AUTOPHAGIC VACUOLES PRODUCED IN VITRO

II. Studies on the Mechanism of Formation of Autophagic Vacuoles Produced by Chloroquine

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ABSTRACT
Continuous phase-contrast observations have been made on macrophages following exposure to chloroquine. The initial abnormality is the appearance in the Golgi region of small vacuoles with an intermediate density between that of pinosomes and granules. Over the course of 1–2 hr these vacuoles grow larger and accumulate amorphous material or lipid. Pinosomes or granules frequently fuse with the toxic vacuoles. Chloroquine derivatives can be seen by fluorescence microscopy; the drug is rapidly taken up by macrophages and localized in small foci in the Golgi region. Chloroquine continues to produce vacuoles when pinocytosis is suppressed. Electron microscopic studies of chloroquine effects on macrophages preincubated with colloidal gold to label predominately pinosomes or granules suggest that toxic vacuoles can arise from unlabeled organelles. Later vacuoles regularly acquire gold label, apparently by fusion, from both granules and pinosomes. L cells also develop autophagic vacuoles after exposure to chloroquine. Smooth endoplasmic reticulum apparently is involved early in the autophagic process in these cells. Information now available suggests an initial action of chloroquine on Golgi or smooth endoplasmic reticulum vesicles, and on granules, with alterations in their membranes leading to fusion with one another and with pinosomes.

INTRODUCTION
As reported in the preceding paper (1), chloroquine induces formation of autophagic vacuoles in vitro in mouse macrophages. We present here results of experiments designed to shed light on the primary intracellular site of chloroquine toxicity and on the mechanism by which this toxicity leads to abnormal vacuole formation. These experiments include study of the effects of chloroquine on another type of tissue culture cell, the L-strain fibroblast.

MATERIAL AND METHODS
Cell Preparations
Mouse peritoneal macrophages were maintained in a tissue-culture system as described previously (2). Unless otherwise stated, culture was in medium 199 with 20% newborn calf serum and 200 U/ml penicillin.

Strain-L (Earle) cells were also studied. These cells were grown in 10% fetal calf serum in Eagle's minimum essential medium, with subculture every 4 days. Cells grown as a monolayer for 3 days in Leighton tubes with flying cover slips or in T flasks were placed in fresh medium containing chloroquine for observations of toxicity.

Cinemicrophotographic Observations
Cell cultures on cover slips in Leighton tubes were washed and inverted over a thin rim of paraffin on glass slides so that chambers were made approximately the depth of a No. 1 cover slip. The chambers were filled with fresh medium with or without
chloroquine and sealed with paraffin. Care was taken so that the specimens were not allowed to dry during mounting. The slides were incubated at 38°C and observed by oil-immersion, phase-contrast microscopy for up to 2 hr. Motion pictures of control and drug exposed cells were also recorded by using techniques described previously (3). The movies were taken at five frames per second. Projection of prints at 24 frames per second thus allowed repeated study of normal and chloroquine-treated macrophages with action speeded up approximately five times.

Fluorescence Microscopy

Chloroquine is detectable by fluorescence in cells or tissues (4), so that it was possible to observe directly its localization in macrophages. Concentrations of chloroquine of 100 µg/ml were required to produce fluorescence in treated cells. These observations were made in slide chambers as described above by employing a dark-field fluorescent system with BG 12 exciter and OG-5 absorption filters.

Metabolic Inhibitors

The following compounds were used (5): 2,4-dinitrophenol (1 X 10⁻⁴ M, Eastman Organic Chemicals, Rochester, N. Y.), iodoacetate (5 X 10⁻⁴ M, Amend Drug and Chemical Co., New York), parafluorophenylalanine (1.0 mg/ml, Nutritional Biochemical Corporation, Cleveland, O.); sodium fluoride (1 X 10⁻³ M, Merck & Co., Inc., New York); puromycin 1 µg/ml (Nutritional Biochemical Corporation). Fresh solutions of inhibitors in culture medium were adjusted, when necessary, to pH 7.3 with NaOH or HCl. The inhibitors were added to 24-hr-old macrophage cultures 1 hr prior to addition of chloroquine 30 µg/ml; evaluation was made 2 hr later.

Pinosome Counts

Cover slip cultures fixed in glutaraldehyde and mounted in water were observed under oil-immersion phase contrast. Numbers of clear pinocytic vesicles in the pseudopods of 100 cells were counted and expressed as pinosomes per 50 cells.

Variation in Tissue Culture Conditions

Some macrophages were cultured for 48 hr in medium containing low (1-5%) concentration of newborn calf serum for production of cells of unusually low dense granule content (6). Addition of chloroquine then allowed assessment of the relationship between granule content and toxic action of this drug.

Labeling of Macrophage Organelles with Colloidal Gold

Colloidal gold (2.5 mg/ml, Abbott Laboratories, Chicago, Ill.) was dialyzed against phosphate-buffered saline pH 7.4 and was added 1:5 or 1:10 to the macrophage cultures. As established in previous studies (7), gold particles enter the cell in pinocytic vesicles and are rapidly concentrated within centrally located pinocytic vacuoles. After incubation with gold in the medium for several hours, gold particles are seen primarily in dense bodies of macrophages. Addition of colloidal gold simultaneously with chloroquine thus allowed observations on morphology of early toxic events in cells with labeled pinosomes. Preincubation of macrophages with gold for 6 hr followed by washing and addition of fresh medium containing chloroquine allowed similar study of cells with gold label primarily in dense bodies.

Fixation and Processing for Electron Microscopy

Fixation and processing for electron microscopy were according to methods described in the accompanying report (1).

RESULTS

Phase-Contrast Microscopy of Macrophages Exposed to Chloroquine

Macrophages growing on cover slips were observed by phase-contrast microscopy and photographed under “fast speed” motion picture conditions in a thin slide chamber. In well-spread cells it was possible to observe clearly various activities (surface membrane ruffling, pinosome streaming, and the like) and organelles (perinuclear Golgi region and pinocytic vacuoles, transitional zone dense granules, lipid droplets, peripheral mitochondria). Control cells not exposed to drug did not change in their activities or morphology during a 1 hr period of observation in the slide chambers. Macrophages exposed to 50 µg/ml of chloroquine under these conditions exhibited initially increased surface-membrane ruffling and some retraction of their pseudopods. 10-15 min after addition of drug numerous tiny (initially of a size at the limit of resolution by light microscopy, i.e. about 200 mp) vesicles appeared in the Golgi region of the cytoplasm. These tiny vesicles appeared to be somewhat more dense than did nearby pinosomes, but they were much more lucent than the cytoplasmic
granules. In the course of the next 10 min or so, the toxic vesicles gradually increased in size and in number, often to occupy most or all of the nuclear hof region. The motion-picture technique was most valuable for studying this very early phase as it was possible to view the changes somewhat speeded up, to rerun the action, or to reverse it at will. Many instances were recorded of apparent fusion between toxic vacuoles and pinosomes or granules. Markedly vacuolated cells continued to exhibit motility and slow or occasionally rapid translocation of the large vacuoles was associated with the motility. Some of the toxic vacuoles appeared to be partially collapsed or "floppy" and irregular in outline. An hour after chloroquine exposure most cells were severely vacuolated, and amorphous, dense material or refractile lipid droplets were often seen in Brownian movement within vacuoles.

The Intracellular Localization of Chloroquine as Observed by Fluorescence Microscopy

Since chloroquine or a derivative compound fluoresces under ultraviolet illumination (4, 19), it was possible to study its localization in macrophages by observing exposed cells in slide culture chambers by fluorescence microscopy. A dose of 100 µg/ml or more of chloroquine was required for adequate visualization of fluorescent drug or its derivative within cells. As rapidly as slide chambers could be examined under the microscope (3-5 min), a fluorescent deposit in the form of fine dust or droplets was seen distributed in the Golgi region. After 10-15 min these droplets had changed in appearance into fluorescent vesicles or vacuoles, which, in size and location, were identical with the toxic vacuoles described above. In this early phase, fluorescent material was never seen associated with lipid or other organelles in the peripheral cytoplasm.

Relationship Between Pinocytosis and Autophagic Vacuoles Induced by Chloroquine

When pinocytosis is stimulated in macrophages by increasing the concentration of serum in the medium or by adding certain polyanions (6, 8, 9), the cells develop over the course of a few hours large, clear vacuoles in the perinuclear region. The general appearance of macrophages vacuolated as a result of increased pinocytosis is similar to that seen after exposure to chloroquine, and thus we investigated the possibility that chloroquine was acting, at least in part, by stimulating pinocytic activity of the cells. As is shown in Fig. 1, macrophage pseudopods contained somewhat increased numbers of pinocytic vesicles 15 min after exposure to chloroquine, but by 30 min the pinoosome count was no greater than that of control cells; thereafter pinocytosis was in fact somewhat suppressed in cells exposed to chloroquine. Pinocytosis remained suppressed to approximately 50% of control values for 24 hr after the cells were washed and placed in drug free medium. Quantitation of pinocytic activity by these methods is not precise, so that no firm conclusions were possible; it seemed quite unlikely, however, that the brief increase in pinocytic activity could be responsible for the extensive vacuole formation in macrophages exposed to chloroquine.

Several inhibitors of metabolism or of protein synthesis have been shown to suppress or block pinocytosis in macrophages (5). Addition to the macrophage cultures of 2,4-dinitrophenol (1 × 10⁻⁴ M), iodoacetate (5 × 10⁻⁴ M), parafluorophenylalanine (1.0 mg/ml), sodium fluoride (1 × 10⁻³ M), or puromycin (1 µg/ml) 1 hr prior to addition of 30 µg/ml of chloroquine did not block the development of vacuoles in the cells. Pinoosome counts established that these agents did suppress pinocytosis in parallel cells not treated with chloroquine.

One additional experimental approach was used for the study of the relationship between pinocytosis and the toxic vacuoles produced by chloroquine. Colloidal gold added to the medium serves as a convenient marker for pinocytic vesicles and
The perinuclear region of a macrophage fixed 30 min after simultaneous exposure to chloroquine and colloidal gold (see text). Pinocytic vacuoles (PV) exhibit characteristic irregular shape, internal vesicles, and generally electron-transparent content and a high concentration of gold particles. Small vesicles and amorphous material as present in these vacuoles may also be seen in pinocytic vacuoles of macrophages not exposed to chloroquine (see reference 23 for a discussion of the mechanism of transport of cytoplasmic residues into pinosomes). A large autophagic vacuole (AV) shows typical tubular extensions and a heterogeneous content of moderately electron-opaque amorphous material, membranous elements and lipid. This autophagic vacuole contains very few gold particles; this indicates that it did not arise from pinocytic vesicles or vacuoles. X 30,000.

Vacuoles (7). Addition of colloidal gold and chloroquine at the same time thus allowed a comparison to be made at intervals thereafter of the gold content of pinocytic and of toxic vacuoles. After 30 min (see Fig. 2) the pinocytic vacuoles were heavily labeled with gold particles, whereas the toxic vacuoles (typified by their heterogeneous content, invaginations, and protrusions) were labeled lightly or not at all. 3 hr after simultaneous exposure to gold and chloroquine, the autophagic vacuoles commonly showed moderately intense gold labeling. The results thus suggested that the autophagic vacuoles did not arise primarily from pinocytic structures but that fusion or some mechanism of interchange between these organelles came into play with passage of time.

Relationship Between Granules and Autophagic Vacuoles Induced by Chloroquine

An obvious possible source of membrane and of hydrolases for the autophagic vacuoles was the population of cytoplasmic dense bodies or granules in macrophages maintained under these conditions. Perhaps chloroquine was acting on these lysosomal structures to bring about fusion with one another or with other organelles leading to vacuole formation.

The granule content of macrophages varies widely depending on age of the culture and composition of the medium (6). Macrophages maintained in a medium of low serum concentration had only a few small granules, but on exposure to chloroquine they formed toxic vacuoles in a
FIGURE 8 A portion of a macrophage which had been preincubated with colloidal gold for 6 hr to label the granules, and then transferred to medium containing chloroquine but no gold. After 30 min the early autophagic vacuoles (AV), identified by their shape and moderately electron-transparent content, contain only a few gold particles, whereas granules (G) nearby and in other parts of the cell are heavily labeled with gold and exhibit a dense matrix. A structure of indeterminate morphology (arrow) has features between those usually seen in granules and toxic vacuoles and is heavily labeled with gold; it may thus have been formed by fusion of a granule with a toxic vacuole. X 30,000.

manner not significantly different from that seen with heavily granulated macrophages which had been cultured in a high serum medium. Fresh mouse peritoneal macrophages or human blood monocytes are relatively immature cells with only small numbers of dense bodies. Chloroquine also induced extensive vacuole formation in these cells; this finding eliminated cultivation in vitro and preexisting large granule content as prerequisites for the toxic effect.

In macrophages cultivated in a suitable medium, the pinocytic vacuoles acquire hydrolases and in the course of several hours mature into dense bodies (6, 7, 10). If colloidal gold is present during this process, the cells show heavy deposits of gold particles in their granules. Macrophages thus labeled were washed and placed in medium containing chloroquine. The toxic vacuoles appearing 15-30 min later contained gold (Fig. 3), but the concentration of gold in the irregularly shaped, large autophagic vacuoles was generally lower than that in typical dense bodies.

The results thus suggest that preformed granules are not the primary site of chloroquine action and autophagic vacuole genesis, but they participate in the process early and deliver their contents to the vacuole, probably by fusing with it.

Effects of Chloroquine on L-Strain Fibroblasts

Studies were made to establish the toxic effects, if any, of chloroquine on a cell type other than the macrophage. The Earle strain of L cell was selected for this purpose, as L cells differ in structure and function from macrophages in many regards. L cells multiply rapidly in culture, for instance, whereas macrophages do not undergo cell division in vitro. L cells display much less phagocytic and pinocytic activity, and they contain fewer dense bodies than do macrophages.

The ultrastructure of L cells cultured on glass for 72 hr and processed by mixed fixation is illustrated in Fig. 4. Nuclei were very large, showing dispersed chromatin and prominent nucleoli. Cytoplasm in the nuclear hof area showed numerous small vesicles, well-developed complexes of Golgi cisternae, and a few large clear vacuoles. Peripheral cytoplasm of L cells contained large, irregularly shaped mitochondria, numerous polyribosomes, a few multivesicular bodies, and a few
FIGURE 4  An L-strain mouse fibroblast cultured on glass for 3 days and processed by mixed fixation. The nucleus (N) exhibits a dispersed chromatin pattern. Golgi (GO) vesicles and cisternae, and a few large clear vacuoles (CV) are seen in the nuclear hof area. Note the large number of very small vesicles in the centrosomal region. Large mitochondria (M), many with irregular shapes, are widely distributed in the cytoplasm. In some places (arrow) rough ER is seen in continuity with dilated smooth ER; the dilated smooth ER often contains a small, round, very electron-opaque body. In cross-sections (double-stemmed arrows) of transitional ER these dense bodies may appear to be in vesicles. The L-cell cytoplasm exhibits many polyribosomes and amorphous moderately electron-opaque material. A few multivesicular bodies (MVB) may be seen; dense bodies or granules are rare or absent. X 24,000.
strips of partly rough, partly smooth endoplasmic reticulum (ER). The smooth ER was dilated, and frequently contained a small, round (600 μm), very electron-opaque body, possibly of viral nature. These dense bodies appeared to be in small vesicles or vacuoles in transverse sections of the dilated smooth ER; they were distributed throughout the cytoplasm but were especially numerous in the transitional zone between the Golgi region and the general cytoplasm.

L cells exposed to 30 μg/ml chloroquine developed large cytoplasmic vacuoles generally resembling those seen in macrophages. Individual L cells seemed to vary markedly in their susceptibility to chloroquine toxicity, in contrast to the fairly uniform response of macrophages. A small percentage of exposed L cells showed extensive autophagic vacuole formation within 15 min, and after 3 hr of incubation with chloroquine, approximately 75% of the cells showed changes ranging from mild to severe, whereas the remaining 25% still appeared normal.

Early toxic changes in L cells exposed to chloroquine appeared under the phase-contrast microscope as clear, round vacuoles about the Golgi region. Electron microscopy of early toxic vacuoles (Fig. 5) showed a distinct limiting membrane and a content of amorphous material, tiny vesicles, and small dense bodies resembling those seen in dilated terminal smooth ER of the normal cell (see above). The vacuoles in other affected cells were larger and highly irregular in shape (Fig. 6). More severe chloroquine toxicity in L cells took the form of large autophagic vacuoles which varied widely in their content (Fig. 7). In one vacuole a well-preserved mitochondrion was demonstrated; other vacuoles contained electron-opaque amorphous deposits, fine granular material, or stacks of membranes in an onionskin pattern. A double limiting membrane with intervening clear space was seen about some of the toxic vacuoles. The cytoplasm of these severely vacuolated L cells showed apparently normal polyribosomes, short strips of ER, and normal elongated mitochondria. Golgi cisternae were sometimes dilated.

**DISCUSSION**

Macrophages exposed to 30 μg/ml of chloroquine develop, within an hour, many large vacuoles in the perinuclear region. Obviously the total surface
FIGURE 6  Illustrates a slightly more advanced state of toxic vacuolization of L cells exposed to chloroquine. Large vacuoles are grouped about the Golgi region in a manner quite similar to that seen in affected macrophages (compare to Fig. 10 in accompanying paper, reference 1). The vacuoles are generally electron transparent and contain amorphous material and small vesicles. Nucleus, Golgi elements, and peripheral cytoplasmic elements are essentially normal. X 20,000.

area of membrane around these toxic vacuoles is considerable. Many of the studies reported here were done to shed light on origin of this vacuolar membrane. The possibility that the membrane is newly synthesized in response to chloroquine exposure seems highly unlikely, in view of the rapidity with which vacuoles appear and in view of the fact that various inhibitors of metabolism and of protein synthesis do not block vacuole formation. If it is assumed then that the vacuolar membrane arises from preexisting cytmembranes, the structures which are possible contributors include the following: (a) Golgi cisternae, (b) perinuclear vesicles or vacuoles deriving from the Golgi complex or smooth ER, (c) pinocytic vacuoles, (d) dense bodies or granules, (e) tubules or cisternae of ER, and (f) cell surface membrane via pinocytic activity. The observations made here seem to eliminate all of these possibilities except (b) the smooth vesicles in the Golgi region and possibly (d) the dense granules. As is pointed out in the accompanying paper (1), Golgi cisternae appear to be unchanged during the early stages of chloroquine toxicity; the Golgi abnormalities seen in some macrophages after several hours of exposure to chloroquine involve dilatation of the
FIGURE 7 Shows various other forms of autophagic vacuoles in an L cell exposed to chloroquine for 2 hr. Some of the vacuoles appear to have a distinct double membrane (double-stemmed arrows). One vacuole contains a well-preserved mitochondrion (arrow) in addition to amorphous material and membranous matter. The content of other vacuoles varies widely, including membranous lamellae in an onionskin pattern, very electron-opaque structureless masses, and areas which appear identical with cytoplasmic matrix. × 20,000.

cisternae, but there is no significant disappearance of the Golgi stacks. The experiments in which pinocytic vacuoles were labeled with colloidal gold show that the early chloroquine-toxic vacuoles do not arise primarily from these structures, for the toxic vacuoles in this circumstance contained little or no gold. Origin of the vacuoles entirely from cytoplasmic dense bodies also seems unlikely from the gold-labeling observations and from the fact that extensive vacuolization appears in cells having but few dense bodies, e.g. macrophages cultured in low serum medium, monocytes, L cells. The macrophages studied exhibit only a few short strips of ER, so that origin of the toxic vacuoles from this potential membrane source is quite unlikely. Finally, pinocytic activity of the cells can be varied by varying the serum concentration in the medium or can be suppressed or blocked by inhibitors (puromycin, amino acid analogues, dinitrophenol, etc), without markedly affecting the vacuolization which appears following chloroquine exposure. Although the evidence thus indicates that the early toxic vacuoles can and probably usually do arise from structures other than pinosomes, granules, and ER, both phase microscopy and ultrastructural observations show that these organelles do contribute their content to the vacuoles with passage of time, probably by fusion.

Incrimination of vesicles of the Golgi region as the likely source of vacuolar membrane fits with fluorescence microscopy showing that the drug is rapidly taken up by macrophages and localized in the Golgi area in tiny foci, and with light and electron microscopic observations showing that the vacuoles make their appearance in this area. Both phase and electron microscopy show the density of the content of the early toxic vacuoles to be somewhat greater than that of pinocytic vacuoles or vesicles, and considerably less than that of the dense granules. We think it likely, then, that chloroquine or its derivative is somehow concentrated in the Golgi vesicles and alters their membranes, with
a resulting tendency of these vesicles to fuse with one another, and with other membrane limited cytoplasmic organelles. Perhaps granules are similarly directly affected.

Previous studies have established the pathway of hydrolase synthesis in stimulated macrophages (7). The enzymes are made in the rough ER, then are transported to the Golgi region, and finally appear in dense bodies. Almost certainly the vesicular elements in and around the Golgi region contain hydrolases, and if we are correct in concluding that these vesicles fuse with one another to form the early vacuoles, the vacuoles would be endowed with hydrolases from the start, and they would likely acquire additional digestive enzymes by fusing with dense bodies. Fusion of the toxic vacuoles with pinocytic vacuoles would then convert them into heterophagic vacuoles (containing extracellular material and digestive enzymes). Incorporation of cytoplasmic components into the vacuoles by invagination or trapping (see Discussion in accompanying paper, reference 1) would in turn convert them into autophagic vacuoles as well.

Our conclusions on origins of the limiting membranes of autophagic vacuoles agree reasonably well with those drawn by others in different systems (discussed in references 11–13). Novikoff and his colleagues have proposed that autophagic vacuoles in nerve and liver cells arise from smooth ER, in the Golgi zone (14, 15). The macrophages and L cells studied here show a highly developed Golgi complex, but contain only a few ER elements, so that it seems likely that most of the vesicles in the nuclear hof of these cells are derived from the Golgi rather than from smooth ER. In any event, both Golgi and smooth ER vesicles represent protolysosomes or primary lysosomes of these cells, and it is difficult or impossible to distinguish between them by techniques currently available.

The mechanism of chloroquine uptake by cells is unknown. Chloroquine or its fluorescent derivative enters the cells within minutes after its addition to the medium. That the rapid uptake and toxic effects are not blocked by various metabolic inhibitors suggests that chloroquine is taken into the cells by some means other than pinocytosis or energy-dependent active transport. The only condition found to inhibit chloroquine uptake or action is low temperature. Cells held at 4°C in the presence of 30 μg/ml chloroquine for 2 hr and then washed in the cold prior to incubation at 38°C do not develop vesicular vacuoles. There are similarities between chloroquine and at least two other agents, neutral red and acridine orange (16–19), each of which is approximately the same molecular weight, is rapidly taken up by certain cells, is apparently concentrated in lysosomes by mechanisms unknown, exhibits or develops fluorescence or photosensitizing reactivity, and produces vacuolization or other cytotoxic effects when added to cells in sufficiently high concentration.

The possible membrane-damaging action of chloroquine is at present unexplained. In bacteria this drug combines with deoxyribonucleic acid and blocks protein synthesis (20–22), but no evidence for a similar action in mammalian cells is available. The mechanism of the antimalarial effects of chloroquine are unknown.

The studies of chloroquine toxicity on L cells establishes that the drug produces autophagic vacuoles in a cell type different from the macrophage. The presence of a small dense body in the smooth ER makes it possible to follow this marker in the L cells exposed to chloroquine, and thus to show that the smooth ER vesicles and cisternae also participate in formation of, or contribute their contents to, the toxic vacuoles. Finally, the cell-to-cell variability in time of toxic response in the L cells, in contrast to the more or less uniform responsiveness of the macrophages, leads us to speculate that dividing cells may be susceptible to chloroquine intoxication only during certain phases of their growth cycle. Further study of this possibility might shed additional light on the mechanisms involved in the toxic response.

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