EFFECTS OF CONCANAVALIN A ON MOUSE PERITONEAL MACROPHAGES

I. Stimulation of Endocytic Activity and Inhibition of Phago-Lysosome Formation*

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Concanavalin A (Con A),[1] is a hemagglutinin originally identified in extracts of the jack bean *Canavalia ensiformis* by Sumner and Howell in 1936 (29). It is a protein composed of four identical 25,500 dalton subunits (8), each containing a saccharide binding site which can accept D-glucopyranose or D-mannopyranose rings (14). There are extensive descriptions of the effects of Con A on lymphocytes of various species (for review, see 7), but the literature is more modest regarding its effect on mononuclear phagocytes. Allen, Cook, and Poole (1), and Mallucci (21) have demonstrated that Con A can bind to the surfaces of mouse peritoneal macrophages, and Lutton (20) has reported that the binding of ^{125}I-labeled Con A to mouse macrophages is reduced 35% after extensive phagocytosis of latex or titanium dioxide particles. Smith and Goldman (27) have reported that multinucleated giant cells develop when human colostral macrophages are exposed to Con A. This effect can be inhibited by α-methyl-D-mannoside or α-methyl-D-glucoside administered simultaneously with the Con A.

We have observed a striking effect of Con A on the endocytic activity of cultured mouse peritoneal macrophages, and on the fate of the pinosomes generated under these conditions. In this paper, we describe the dramatic vacuolization which develops in mouse peritoneal macrophages after their exposure to Con A, the origin of these vacuoles, and their fate within the cell cytoplasm as determined by light and electron microscopy.

Materials and Methods

Macrophages. Resident macrophages were washed from the peritoneal cavity of NCS mice of either sex, weighing 25–30 g, in 2 ml of PBS without divalent cations, pH 7.4 (PD) containing 250 U of

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; Con A, concanavalin A; 199-FCS, Medium 199 supplemented with 20% fetal calf serum; HRP, horseradish peroxidase; αMM, α-methyl mannoside; PBS, phosphate-buffered saline with divalent cations, pH 7.4; PD, PBS without divalent cations, pH 7.4.

heparin (Bio-Heprin, Ries Biologicals, Los Angeles, Calif.) per milliliter. The cells were washed once and resuspended in medium 199 supplemented with 20% fetal calf serum (199-FCS) at a concentration of 2×10^6 cells/ml. In various experiments, the following volumes of cell suspensions were used: 1.0 ml for 35-mm plastic disposable tissue culture dishes (Nunc, Roskilde, Denmark); 3.0 ml for 60-mm plastic disposable tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.); 0.5 ml for 22-mm glass cover slips (Corning Glass Works, Corning, N. Y.), and 0.15–0.2 ml for 18-mm glass cover slips (Corning Glass Works). The cells were incubated at 37°C in 5% CO_2 for several hours, washed to remove nonadherent cells, and then reincubated in fresh 199-FCS for 48 h before their use.

Lectins. Concanavalin A, crystallized three times and lyophilized, was purchased from Miles Laboratories, Kankakee, Ill., and stored in a desiccator as a powder. Concentrated solutions in phosphate-buffered saline (PBS) were prepared at least weekly and kept at -20°C until used. Phytohemagglutinin prepared from *Phaseolus vulgaris* seeds (purified phytohemagglutinin) was purchased from Burroughs-Wellcome Co., Research Triangle Park, N. C.

Phase-Contrast Microscopy. Cover slip preparations were washed and fixed in 2.5% glutaraldehyde in PBS for 15 min either at room temperature or in the cold. The cover slips were then rinsed in distilled water, inverted over glass microscope slides, and sealed over a drop of distilled water with melted paraffin. Cells were examined with a Zeiss microscope equipped with phase-contrast optics (Carl Zeiss, Inc., New York). Photographs were taken with a Zeiss Ultraphot II on Kodak 4×5-in Ektapan film (Eastman Kodak Co., Rochester, N. Y.).

Enumeration of Cytoplasmic Vesicles. Fixed cover slip preparations were examined by phase-contrast microscopy, at a magnification of 1,000. Generally, over 100 consecutive cells were examined, and phase-lucent vesicles larger than 2 μm in diameter were counted in duplicate preparations, and the results averaged.

Electron Microscopy. Cells were prepared for electron microscopy using the method of Hirsch and Fedorko (17). Monolayers were washed several times in serum-free medium, and in 0.9% NaCl, and then fixed on ice for 30 min in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After this, the cells were washed, scraped, pelleted, and postfixed for 30–75 min on ice in a mixture of one part 2.5% glutaraldehyde in cacodylate buffer; two parts 1% osmic acid in cacodylate buffer freshly prepared immediately before use. The cell pellets were then washed, and resuspended in 0.5% magnesium uranyl acetate in 0.9% NaCl for 30 min at room temperature, or overnight in the cold. They were then rewashed, embedded in 2% Nobel agar (Difco Laboratories, Detroit, Mich.), dehydrated in graded alcohols, and then in propylene oxide, and finally embedded in Epon. Thin sections were cut on a Porter-Blum microtome (Ivan Sorvall, Inc., Newtown, Conn.), mounted on Formvar-coated carbonized grids and examined in a Siemens-Elmiskop I electron microscope (Siemens Corp., Iselin, N. J.) with or without the aid of alcoholic uranyl acetate and lead citrate. In some cases, monolayers were fixed and embedded in situ. Epon-filled plastic capsules were inverted over the monolayers, hardened, and then snapped away from the plastic dish. The cells adhering to the lower surface of the capsule were then sectioned in the usual way and examined as above.

Acid Phosphatase Histochemistry. Acid phosphatase was localized using β-glycerophosphate as substrate, and lead nitrate as the capture reagent (10). For light microscopy, cover slips were rinsed in PBS, and fixed in 1.25% glutaraldehyde in PBS for 15 min at 4°C. The cells were then incubated on ice in 0.88 M sucrose for 15 min, and then exposed for 30 min at 37°C to a solution of 0.01 M sodium β-glycerophosphate and 3.6 mM lead nitrate in 0.05 M acetate buffer, pH 5.0, which was freshly prepared and prewarmed and filtered immediately before used. The cells were then washed once, exposed to 1% acetic acid for 1 min, and briefly exposed to ammonium sulfide vapor before being rinsed and mounted.

Preparations for electron microscopy were treated exactly as described by Jones and Hirsch (18). Cells were fixed in 2.5% glutaraldehyde in cacodylate buffer for 30 min on ice, treated with 0.88 M sucrose for 15 min on ice, and then exposed to substrate for 30 min at 37°C. They were then extensively washed in 5% sucrose in cacodylate buffer, scraped, and processed in a mixture of glutaraldehyde and osmic acid, and then in uranyl acetate before dehydration and embedding.

Horseradish Peroxidase Histochemistry. For either light or electron microscopy, cells were fixed in glutaraldehyde and then stained by the technique of Graham and Karnovsky (16) using 15 mg diaminobenzidine dissolved in 30 ml of Tris 0.05 M, pH 7.6 to which 0.1 ml of 3% H_2O_2 was added immediately before use. Cells were stained for 5–15 min and then processed for microscopy.

Adenosine Triphosphatase Histochemistry. Cells were treated according to the technique of Gordon and Cohn (15). Monolayers were fixed in 0.6-1.25% glutaraldehyde in PD for 10 min on ice, washed, and then incubated for 30 min at 37°C in a solution containing 5 mM ATP, 5 mM MgSO$_4$, 100 mM NaCl, 30 mM KCl, 2.4 mM Pb(NO$_3$)$_2$, and 80 mM Tris-Maleate, adjusted to pH 7.2. Cells were then rinsed and exposed sequentially to 1% acetic acid and dilute ammonium sulfide before mounting.

Enzyme Assays. Acid phosphatase in 0.1% Triton X-100 cell lysates was assayed as previously described (3) using sodium α-napthol acid phosphate (Sigma Chemical Co., St. Louis, Mo.) as substrate, in 0.1 M acetate buffer at pH 5.0. Color was developed with Fast Red ITR (diazotate-N,N'-diethyl-4-methoxymetanilamide, practical grade, Sigma Chemical Co.), at pH 8.0, and the absorbance at 545 nm was measured and compared with a standard curve prepared with α-naphthol reacted with the coupling reagent.

5'-nucleotidase was assayed by the method of Avruch and Wallach (2), using adenosine-2-[³H]-5' monophosphate, ammonium salt (Amersham/Searle Corp., Arlington Heights, Ill.) as substrate. The reaction was carried out at pH 9.0, in the presence of magnesium. Apparent enzyme activity in macrophage lysates was decreased 6% by the addition of β-glycerophosphate (10 mM). Protein was assayed by the Lowry method (19) in a concentration of Triton X-100 of 0.05% or less.

Electron Microscope Markers. Thorotrast (Fellows-Testagar, Detroit, Mich.), was added directly to cell cultures at a 1:40 dilution (11). Colloidal gold (2.5 mg/ml), Abbot Laboratories, North Chicago, Ill.) was dialyzed several times against medium-199 and added as a 1:5-1:10 dilution to cell cultures (12).

Phagocytosis of Immunoglobulin-Coated SRBC. Sheep red blood cells (SRBC) in Alsever's solution (Microbiological Associates, Bethesda, Md., or Animal Blood Center, Syracuse, N. Y.) were washed and suspended in PD to a dilution of 5%. A portion of these cells was incubated for 30 min at 37°C with an IgG rabbit antisheep red blood cell antibody at a subagglutinating dilution, in the absence of hemolytic complement. The red cells were then washed several times in PD, and added at a concentration of 5 × 10⁷ cells/ml to macrophage cultures and incubated for 15-60 min. Cover slips were removed from culture, washed, fixed in glutaraldehyde, and examined by phase microscopy. Red cells could be identified as being unassociated with, attached to, or interiorized by macrophages. 100 or more macrophages in duplicate preparations were scored for attachment or ingestion, and the results averaged, and compared with macrophages similarly treated with unopsonized SRBC.

Phagocytosis of Latex Particles. Latex particles (1.099 μm diamter, Dow Chemical Corp., Midland, Mich.) were washed several times in serum-free medium, suspended at 10 times their original volume in medium, sterilized with a UV lamp, and stored refrigerated until use. For most experiments, the diluted latex was added to an equal volume of 199-FCS before being incubated with cell monolayers.

Results

When macrophages maintained in culture for 48 h are exposed to Con A at a concentration of 4-100 μg/ml, numerous large, phase-lucent vesicles develop in the cell cytoplasm (Table I and Fig. 1). The effect is rapid, with some vesicles readily apparent after 15 min of exposure. The number of vesicles increases linearly with time for at least 60 min when the cells are maintained at 37°C (Fig. 2). No vesicles are formed at 4°C, but, exposure of the cells for 10 min at 4°C, followed by thorough washing, and incubation at 37°C demonstrates the full effect (Fig. 3). No effect is seen when the cells are exposed to Con A in the presence of α-methyl mannoside (αMM) at a concentration of 100 mM. The phenomenon is not dependent on any serum components, and may be seen with serum-free medium, or with PBS.

The length of time during which the cells were cultured was irrelevant to their response to Con A, and similar effects were seen using freshly harvested

TABLE I

Development of Cytoplasmic Vesicles in Mouse Peritoneal Macrophages Exposed to Con A for 60 min at 37°C in 199-FCS

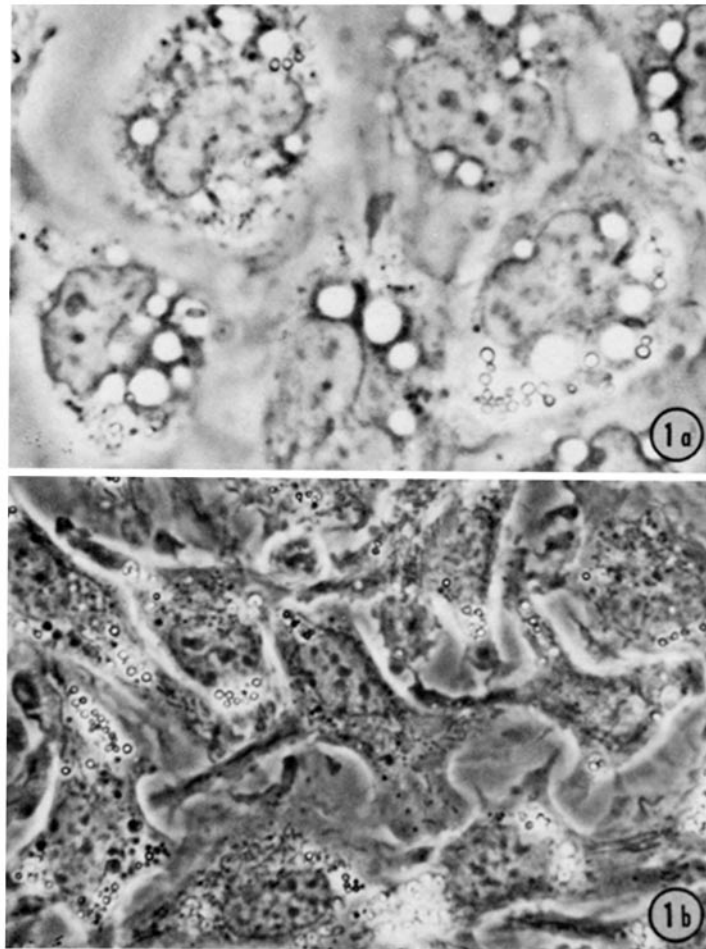| Concentration of Con A	Average no. of vesicles/cell
μg/ml	
0	2.10
10	4.20
20	5.40
50	7.35
20 + αMM (50 mM)	2.18
50 (4°C)	1.03

FIG. 1. Phase-contrast photomicrographs of mouse peritoneal macrophages cultivated in 199-FCS. (a) Con A (50 μg/ml) for 60 min at 37°C. Cells are extensively vesiculated. × 1,600. (b) Con A (50 μg/ml) and αMM (100 mM). Cells show an inhibition of the Con A effect. × 1,000.
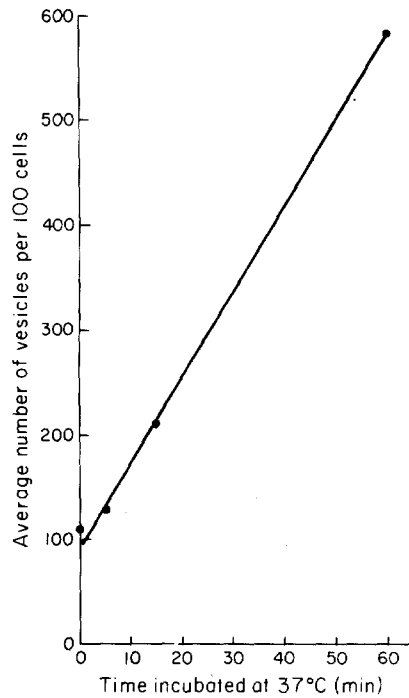
FIG. 2. Average number of cytoplasmic vesicles present at various times following addition of Con A (50 μg/ml) to 199-FCS, at 37°C.
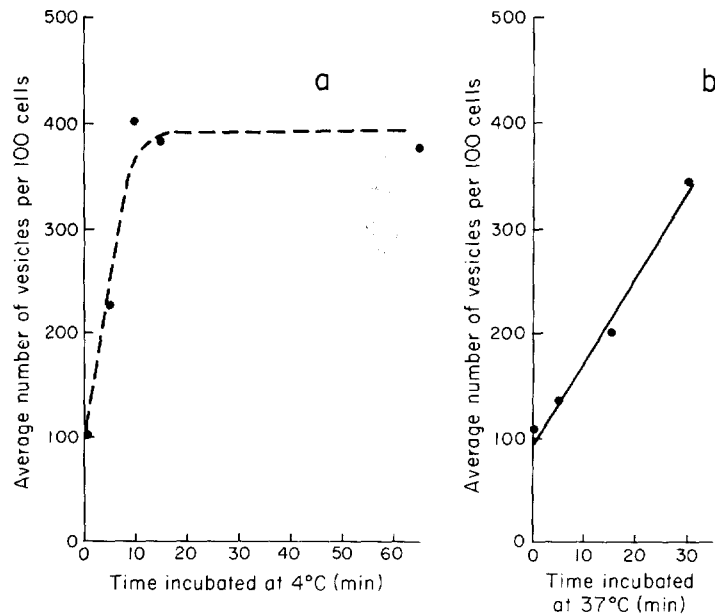

FIG. 3. Development of cytoplasmic vesicles following exposure of cells to Con A (50 μg/ml) at 4°C. (a) Cells were exposed to Con A at 4°C for 0-60 min, washed, and incubated in 199-FCS for 30 min at 37°C, fixed, and the cytoplasmic vesicles counted. (b) Cells were exposed to Con A at 4°C for 30 min, washed, and incubated in 199-FCS for up to 30 min at 37°C before being fixed, and the cytoplasmic vesicles were counted.

peritoneal cells exposed to Con A in suspension, or in cells cultured up to 1 wk preceding their exposure to Con A. Once formed, the phase-lucent vesicles may persist in the macrophage cytoplasm for up to 2 days with little change in their appearance and no apparent effect on the viability of the cells (Table II).

Ultrastructural Features of Con A-Treated Macrophages. When Con A-treated cells are examined by the electron microscope, the large clear vesicles are seen to be surrounded by a classic unit membrane about 80–100 Å wide (Fig. 4). Amorphous material is occasionally seen adhering to the inner surfaces of some of these vesicles, and in some, smaller-bounded vesicles are enclosed, suggesting a multivesicular body. No other effects on the cell were noted.

The characteristic ultrastructural appearance of the vesicles and the sensitivity of their generation to temperature suggested that they are pinosomes generated by endocytosis from the plasma membrane. Conclusively to demonstrate this, various electron microscope markers were added to the cell culture

TABLE II

Persistence of Vesicles in Macrophages Following Exposure to Con A (50 μg/ml) for 60 min and Subsequent Transfer to 199-FCS

Time following exposure to Con A	Average no. of vesicles per cell
h	
0	5.81
4	5.24
8	3.81
11	3.79
20	2.12
Control	0.62

Control cells were maintained in 199-FCS and fixed at 0 hours.

medium simultaneously with the Con A. In Fig. 4, we show the result of including colloidal gold in the incubation medium. The gold is present within many of the otherwise clear membrane-bound cytoplasmic vesicles, indicating that these vesicles have been generated from the plasma membrane. Similar results were obtained by incubating the cells in either Thorotrast or horseradish peroxidase (HRP) simultaneous with their exposure to Con A.

Identification of the Vesicle Membrane as Derived from Plasma Membrane. To show that the vesicle membrane itself, and not merely its contents, was a result of endocytic activity, we histochemically examined the distribution of the plasma membrane enzyme ATP (15). In resting cells in culture, this enzyme appears to generate reaction product only in association with the plasma membrane. However, when cells which have been exposed to Con A for 60 min at 37°C are stained for the enzyme, reaction product is seen outlining cytoplasmic vesicles as well as parts of the plasma membrane (Fig. 5), indicating the plasma membrane origin of at least some of the membranes surrounding the cytoplasmic vesicles generated after incubation with Con A.

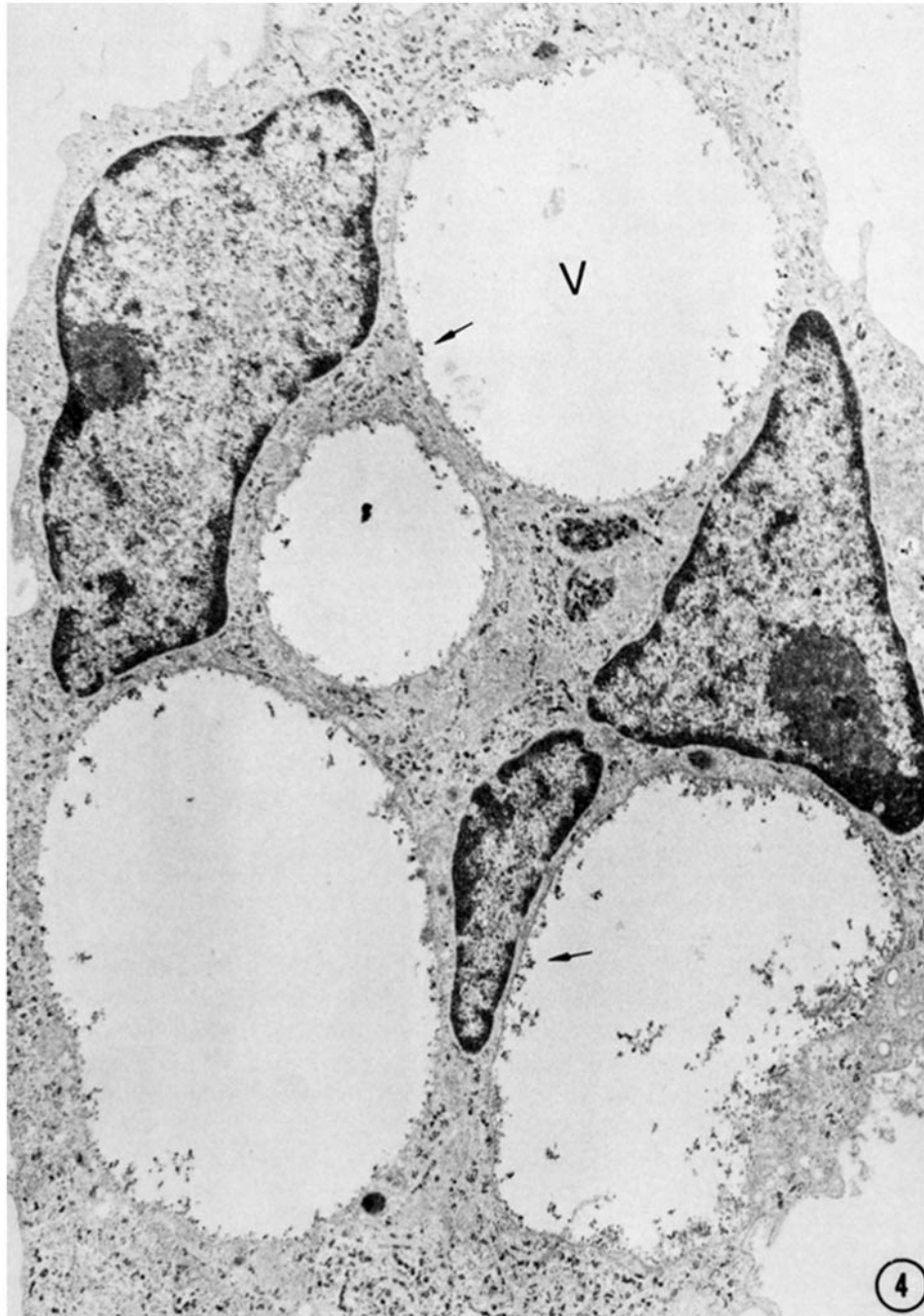*Localization of Con A to Pinosome Membrane.* As previously reported by

FIG. 4. Electron micrograph of a macrophage exposed to Con A (50 µg/ml) at 37°C in the presence of colloidal gold. Four large cytoplasmic vesicles (V) are evident. Each is bounded by a typical unit membrane and each includes many electron-dense gold particles (arrows) both adjacent to the membrane, and more centrally in the vesicles. × 11,000.

FIG. 5.   Adenosine triphosphatase staining reaction in a macrophage exposed to Con A (50 µg/ml) for 60 min at 37°C. Reaction product is evident both over the macrophage plasma membrane, and in association with the borders of numerous cytoplasmic vesicles. × 1,200.

Allen et al. (1), and by Mallucci (21), Con A can be shown to bind to the macrophage plasma membrane. Cells were chilled on ice and exposed for 30 min to Con A (50 µg/ml) in complete medium, and subsequently to HRP. The cells were then washed extensively in iced medium, fixed on ice with 2.5% glutaralde-hyde in cacodylate buffer, and then stained for peroxidase with diamino-benzi-dine (16) for 15 min at room temperature. After this, the cells were prepared for electron microscopy as described under Materials and Methods, and examined. As shown in Fig. 6, there was a dense coat of reaction product outlining the cell surface. No such product was seen when Con A was omitted, or αMM (50 mM) was included.

If the cells were incubated at 37°C for 1 h after exposure to Con A and HRP at 4°C, no reaction product was evident at the cell surface, but many vesicles showed reaction product lining their inner surfaces (Fig. 7). Thus, when the cells were maintained at 4°C, Con A could be localized through its binding to HRP (6), to the plasma membrane, while after 1 h at 37°C, the Con A was now associated with the inner surfaces of many cytoplasmic vesicles.

Quantitation of Pinocytic Rate in the Presence of Con A.   Qualitative studies, and estimation of vesicle numbers in macrophages exposed to Con A, suggested that a large number of pinocytic vesicles were accumulating in the macrophage cytoplasm. This could represent an increase in the rate of formation of pinocytic vesicles, a decrease in their rate of disappearance, or a combination of both changes. To clarify this aspect of the phenomenon, we measured the rate of pinocytosis of cultured macrophages exposed to culture medium with 20% FCS, 199-FCS supplemented with Con A (50 µg/ml), or 199-FCS containing Con A (50 µg/ml) and αMM (50 mM). The pinocytic rate was measured as the rate of uptake of HRP, which was added to all three media at a concentration of 1 mg/ml. As shown in Fig. 8, in all three cases uptake of horseradish peroxidase was linear for up to 2 h of incubation.
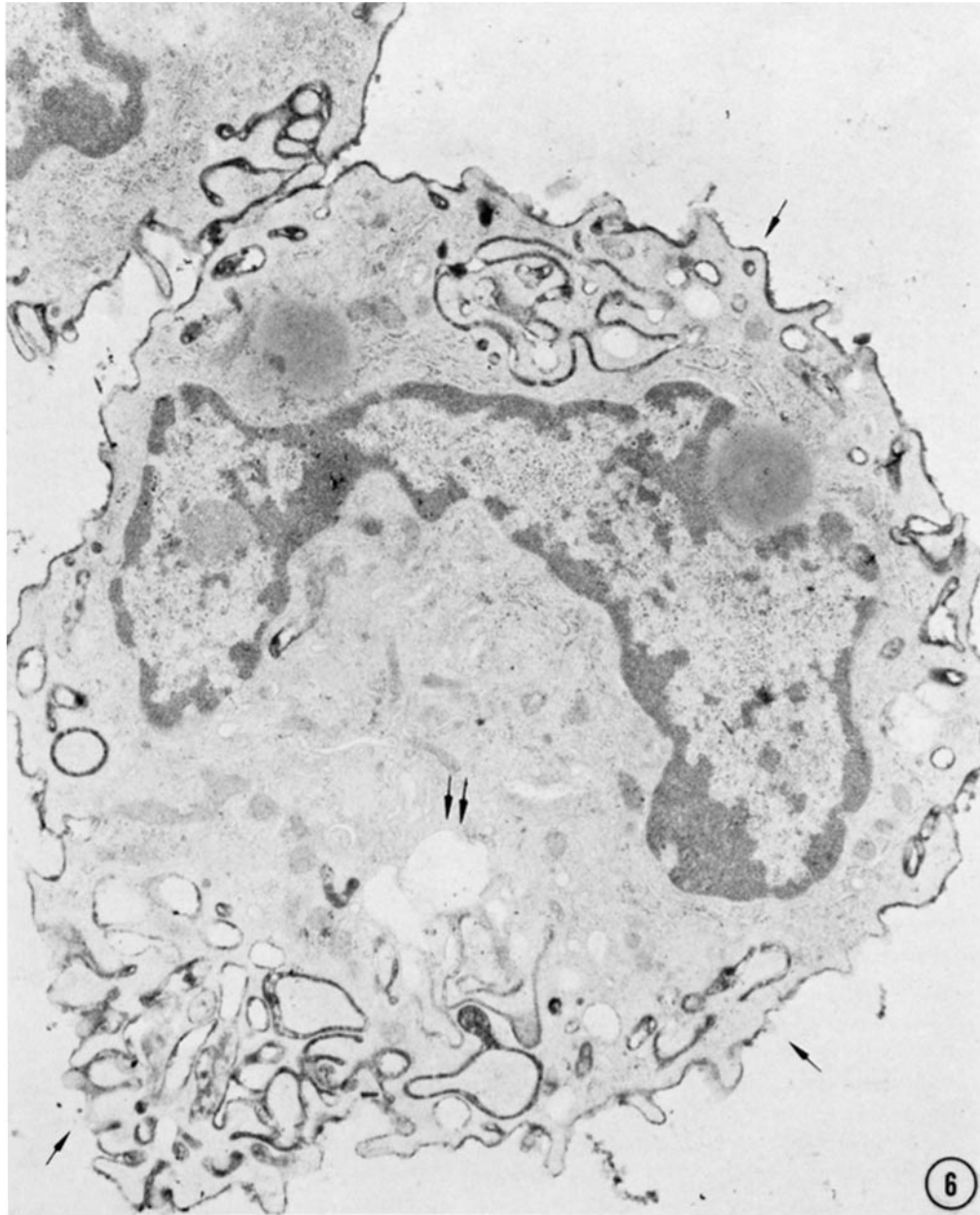
Fig. 6. Electron micrograph of a cell exposed sequentially to Con A and HRP, at 4°C, and then immediately fixed and stained for peroxidase. Note reaction product (single arrows) outlining cell surface, including highly fenestrated ruffled membrane, but excluding any cytoplasmic vesicles (double arrow). × 13,000.

As shown in Table III, however, the rate of uptake of HRP was 3.4 times greater for Con A-treated cells than for control cells. Inclusion of αMM returned the rate of uptake to control values.

Because HRP is a glycoprotein (25) which can be bound by Con A, the

Fig. 7. Macrophage exposed sequentially to Con A (50 µg/ml), and then HRP (1 mg/ml), both at 4°C, before rinsing, and re-incubating at 37°C for 60 min. Note large pinosome (P) bordered by dark reaction product (arrow) indicating location of HRP, and by inference, of Con A. Diamino-benzidine stain for HRP. × 12,400.

apparent rate of pinocytic uptake of HRP in the presence of Con A may be inflated as a result of binding of HRP to plasma membrane-bound Con A, with subsequent endocytosis of the HRP-Con A complexes. We therefore tried to estimate the magnitude of this binding effect to judge its relative significance.

Macrophage monolayers were exposed to Con A for 60 min at 4°C, washed twice with iced saline, and then exposed to HRP (1 mg/ml) for 30 min, again at 4°C. The cells were then washed, lysed in detergent, and assayed for HRP activity. Some cells were washed with αMM (100 mM) before lysis to permit estimation of the amount of HRP which was bound specifically to the cells via Con A. Thus, under conditions designed to maximize the ability of HRP and Con A to interact at the cell surface, the cell-bound Con A amounted to only about 13.4% of the total HRP interiorized in the presence of Con A at 37°C.(Table IV).

We further examined the effect of Con A on the endocytic rate of peritoneal
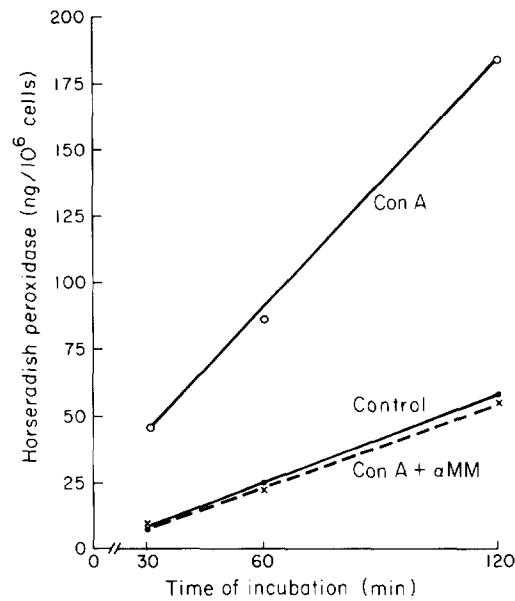
FIG. 8. Uptake of HRP by macrophages in 199-FCS (●—●), 199-FCS supplemented with Con A (50 μg/ml) (O—O), or 199-FCS supplemented with Con A and αMM (100 mM) (×---×).

TABLE III

Rate of Uptake of Horseradish Peroxidase by Mouse Peritoneal Macrophages Exposed to 1 mgm/ml HRP in 20% FCS, Supplemented as Indicated

Condition	Rate of uptake of HRP	Fractional uptake
	$ng/h/10^6$ cells	% load/h
Control	27.3	0.0037
Con A (50 μg/ml)	92.2	0.0126
Con A (50 μg/ml) + αMM (50 mM)	27.0	0.0036

TABLE IV

Comparison of Total Amount of HRP Bound by Con A to the Macrophage Surface, and Amount Susceptible to Removal with αMM, with Amount of Enzyme Interiorized in 2 h as Measured in a Previous Experiment

Experiment	Rinse conditions	Cell-associated HRP
		$ng/10^6$ cells
Surface-bound HRP	0.9% NaCl	29.7
Surface-bound HRP	0.9% NaCl + αMM (100 mM)	5.2
Interiorized HRP	—	92.2

macrophages, using [^{125}I]BSA as the pinocytic marker. Bovine serum albumin (BSA), which is not glycosylated, has no potential for artifactually increasing its apparent rate of uptake by interacting with Con A. [^{125}I]BSA, at an activity of 37 × 10^6 cpm/mg protein, was diluted 1:10 with unlabeled BSA and the mixture was added to 199-FCS at a concentration of 10 mg/ml. Macrophage monolayers were exposed to the [^{125}I]BSA for 2 h at 37°C in either the presence or the absence of Con A (50 μg/ml), washed, lysed, and assayed for TCA-precipitable radio-iodine. For reproducible results, it was found important to postincubate the cells in 199-FCS for 30 min after their incubation with the albumin, presumably to permit the endocytosis of surface-adherent protein. As shown in Table V, Con A increased the rate of uptake of [^{125}I]BSA 4.7-fold as compared with control conditions.

TABLE V

Rate of Uptake of [^{125}I]BSA by Mouse Peritoneal Macrophages
Exposed to 1 mgm/ml [^{125}I]BSA in 20% FCS, or [^{125}I]BSA and
Con A

Condition	Rate of uptake of [^{125}I]BSA	Fractional uptake
	ng/h/10^6 cells	% load/h
Control	47	0.0047
Con A (50 μg/ml)	212	0.0212

Effect of Con A on Phagocytosis of Latex or IgG-Coated SRBC. Because of the striking effect of Con A on macrophage endocytic activity we examined the effect of Con A treatment on the ability of these cells to interiorize either latex, a paradigm of "nonspecific" uptake, or IgG-coated SRBC, an example of uptake involving recognition of the Fc-portion of the opsonizing immunoglobulin molecule. Extensive ingestion of latex particles was accomplished by macrophages which had either been preincubated for 60 min at 37°C in medium containing Con A (50 μg/ml) (Fig. 9), or which were simultaneously exposed to both latex and Con A.

As the results in Table VI show, Con A also had no effect on the capacity of macrophages to ingest opsonized SRBC. In other experiments, macrophages exposed to Con A (50 μg/ml) for up to 12 h continued to ingest opsonized SRBC normally.

Effect of Con A on the Macrophage 5'-Nucleotidase. Macrophages were exposed to Con A (50 μg/ml) for 1 h, washed, and reincubated in fresh 199-FCS for up to 9 h, along with untreated controls and other cells which had been allowed to phagocytize polystyrene beads. At various times, cells were harvested in 0.05% Triton, and the lysates were assayed for 5'-nucleotidase activity. As shown in Table VII, specific enzyme activity was unchanged in the Con A-treated cells, while it showed a decrease to less than 60% of its original value in cells exposed to latex, with a gradual recovery of activity with further incubation, as has previously been described by Werb and Cohn (30).
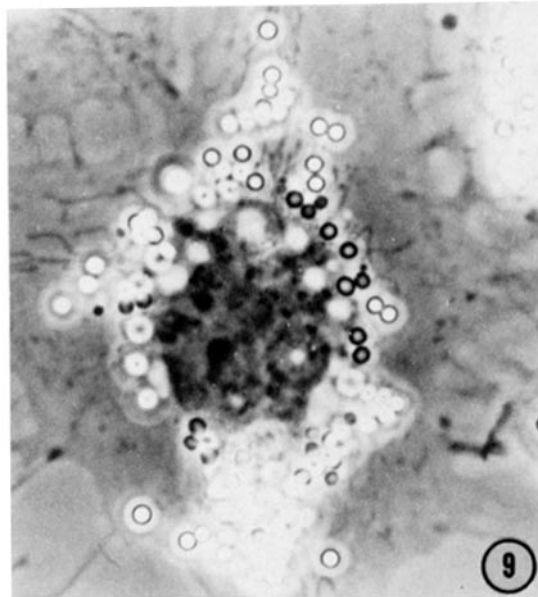
FIG. 9.   Phase-contrast photomicrograph of a macrophage exposed to Con A (50 μg/ml) for 60 min at 37°C, and then offered polystyrene beads. Note phase-lucent cytoplasmic vesicles due to the Con A, and numerous ingested latex particles clustered throughout the cytoplasm. × 1,600.

TABLE VI

Time-course of Ingestion of Opsonized SRBC by Cells Pretreated for 1 h with Con A, and by Untreated Cells (Control)

Time		% Cells phagocytis-ing SRBC	Average no. of SRBC ingested/ 100 cells	Average no. of SRBC phagocytis-ing cell
min				
15	Control	14.0	40	2.81
	Con A	13.0	19	1.36
30	Control	95.0	497	5.23
	Con A	93.5	548	5.86
60	Control	95.3	689	7.24
	Con A	99.0	709	7.16

Once Con A-stimulated pinosomes are formed, they can persist in the cell cytoplasm for many hours. Cells exposed to Con A (50 μg/ml) for 60 min at 37°C, washed, and then placed in fresh 199-FCS without Con A and reincubated at 37°C will still show large phase-lucent cytoplasmic vesicles for at least 20 h (Table II). This unusual persistence of Con A vesicles raises a question about the capacity of these vesicles to be incorporated in the normal way into phagolyso-

TABLE VII

5' Nucleotidase Activity of Macrophage Lysates Following
Exposure to Con A, or Latex, and Re-incubation in 199-FCS

Period of post-in-cubation	5'-nucleotidase activity		
	Control	Con A	Latex
h	nm AMP hydrolysed/min/mg protein		
0	111.7	141.0	140.8
3	123.5	143.1	85.4
5	141.2	142.8	75.8
7	150.2	146.3	89.7
9	138.0	137.3	95.2

somes in which digestion of their contents would then take place. Therefore, we examined the ability of Con A vesicles to fuse either with primary or secondary macrophage lysosomes.

Distribution of Acid Phosphatase in Con A-Treated Macrophages. The distribution of acid phosphatase in macrophages exposed to Con A was examined both by light and electron microscopy. By light microscopy (Fig. 10 a and b) areas of lead reaction product are evident in small membrane-bounded foci throughout the cytoplasm of Con A-treated macrophages, as they are in control cells. However, the large, phase-lucent cytoplasmic vesicles are free of reaction product, although they appear in some cases to show areas of reaction product close, to, but distinct from, the cytoplasmic vesicles. Electron microscopy (Fig. 11) confirms the impression that the large cytoplasmic vesicles are in fact free of reaction product, although acid phosphatase activity is readily demonstrated in small cytoplasmic granules, presumably primary lysosomes.

The failure to demonstrate acid phosphatase reaction product in presumptive Con A vesicles could be due to an inhibition by Con A of the enzymatic activity of acid phosphatase. This possibility was excluded by measuring the acid phosphatase activity of triton extracts of cultured macrophages in the presence of Con A up to 3,000 μg/ml. There was no effect on enzyme activity as compared with controls.

Distribution of Markers Included in Secondary Lysosomes. Macrophages were exposed to one of several electron microscopic markers, and, following exposure to Con A, the relation of these preloaded markers to the Con A-induced pinosomes was examined. The cells shown in Fig. 12 were exposed to thorotrast for 2 h, washed, postincubated for 30 min, exposed to Con A (50 μg/ml) for 60 min, and then examined by phase microscopy. After the cells are exposed to thorotrast for some time, numerous phase-dense granules are evident in the cytoplasm presumably representing secondary lysosomes. Cells subsequently exposed to Con A for 1 h at 37°C show two distinguishable populations of vesicles. One group appears identical to the thorotrast-laden secondary lysosomes seen in cells fixed without exposure to Con A. The second, phase-lucent, vesicle group, appears identical to the Con A-induced pinosomes.
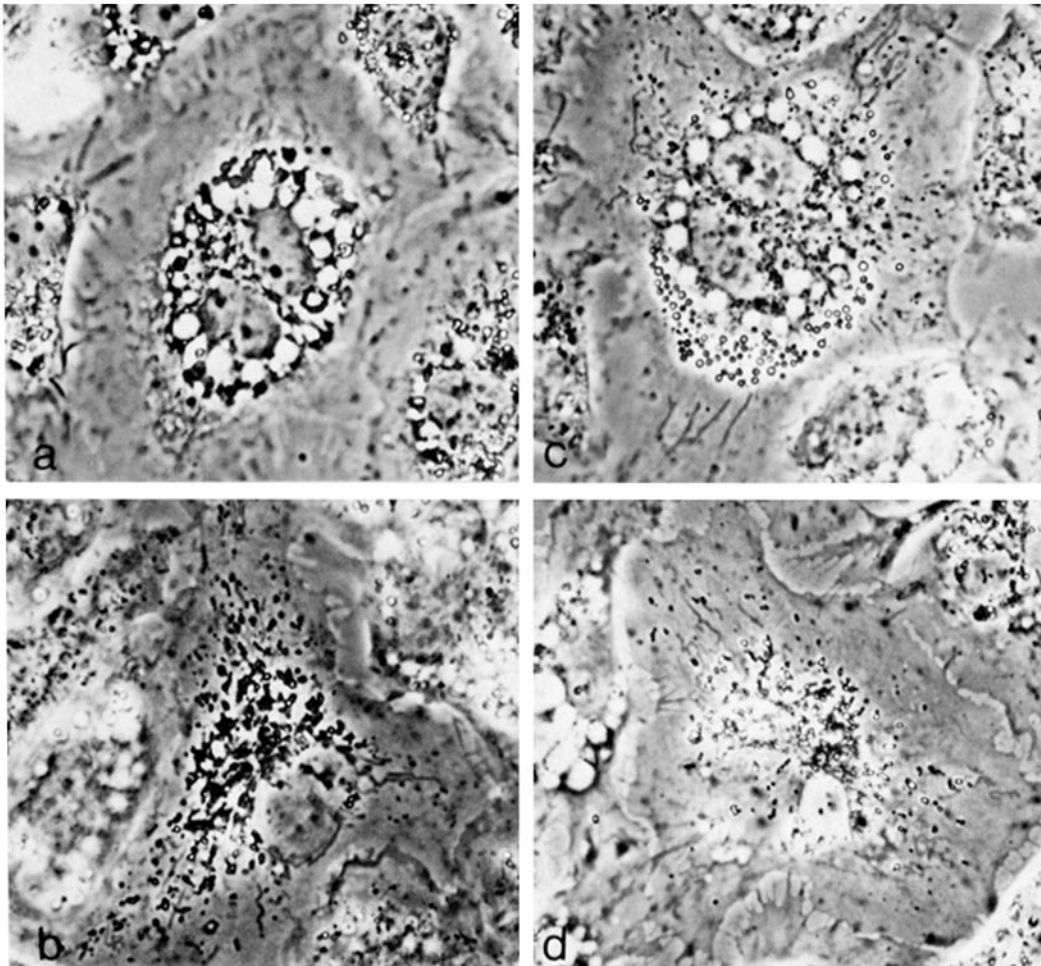
FIG. 10.   Light photomicrographs of cultivated macrophages. (a) Con A (50 μg/ml) at 37°C, staining for acid phosphatase. Note lucent Con A-induced pinosomes, free of reaction product, and separate granules showing dense reaction product. (b) Control cells stained for acid phosphatase, showing only the population of acid phosphatase-positive granules. (c) Macrophages exposed to HRP (1 mg/ml) for 2 h before exposure to Con A, and then stained for HRP with diamino-benzidine. Again, note distinguishable Con A pinosomes, and peroxidase-positive granules. (d) HRP control, showing only peroxidase-positive granules. × 1,000.

The same results are obtained when macrophages are preloaded with HRP and subsequently exposed to Con A. Again two population of vesicles can be identified, both by light microscopy (Fig. 10 c and d) and by electron microscopy (Fig. 13).

Colloidal gold is also an effective marker to be used in such preloading experiments. As it is possible that the large size of some Con A pinosomes makes fusion with secondary lysosomes undetectable by extensively diluting the transferred marker particles, we examined the distribution of preloaded gold particles in cells in which large autophagic vacuoles are present as the result of

exposure to chloroquine or Tris buffer (28). Fig. 14 shows a cell loaded with gold, and then exposed to chloroquine (30 μg/ml). Gold particles are clearly detectable in vacuoles which are comparable in size to the larger Con A pinosomes. However, no gold is detectable in the clear pinosomes of similarly loaded cells which were exposed to Con A.

Discussion

Con A can interact with a variety of phagocytic cells, often with a considerable effect on their subsequent structure and function. For example, Smith and Goldman reported that the formation of multinucleated giant cells was encouraged when human colostral macrophages were exposed to Con A at concentrations of 10–20 μg/ml for 6–48 h (26). This effect was sensitive to the

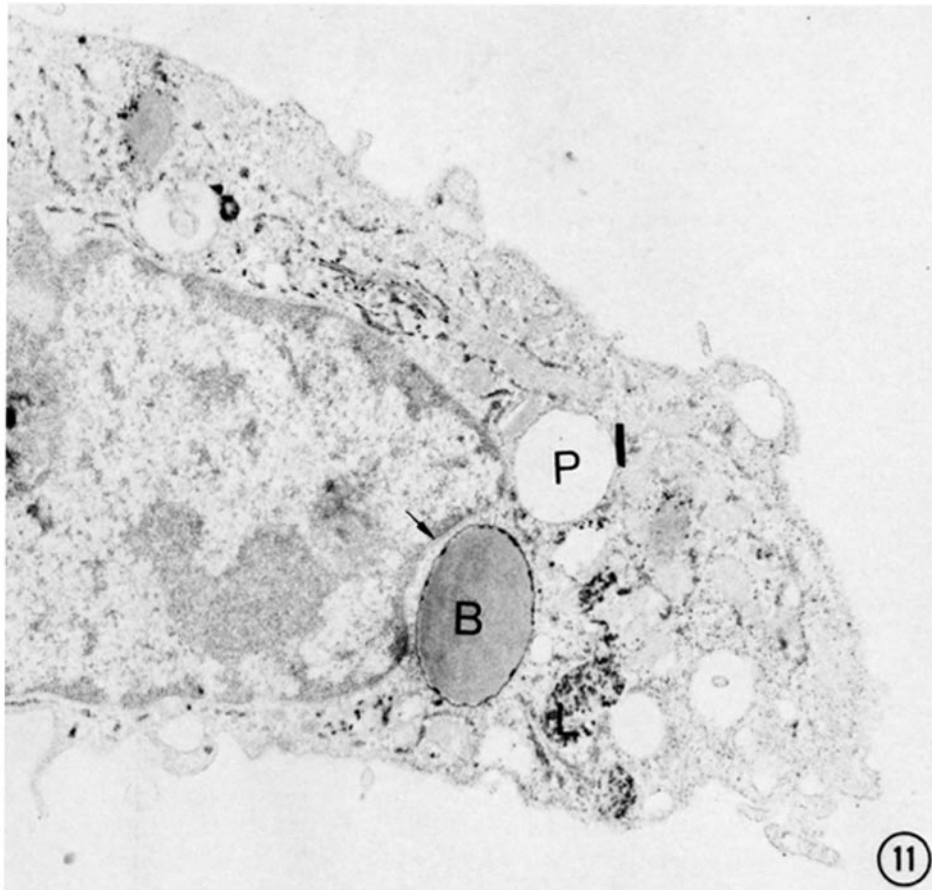FIG. 11. Electron micrograph of a macrophage incubated in 199-40% FCS for 24 h, exposed to Con A (50 μg/ml) for 60 min at 37°C and simultaneously to polystyrene beads, and then stained for acid phosphatase activity. Note black reaction product over primary or secondary lysosomes (L), while clear pinosome induced by Con A (P) is free of reaction product. Reaction product (arrow) is, however, evident adjacent to latex bead (B). × 5,500.
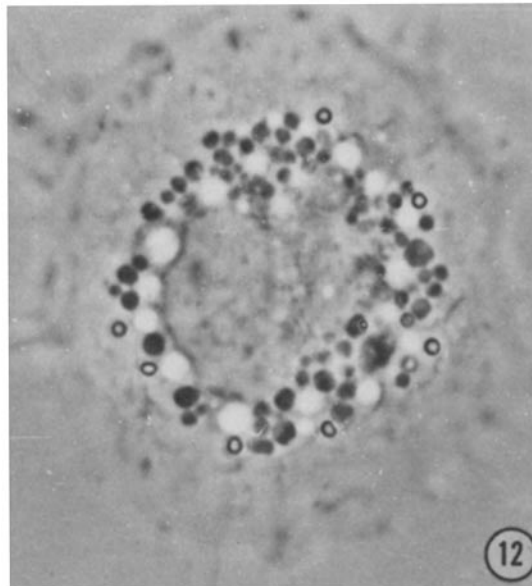
Fig. 12. Phase-contrast photomicrograph of a cell preloaded with thorotrast, present in the dark phase-dense granules, and then exposed to Con A (50 μg/ml) for 60 min at 37°C. Phase-lucent Con A pinosomes are free of thorotrast. × 1,000.

appropriate sugars, indicating that Con A was probably specifically interacting with mannose residues on the macrophage surface. Mouse peritoneal macrophages were reported to be resistant to the enhancement of giant cell formation by Con A in concentrations as high as 50 μg/ml.

Allen, Cooke, and Poole (1), however, have shown that Con A can be bound to sugars on the surface of mouse peritoneal macrophages. They reported that following a 10-min exposure of the cells to a fluorescein conjugate of Con A, the cell surface was fluorescently marked and that this marking was sensitive only to the appropriate sugars. With longer incubation periods, cytoplasmic fluorescence was observed in association with vacuoles. This fluorescence was attributed by the authors to endocytic activity, but it is not clear whether this represented internalization of bound Con A, or pinocytosis of Con A dissolved in the bulk phase, or perhaps, both.

Lutton (20), using [^{125}I]Con A, has confirmed that Con A can bind to mouse peritoneal macrophages. Further, he reported that following extensive ingestion of either polyvinyltoluene beads or titanium dioxide particles, the degree to which Con A was bound by the cells could be reduced as much as 35%. Additional incubation of the cells for 8 h led to a full recovery of binding capacity. This recovery was not dependent upon serum supplementation of the incubation medium, but was reported to be sensitive to cycloheximide.

Con A can agglutinate rabbit polymorphonuclear leukocytes (28), and Smith and Revel (27) have studied the surface distribution of Con A binding sites on these cells, using Con A-*Busycon canaliculatum* hemocyanin complexes which
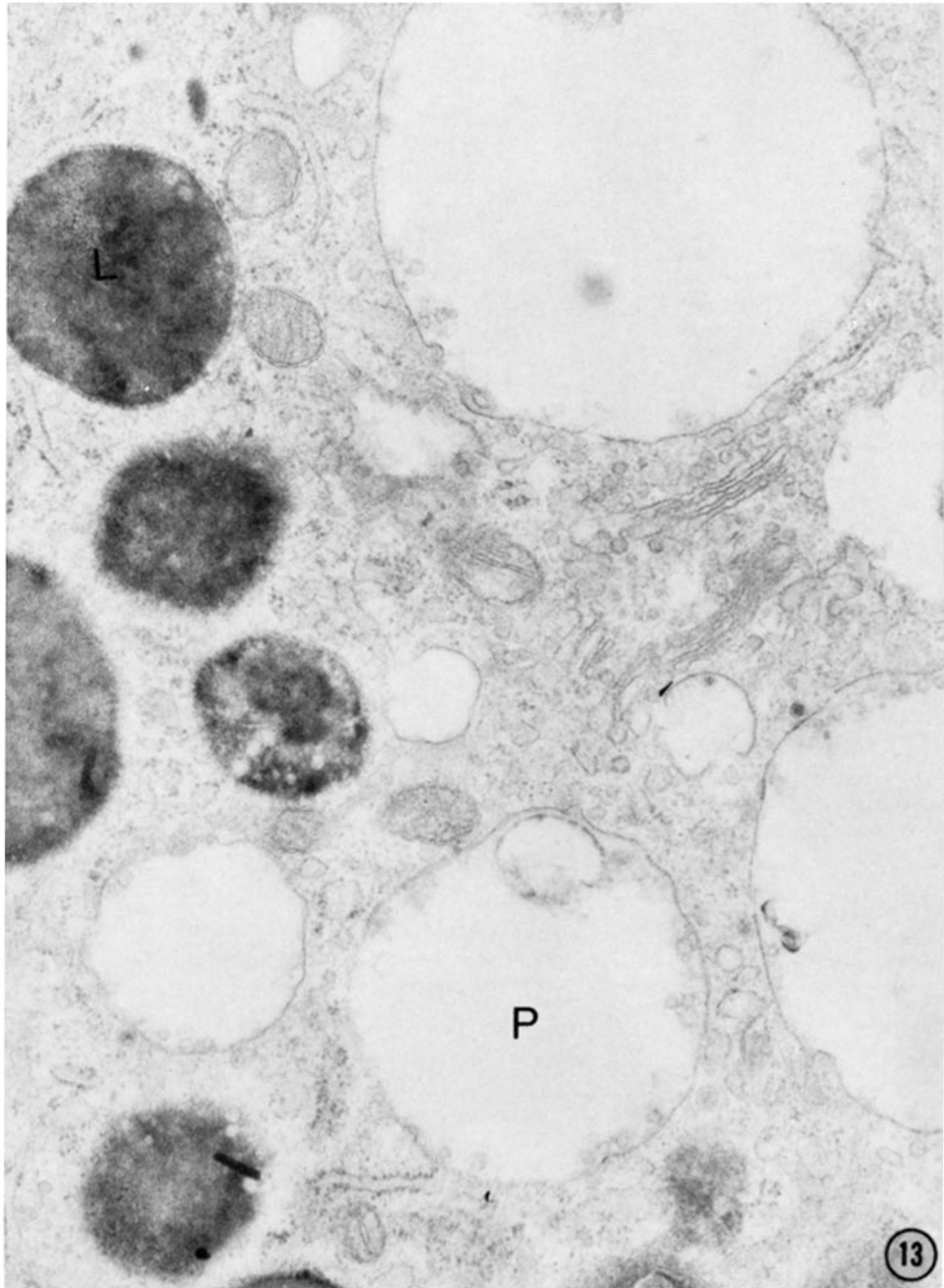
FIG. 13. Electron micrograph of macrophage pre-loaded with HRP (1 mg/ml) for 2 h, before exposure to Con A (50 μg/ml) for 60 min at 37°C. Note peroxidase-positive secondary lysosomes (L), and peroxidase-negative con A pinosomes (P). Cells stained with diamino-benzidine. × 30,900.
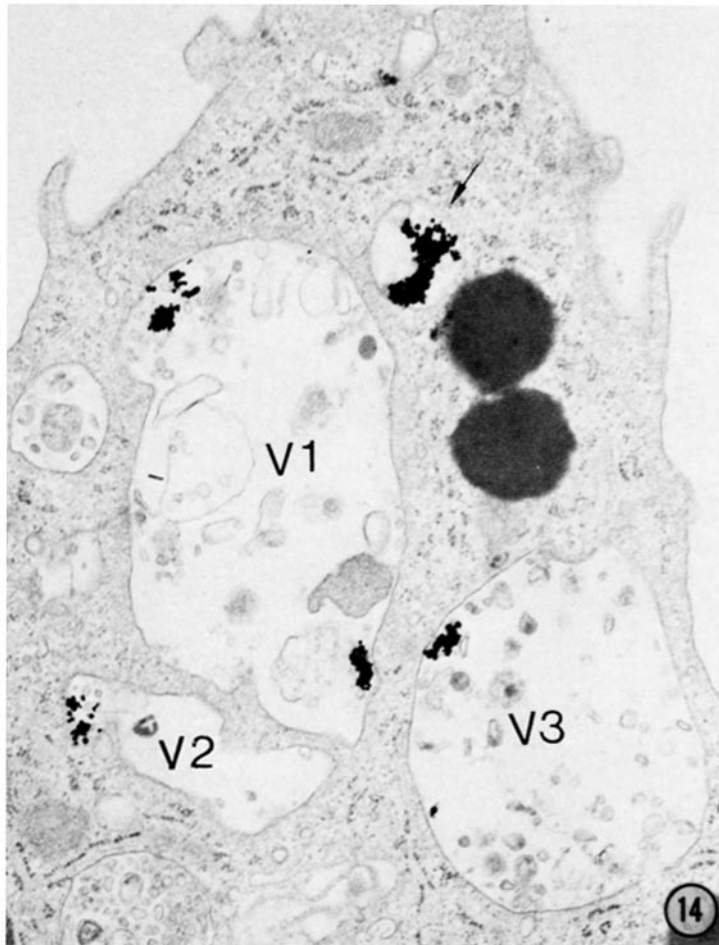
FIG. 14. Electron micrograph of macrophage preloaded with gold, and then exposed to chloroquine (30 μg/ml) for 60 min. Gold particles are seen stored within secondary lysosomes (arrow), as well as within some chloroquine-induced autophagic vacuoles (V1-3). × 23,200.

can be recognized on surface replica preparations examined by transmission electron microscopy. They found that at 37°C hemocyanin molecules were collected into a wide band located between the nucleus and the cell periphery. However, at 4°C, the molecules were distributed homogeneously over the entire cell surface. Unfortunately, no information is given on the distribution of hemocyanin molecules on cells fixed before exposure to Con A, making it uncertain to what extent the process of binding the Con A may itself influence the distribution of binding sites. Berlin, however, has described a dramatic functional effect of adding Con A to rabbit neutrophils (5). When neutrophils are incubated with increasing concentrations of Con A, up to 100 μg/ml, their ability to ingest polyvinyltoluene beads which are offered simultaneously is progressively lost.

Our studies show that Con A, once bound to the macrophage plasma

membrane, is rapidly interiorized by classic pinocytic mechanisms to form a membrane-bounded cytoplasmic vesicle in which the Con A is associated with the inner membrane surface. Binding of Con A to the macrophages at temperatures at which pinocytosis does not occur is sufficient to lead to the development of these vesicles when the cells are transferred to lectin-free medium and warmed. Binding is rapid, proceeds apparently normally at 4°C, and is entirely inhibited by αMM, but not by sugars which cannot be bound by Con A.

Con A increases the endocytic rate of cultured macrophages between 3.5- and 4.5-fold. This stimulation again requires interaction with saccharide residues on the cell membrane, as inferred from the ability of αMM to prevent this stimulation. Several experiments were performed to examine the possibility that this apparent increase in endocytic activity was not accounted for simply by an artefactual binding of the Con A to the marker used for making the uptake measurements. Although horseradish peroxidase, a glycoprotein, does bind to Con A (P. J. Edelson, unpublished observations), such binding could not, under the most favorable conditions, account for more than approximately 14% of the total HRP interiorized during the course of the experiment. In addition, we obtained similar results using [^{125}I]BSA as the endocytic marker. Bovine serum albumin is not a glycoprotein, and there is no reason to expect that it could bind to Con A, and in that way increase its apparent rate of endocytosis.

Con A does not appear to preferentially bind to areas of the macrophage membrane specialized for ingestion of IgG-coated particles, the Fc-receptor (22), and, in fact has no effect on the phagocytosis of either IgG-coated or latex particles by the macrophage. In addition, Con A did not modify the activity of 5'-nucleotidase, an exteriorly disposed plasma membrane enzyme.

Once formed, the Con A pinosomes have a remarkable natural history, as compared with most macrophage endocytic vesicles. While most pinosomes fuse with pre-existing primary or secondary lysosomes forming hybrid "phagolysosomes", this process is markedly impaired in the case of Con A pinosomes. Neither acid phosphatase, an endogenous marker of primary and secondary lysosomes, nor gold, HRP, or thorotrast, accumulated in secondary lysosomes, could be detected in Con A pinosomes. Our inability to detect these markers could not be explained as the result of their dilution within the large volumes of some of the Con A pinosomes, as these markers could easily be detected in vesicles of comparable size which were formed after exposure to Tris buffer, or to chloroquine.

The simplest explanation of our results is that Con A initially binds to sugar residues, probably mannose, present on the plasma membrane. As mannose does not occur in glycolipids of mammalian cells (13), Con A is presumably bound to one or several membrane glycoproteins. This binding, under permissive conditions, leads to interiorization of the Con A-membrane complex in pinocytic vesicles which are evident as phase-lucent vacuoles throughout the macrophage cytoplasm. In spite of the rather extensive interiorization of membrane, this process has no apparent effect on the ability of the macrophage to either bind or ingest either latex particles, or opsonized SRBC. Once formed, these pinosomes show an exceptional ability to persist in the cytoplasm, with little microscopic

evidence of fusion with either primary lysosomes or other preformed secondary lysosomes.

The capacity of Con A to stimulate pinocytosis is not unique to the macrophage. Endocytosis of Con A has probably been observed previously in lymphoid cells, for example by Pauli, et al. (23). They reported that, when a mixture of human peripheral blood lymphoid cells is exposed to Con A, a progressively greater fraction of the Con A bound by the cells becomes resistant to elution with αMM. In addition, this fraction is no longer detectable on the cell surface by a fluoresceinated anti-Con A antibody, suggesting indirectly that this fraction is within the cell interior. We have studied the effect of Con A on the endocytic rate of human lymphoid cells 8866 (P. J. Edelson and G. J. Hoffman, unpublished observations), and have found that, as with the macrophage, the pinocytic rate of 8866 cells is also increased about fourfold, and that fluoresceinated Con A can be detected in the cell cytoplasm.

It has also been reported that exposure of rabbit alveolar macrophages, as well as guinea pig neutrophils, to Con A at concentrations from 30-160 μg/ml, can stimulate hexose monophosphate shunt activity (24). This, too, may be a result of increased endocytic activity stimulated by Con A. The authors report that a fluoresceinated Con A preparation was found to be localized to the cell surface and was not observed in the cytoplasm, but this may be due to characteristics of the conjugate used, or to the length of time for which the cells were observed following exposure to Con A.

Con A, by directly interacting with the pinosome membrane, has a striking effect on the fate of these vesicles by preventing their fusion with lysosomes to form a phagolysosomal complex. Bachi, Aguet, and Howe (4) have previously noted that Con A can inhibit the Sendai-virus induced fusion of human erythrocytes, and this may well represent a similar or identical phenomenon.

In the following paper, the relation of the Con A to the endocytized plasma membrane will be more directly examined, using a fluoresceinated Con A derivative. In addition, the fate of proteins incorporated into the Con A vesicles will be described, and the reversibility of the inhibitory effects of Con A on phagolysosome formation will be detailed (9).

Summary

Concanavalin A (Con A) binds to saccharide residues on the mouse peritoneal macrophage plasma membrane and stimulates extensive pinocytic interiorization of the membrane. The overall pinocytic rate is increased 3.5-4.5 times by the addition of Con A, and the surface marker enzyme adenosine triphosphatase can be identified histochemically in association with the cytoplasmic vesicles generated after exposure of the cells to Con A. Once formed, these pinocytic vesicles may persist for several days and fail to show morphologic evidence of fusion with primary or preformed secondary lysosomes. There is no apparent effect on the capacity of the macrophage to ingest either latex particles or IgG-coated SRBC administered either simultaneously with or subsequent to the Con A.

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