play some role in these actions.

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