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Studies on the mechanism of phagocytosis. I. Effect of metabolic inhibitors on the phagocytosis of iron colloid particles by ascites macrophages

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Abstract

For the purpose to clarify the mechanism of phagocytosis or pinocytosis, the observations on the tumor ascites, including the macrophages as well as the tumor cells, were carried out by incubating with the iron colloid with or without pretreatment by several inhibibitors of glycolysis, oxidative phosphorylation and respiration, or under hypotonic or cold environments. The results have demonstrated that there are three steps in the phagocytosis. The first step is the adhesion of the substance to the cell surface, which is not an energy-requiring process. The second step is the engulfing which proceeds by using the energy supplied by glycolysis. The third is the accumulation of the substance into the vesicles through the canaliculi connecting the cell surface with the vesicles. The discussion was made on the existence of the active site on the cell surface to which the substance can be adhered, and the accumulation mechanism of the material into the phagocytic vesicles by the membrane flow, the flowing movement of the outer lipid layer of a unit membrane through the canaliculi which connect the cell surface to the phagocytic vesicles.

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STUDIES ON THE MECHANISM OF PHAGOCYTOSIS

I. EFFECT OF METABOLIC INHIBITORS ON THE PHAGOCYTOSIS OF IRON COLLOID PARTICLES BY ASCITES MACROPHAGES

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Phagocytosis means the uptake of solid material by cell and can be seen distinctly in macrophages whose special function is the removal of solid foreign bodies and metabolic products as the scavenger cells. Later on, the concept of pinocytosis was proposed by LEWIS[1] (1931) as the drinking-process of cells in contrast to phagocytosis, the uptake of some soluble substance. However, the mechanism should be identical in both cases as suggested by HOLTER[2] (1959) from the observation of amebae. Therefore, these two have originally been developed in lower animals as the process to take up intermittently the nutrient from external milieu in quanta. For this reason the term phagocytosis is used as the one including the concept of pinocytosis in this paper. Concerning the mechanism, it is thought that the change in the equilibrium in the surface-tensions of the cell surface, the medium and the particles should be responsible for inducing the phagocytic engulfment of the cell surface by which the particles can be ingested (FENN[3], 1921; PONDER[4], 1928).

Recently, the evidences have been accumulated that phagocytosis is an energy-requiring process which is supported by the energy yielded by glycolysis (BECKER *et al*[5]., 1958; SBARRA and KARNOVSKY[6], 1959; COHN and MORSE[7], 1960; OREN *et al.*[8], 1963), because phagocytosis is suppressed in the presence of glycolytic inhibitors. A refutation to this, however, is that the suppression of phagocytosis may not result from the inhibition of energy metabolism but from the swelling of the cell, which inevitably ensues in the presence of inhibitors (WOODIN[9], 1963). Against this contradictory opinon we have few evidences that the suppression of phagocytosis in the presence of the glycolytic inhibitors is solely due to the inhibition of the energy supply for phagocytosis. For the purpose to solve this problem, the authors tried to clarify whether phagocytosis is an energy-dependent process or not, and revealed that phagocytosis proceeds

in three steps: the first step is the adhesion of a substance to the cell surface, which is energy-independent reaction, the second is the engulfing at the site of the adhesion with or without formation of pseudopodes in the surounding area, which is energy-requiring process, and the third is the accumulation of the substance into vesicles.

MATERIALS AND METHODS

Macrophages in Ehrlich tumor ascites of ddN mice served as materials. The macrophages and ascites tumor cells were harvested a week after the inoculation of Ehrlich ascites tumor cells. After washing five times with cold Hanks' solution, the cells were suspended in Hanks' solution. The final cell population was about 2.5×10^7 per ml and two ml each of the suspensions were used for one observation. Some series of these cell suspensions were incubated with iodoacetate, fluoride, 2,4-dinitrophenol, cyanide, and antimycin A of varying concentrations for thirty minutes. One series of the cell suspensions was then reincubated in Hanks' solution containing no inhibitors and served as control. After incubation all the samples were added with 0.5 ml of iron chondroitin sulphate (CS-Fe) and then reincubated for ten minutes at 37°C, while shaking gently (90 cycles/minute). To check the effect of low temperature, the same cell suspensions were exposed to about 0°C (ice cold) for thirty minutes and 24 hours, and then they were reincubated with CS-Fe for ten minutes at 0°C or 37°C. To check the effect of cell-swelling on phagocytosis, hypotonic Hanks' solutions diluted with six, four and two volumes of water were used as incubating media. In some series of experiments the cells suspended in ascites fluid were observed after treating them in the same manner as with the cells suspended in Hanks' solution. Further, some ddN mice bearing tumor ascites were given intraperitoneal injection of CS-Fe (2 ml/animal) and 10 minutes later the cells of ascites were observed as those incubated *in vitro*.

After 10-minute incubation with iron colloid, a droplet from each cell suspension was smeared, dried, fixed with methanol, stained by Perls' reaction for iron with nuclear stain by Kernechtrot and observed under light microscope, and the rest were observed under electron microscope.

For the electron microscopy the cells were mixed with an excess amount of cold fixative, usually 2.5% glutaraldehyde (SABATINI *et al.*[10], 1963) in 0.1M or Millonig's phosphate buffer (pH 7.4) (MILLONIG[11], 1962) for ten minutes, washed with the same buffer, and post-fixed with 1% OsO_4 in phosphate buffer. Only the materials pretreated with hypotonic medium were fixed with 2.5% glutaraldehyde solution in the buffer of the same tonicity as the hypotonic medium used at incubation. After the post-fixation, they were washed with cold water several

times by repeated centrifugation and finally suspended in a small amount of water. The cell suspensions were transferred into polyethylene tubes sealed at one end of two millimeters in diameter and three centimeters long and centrifuged at 7,000 *g* for 20 minutes. The cell pellets in the tubes were obtained by cutting the tubes into a few fragments, about three millimeters in length, and they were dehydrated through ethanol series. At an advanced dehydration the cell pellets detached themselves from tubes and they were embedded in Epon 812 mixture (MARUYAMA[12], 1963) through propylene oxide (LUFT[13], 1961). Thin sections were prepared with a Porter-Blum MT-1 microtome, mounted on naked 300-mesh copper or nickel grids, stained with 5% uranyl acetate in 70% aqueous solution of ethanol and/or alkaline lead solutions (MILLONIG[14], 1961; or KARNOVSKY[15], 1961) and observed under electron microscope, Hitachi HU-11A.

CS-Fe was supplied by Dainippon Seiyaku Co. It was a colloidal solution of a dark red color, pH 7.21 and 467 mosmol measured by cryoscopy, 1 ml of which contained 4 mg of iron. The size of its colloidal particles estimated by electron microscopy ranged from about 30 Å in the smallest unit to about 200 Å in the aggregated group particles, of which the latter were observed in this study. The colloid particles in Hanks' solution and ascites fluid were charged negative as revealed by the paper electropheresis in veronal-acetate buffer (pH 7.4). Iron was combined so firmly with chondroitin sulphate that the two components could not be separated by electrophoresis.

The inhibitors used were iodoacetate of Wako Junyaku Co., sodium fluoride of Okayama Yakuhin Co., 2, 4-dinitrophenol of Wako, potassium cyanide of Wako, and antimycin A of Kyowa Hakko Co.

RESULTS

The tumor ascites obtained 7 days after inoculation contained a number of Ehrlich ascites tumor cells and a moderate number of macrophages or monocytes (about ten per cent of all the ascites cells). After ten minute incubation of the ascites with colloid iron *in vitro* there was observed a striking phagocytosis of colloidal particles but no phagocytosis in the case of the tumor cells as revealed by Giemsa stain and Perls' reaction, *i. e.*, the tumor cells were completely free of iron particles. The same could be observed with those in the tumor ascites cells suspended in Hanks' solution and incubated with the iron colloid for ten minutes at 37°C.

Electron microscopy of the ascites cells incubated with the iron colloid revealed that macrophages ingested a number of iron colloid particles of about 200Å in diameter by the 10-minute incubation but the tumor cells did not. Besides these, the iron colloid particles were found to be adhered solely to the cell

surface of macrophages but not the tumor cells. Careful observations on macrophages revealed that each iron colloid particles adheres to the cell surface with a certain distance from the neighboring particles. No massive colloid adherence covering whole area of cell surface has ever been encountered, though in some phagocytic vacuoles a massive accumulation of the colloid particles can be seen. In some cells the phagocytic vesicles containing a mass of the colloid particles were distributed densely in the area surrounding Golgi apparatus, but in others these vesicles were near the cell surface. The findings are identically the same in both cases; the cells suspended in ascites fluid and those washed and suspended in Hanks' solution (Figs. 1, 2 and 3).

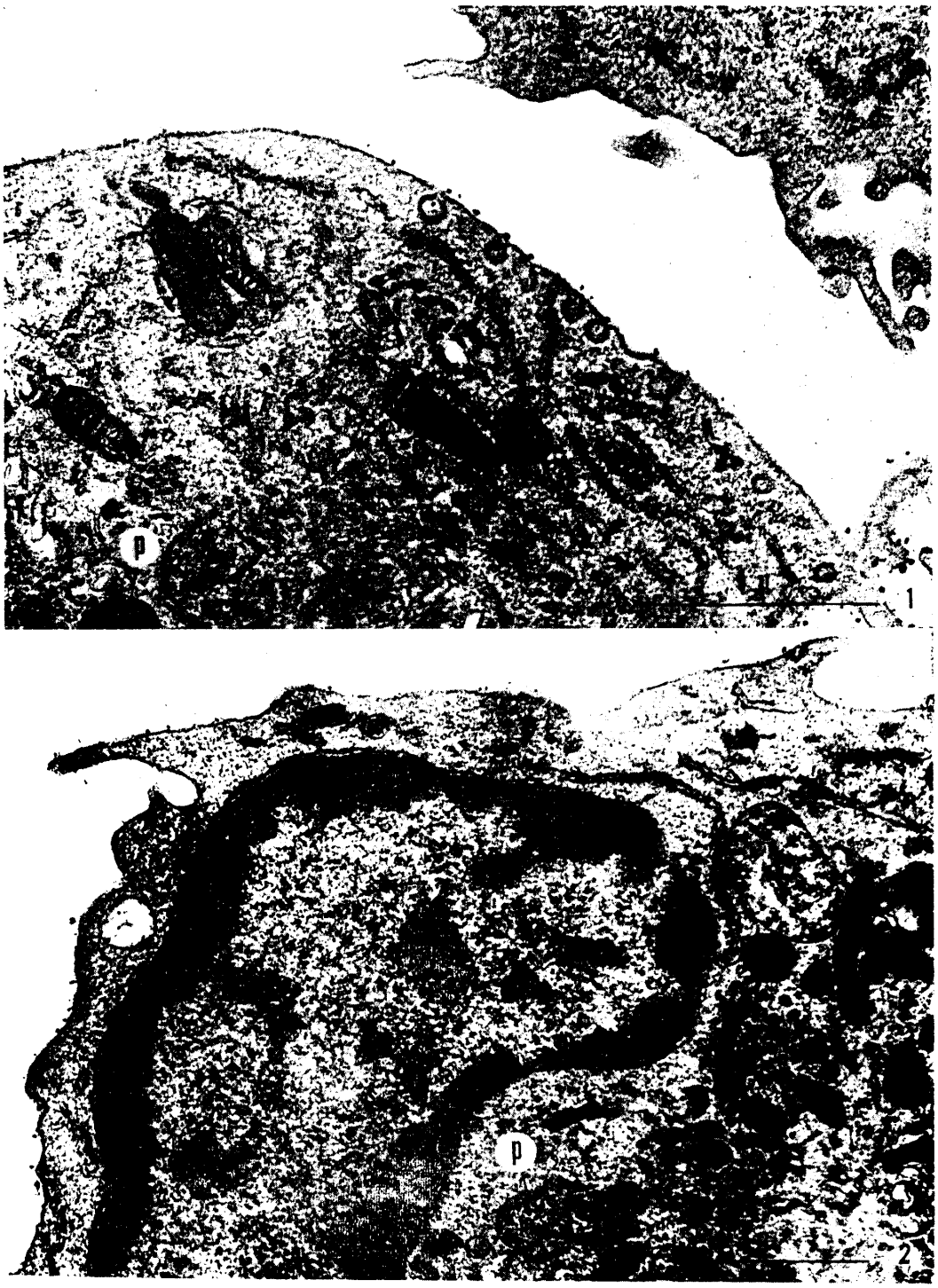
Some phagocytic cells revealed small invaginations on the cell surface just at the sites where the colloidal particles were attached (Fig. 1). The picture is similar to that of rhopheocytic vesicles described by POLICARD and BESSIS[16] (1958), but in this instance it should be the initial stage of phagocytosis. All the ingested colloid particles were found to be situated in the vacuoles or tubules having a single membrane but not in the cytoplasmic ground substance (Figs. 1, 2 and 3). The vesicles has a mass of iron granules but the tubules contained only a few granules. In the phagocytic vesicles some membraneous structures which appear to be desquamated elements comprise a part of the membrane composing the phagocytic vesicles. There were observed some small vesicles of about 0.1 μ in diameter containing one or two colloidal particles which appeared to be the cross sections of tubular structures. Some sections of these tubular structures seem to indicate that the tubules communicate the surface with the phagocytic vesicles situated deep in the cytoplasm (Fig. 3). These tubular structures were quite similar to the smooth surfaced endoplasmic reticula in their apperance except for a few colloidal granules.

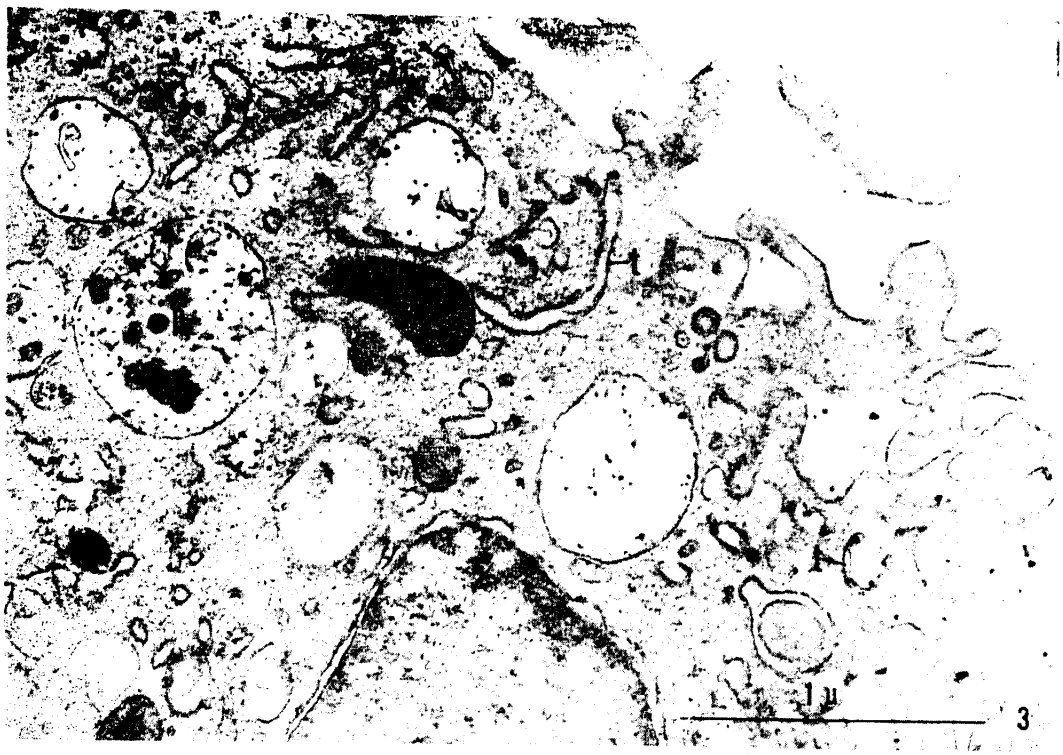
On the basis of these observations of phagocytosis the effects of various energy inhibitors on the process of phagocytosis were studied with the cells washed and preincubated with inhibitors for 30 minutes at 37°C. As the result, it was found that the preincubation with iodoacetate at the concentrations of 10^{-4}M, 10^{-3}M and 10^{-2}M and fluoride at 10^{-2}M and 2×10^{-2}M caused a marked swelling of both macrophages and Ehrlich ascites tumor cells. The former cells

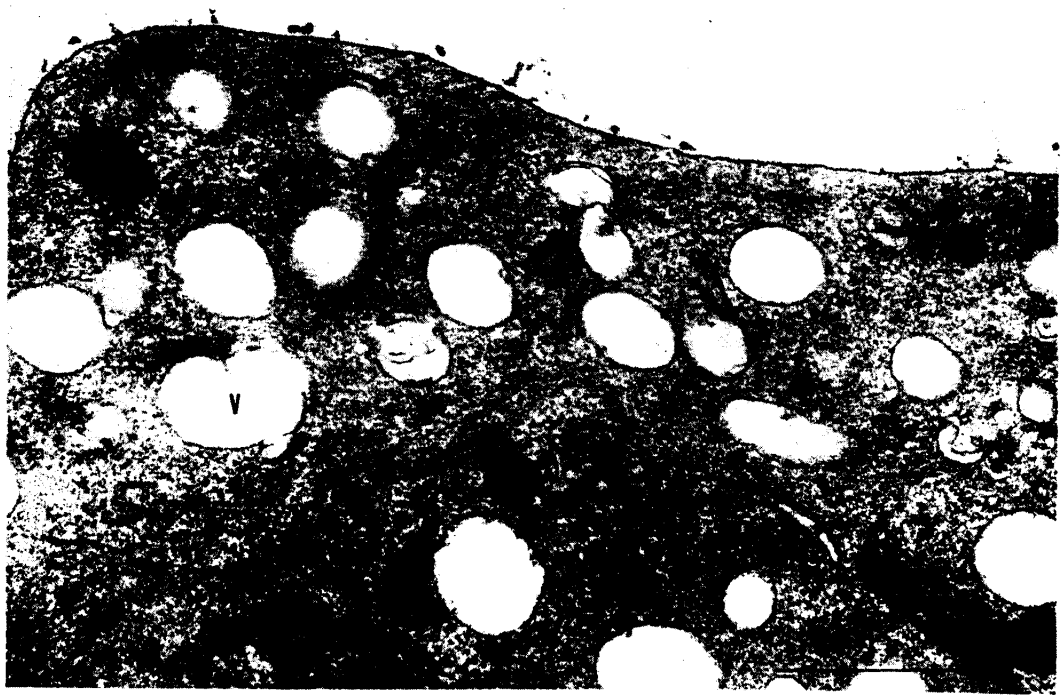
Figs. 1~3 Phagocytic Cells Incubated with Chondroitin Sulfate Iron Colloid (CS-Fe) at 37°C for Ten Minutes after Washing with Hanks' Solution Five Times. The colloidal particles are adhered onto the cell surfaces.

Fig. 1 On the cell surface of the phagocytic cell the phagocytic vesicles containing the colloidal particles are forming. In the cytoplasm large phagocytic vesicles (p) containing colloidal particles are also seen. Note no colloidal particles on the surface of Ehrlich ascites tumor cell (E). M, macrophage. ×35,000

Fig. 2 Colloidal particles are ingested in the phagocytic vesicles (p) which are located in the Golgi area. ×30,000


P
1
P
2


1 μ
3


V

were more markedly swollen than the latter. The cell grew rather round with retarded pseudopod formation, the vacuoles or vesicles of varying sizes were conspicuous in cytoplasm, and mitochondria with the inflated cristae of denser matrices were frequently encountered. These cells were added with iron colloid and then incubated further for ten minutes. However, the cells failed to take up the colloid particles into the cytoplasmic vesicles, though the particles were adsorbed to the cell surface (Figs. 4 and 5).

The swelling of the cells was effectively induced by incubating them in the hypotonic media for thirty minutes ; namely a moderately swelling in the medium diluted three times and a rather marked in that diluted five times. Ehrlich ascites tumor cells were relatively more resistant to the swelling in the diluted media than macrophages, but in the medium diluted five times they were swollen forming tongue-like processes on the cell surface and vacuoles in the cytoplasm. Incubation in the medium diluted seven times resulted in total disruption of the cells. The swollen cells incubated for 30 minutes in hypotonic media diluted 3 or 5 times retained the activity of phagocytosis for iron colloid particles as revealed by further incubation with colloidal iron for ten minutes. The cells adsorbed colloidal iron particles on their surface and ingested the particles into the phagocytic vesicles, in contrast to the case preincubated with fluoride or iodoacetate. The swollen macrophages also ingested some fragments of disrupted cells (Fig. 6), and these phagocytic cell fragments themselves also adsorbed iron particles (Fig. 7). However, Ehrlich ascites tumor cells neither adsorbed nor took up the colloidal particles.

The preincubation with 2, 4-dinitrophenol at the concentrations of 10^{-6} M, 10^{-5}M, 10^{-4}M and 10^{-3}M, cyanide at 10^{-4}M, 10^{-3}M and 5×10^{-3}M and antimycin A at 5γ/ml had no significant effect on the phagocytic activity of macrophages. That is, even after the preincubation these phagocytes adsorbed the colloidal particles on the cell surface and ingested the particles to the same extent as control cells incubated without the agents, though there was a slight change in the configuration of ultrastructures of some organellae (Figs. 8 and 9).

The cells kept at 0°C for thirty minutes and then incubated with colloidal iron at 0°C for ten minutes were slightly swollen in appearance and they adhered the colloidal particles on the cell surface, but they showed a markedly lower phagocytic activity containing only a few colloidal particles in some phagocytic

Fig. 3 Tubules (t) seem to transport the colloidal particles. Phagocytic vesicles (p) also contain the colloidal particles. ×40,000

Figs. 4～9 Phagocytic Cells Incubated with CS-Fe at 37°C for Ten Minutes after Various Pretreatments (at 37°C) for Thirty Minutes.

Fig. 4 Pretreated with 10^{-3}M iodoacetate. The cell is swollen and its contour smooth. The colloidal particles are adhered onto the cell surface and not localized in vacuoles (v). ×30,000

vesicles (Fig. 10). Mitochondria were large and less dense in matrix with narrow intracristal spaces. When the cells were warmed up to 37°C after being incubated with colloidal iron at 0°C and kept at this temperature for ten minutes, they ingested the colloidal particles just as in those incubated at 37°C without preincubation at 0°C. Even the cells stored in refrigerator (about 5°C) for 24 hours were able to engulf the colloidal particles when they were incubated with colloidal iron at 37°C for ten minutes. The cells kept in cold retained the activity of pseudopod formation, differing from those treated with iodoacetate or fluoride.

Ehrlich ascites tumor cells also showed no specific reaction to the iron particles in all the cases observed. The results are summarized in Table 1.

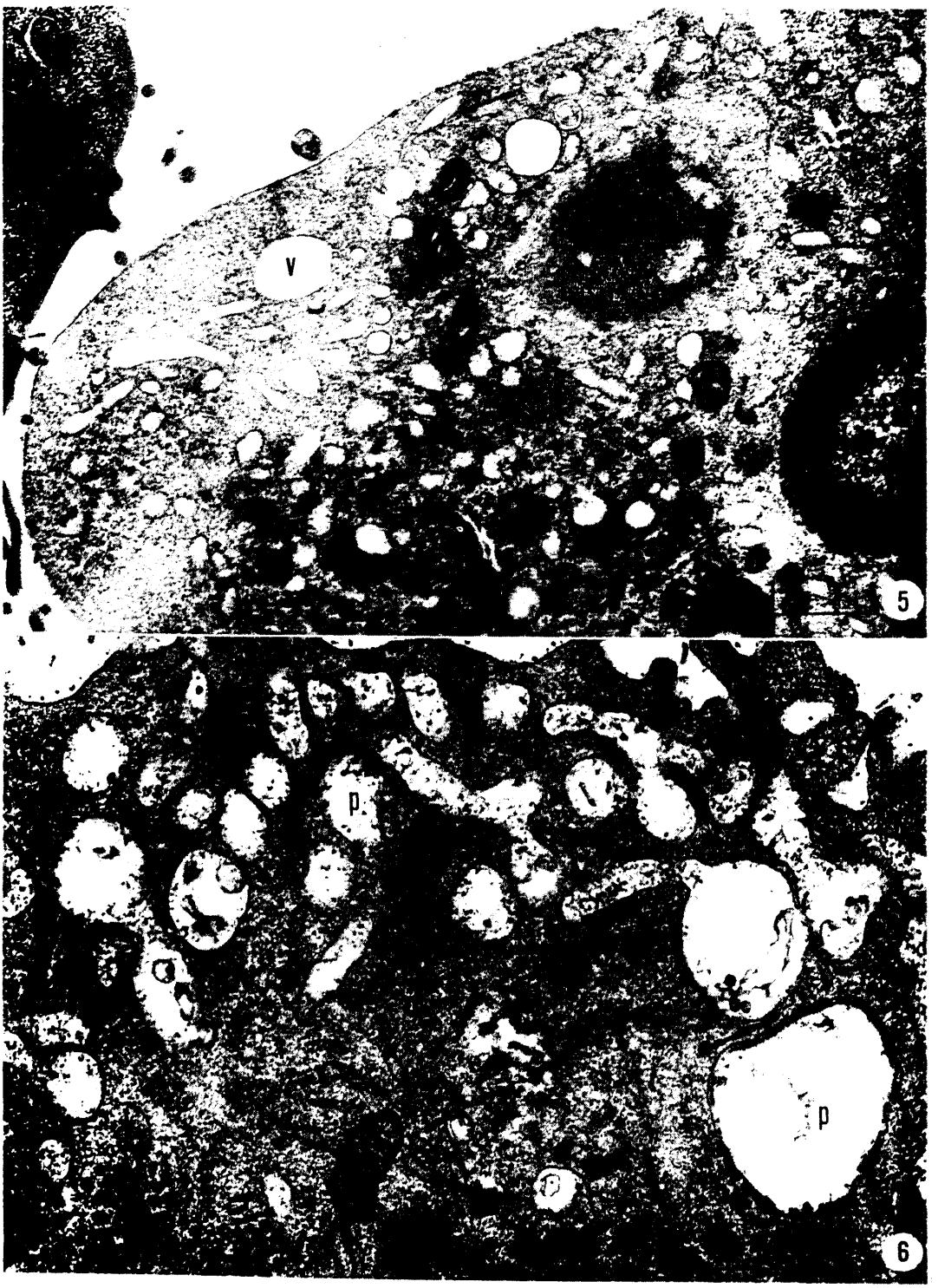
Table 1 Effects of Various Treatments on Phagocytosis

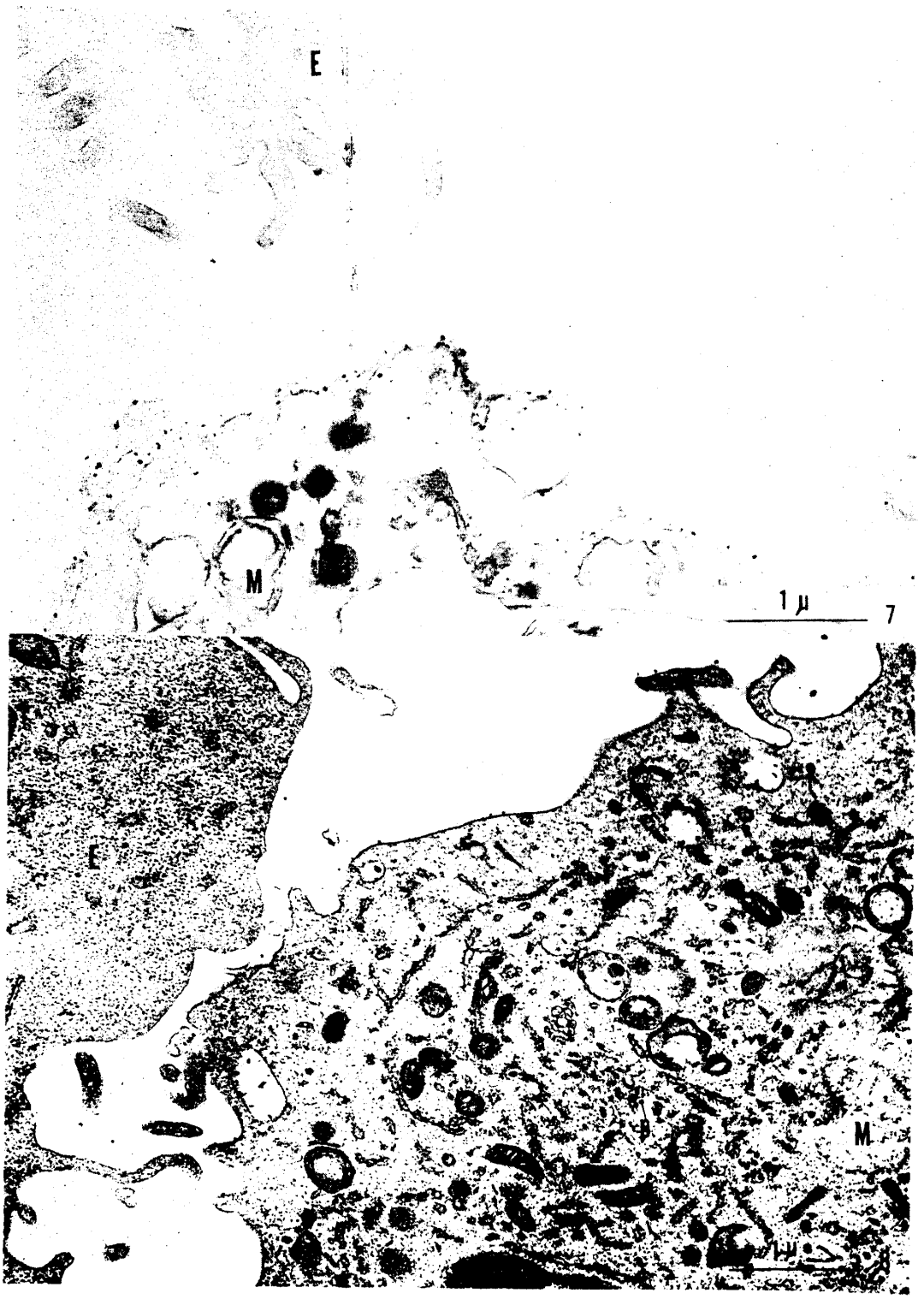
		Adhesion of the colloidal particles to cell surface	Formation of vesicles containing the colloidal particles
Without pretreatment			
Not washed with Hanks' solution before incubation with the colloid		+	+
Washed before incubation		+	+
Pretreated after washing			
Iodoacetate	10^{-4}M	+	−
	10^{-3}M	+	−
	10^{-2}M	+	−
NaF	10^{-3}M	+	±
	10^{-2}M	+	−
	2×10^{-2}M	+	−
2, 4-dinitrophenol	10^{-6}M	+	+
	10^{-5}M	+	+
	10^{-4}M	+	+
	10^{-3}M	+	+
KCN	10^{-4}M	+	+
	10^{-3}M	+	+
	5×10^{-3}M	+	+
Antimycin A	5 γ/ml	+	+
Cold	0°C	+	−
Hypotonic media			
Hanks': H_2O	2 : 1	+	+
	4 : 1	+	+
	6 : 1	all cells disrupted	

Cells were incubated with chondroitin sulfate iron colloid for ten minutes at 37°C except the cells incubated at 0°C. For the details refer to the text.

Fig. 5 Pretreated with 10^{-2} M NaF. Observations are the same as in Fig. 4. ×25,000

Fig. 6 Pretreated with the hypotonic medium (Hanks': H_2O = 4 : 1).
The cell is swollen but its outline irregular because of pseudopodia. The colloidal particles are attached onto the cell surface and ingested in the phagocytic vesicles (p). ×30,000


V
5
P
P
6


E
M
1 μ
7
E
M
1 μ

DISCUSSION

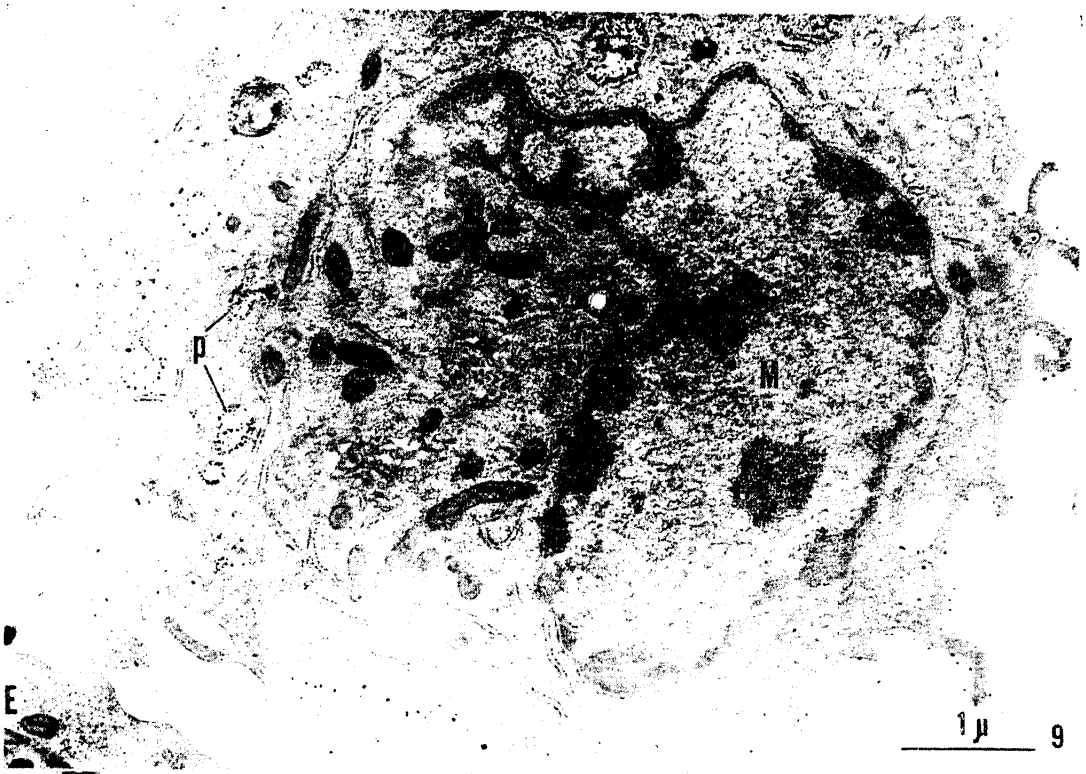
It is quite strange why the tumor cells do not take up the iron colloid particles in the environments where macrophages show a marked phagocytic activity, because the tumor cells take up proteins with pinocytosis as revealed under fluorescent microscopy (HOLTER and HOLTZER[17], 1959) and the colloidal particles are small enough to be taken up by pinocytotic mechanism. For the clarification of this curious phenomenon, more profound knowledge for the phagocytic mechanism is needed. The present experiments seem to give some plausible evidences for this phenomenon.

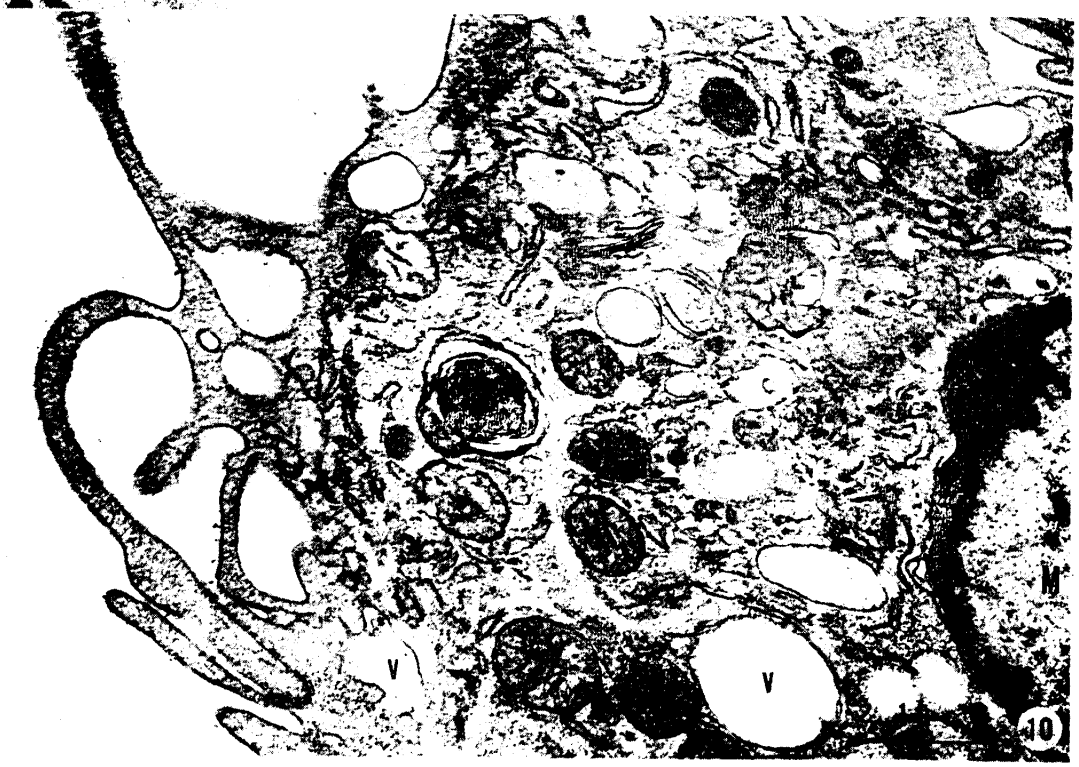
The first to be noted is that there is distinct difference betweeen the adhesiveness of the colloidal iron particles to their cell surface; namely, the particles adhere to the macrophages but not to the tumor cells. Therefore, the cell surface of macrophages should have some chemical groups of specific affinity to these colloid particles, which will be lacking on the tumor cell surface. As the colloid particles are known to be charged negative, the supposed groups on macrophages having the affinity to the colloid particles will be charged positive. Their positive charge will not be so strong as to govern the charge of the cell, because negatively charged tumor cells (LANGLEY *et al.*[18], 1958) never adhere to the macrophages. The electron microscopic pictures show that the colloidal iron particles are adhered to the cell sourface of macrophage at certain distance (50 to 200 mμ) apart but never covering the entire area of the cell membrane. This seems to indicate that the groups having the affinity to the colloid particles are distributed sparsely on the cell surface. Supposing that the colloid particles would adhere to the tumor cell surface being mediated by histone or other basic proteins, the tumor cell should take up the particles by phagocytosis (SENO *et al.*[19], 1967). In any event, the adhesion of the particles should be a definitive and indispensable factor for the phagocytosis and pinocytosis (HOLTER and HOLTZER[17], 1959; RYSER *et al.*[20,21], 1962a, 1962b), and their surface would have the groups of specific affinity to the proteins and no groups to adhere the iron colloid particles.

The adhesion of the colloidal particles to the cell surface should be a simple chemical reaction. It is no energy-requiring process as has been clearly demonstrated in this experiment by using various inhibitors for glycolysis and respiration, uncoupler for oxidative phosphorylation, and cold environments. None of these

Fig. 7 Pretreated with the hypotonic medium. The colloidal particles adhered onto the cell surface of a disrupted phagocytic cell (M), but none on EAT cell (E). ×25,000

Fig. 8 Pretreated with 10^{-4} M 2,4-dinitrophenol. The colloidal particles are adsorbed onto the cell surface and ingested into the phagocytic vesicles (p) as in the cell without pretreatment. ×20,000


D
M
E
1μ
9


V
V
M
10

pretreatments induced the inhibition of the adherence of the colloid particle onto the cell surface of macrophages.

As the second step, the ingestion of the adhered particles is another thing from adhesion. The uptake of the adhered particles into the phagocytic vesicles will be an energy-dependent process, the energy produced by glycolysis. Fluoride and iodoacetate as well as the cold environments inhibit strikingly the uptake of the particles, while 2, 4-dinitrophenol, cyanide and antimycin A do not inhibit the ingestion of the adhered particles. The swelling of the cell induced by fluoride or iodoacetate will not be the factor for the inhibition of phagocytosis, as the swollen cells in hypotonic solution take up the colloid particles actively, and the cells in low temperature environments, which do not induce the cell swelling, ceased to take up the colloid particles.

The uptake of the colloid particles may be initiated by the small envaginations formed on the cell surface just at the site where the particles adhered. In morphologic picture these small envaginations are similar to the rhopheocytic vesicles (POLICARD and BESSIS[16], 1958). The small envagination will develop to a large phagocytic vesicle by the local lysis of the gel structure of cytoplasm, as the developing step can be seen morphologically. The local lysis of the gelous cytoplasm will be due to the disturbance of the molecular structure resulting from the breakage of chemical bonds among molecules to be induced by adsorbing some charged substance to the cell surface. The restoration of the lysed structure to gelous one will require energy, though the possibility that some contractile protein also would participate in the engulfing mechanism, as one of the energy-consuming processes cannot be denied. Policard is of the opinion that the rhopheocytosis is a process other than phagocytosis, but the present observation supports the view that the rhopheocytosis is nothing but the initial stage of phagocytosis as far as the macrophage is concerned.

The finished phagocytic vesicles contain a large number of the colloid particles, too numerous to be understood as that they were taken up by the simple engulfing mechanism. Therefore, as the third step of phagocytosis, there should be some mechanism for the accumulation of the colloid particles into the vesicles. The observation in this study revealed that some phagocytic vesicles are connected with the tubules containing the colloid particles. The small vesicles containing the colloid particles seem to be rather the cross-sections of the tubules than the pinching-off products of the envagination of the cell surface. These tubules

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Fig. 9 Pretreated with 5×10^{-3}M KCN. Observations the same as in Fig. 8. ×17,500

Fig. 10 A phagocytic cell pretreated in cold environment for thirty minutes, then incubated with CS-Fe in cold for ten minutes. The colloidal particles are adhered onto the cell surface but not ingested in vacuoles (v). The cell is rather swollen but its contour irregular. ×20,000

may be the pathway to carry the particles into the phagocytic vesicles. The accumulation mechanism is difficult to be explained by the findings obtained up to date, but the membrane flow theory proposed by BENNETT[22] (1956) may give an information for the mechanism, which explains the accumulation of the colloid particles by a possible conveying mechanism by the aid of the cell membrane moving inward the phagocytic vesicles. This theory may be somewhat difficult to accept because of possible disturbance of the cytoplasmic structure caused by the movement of membrane which has a number of vesicular structures deeply engulfed inside the cytoplasm. However, the outer layer of the unit membrane structure would move independently of the inner layer, as the two molecular layers of lipid attached to their non-polar tails may have smooth sliding surfaces, the sliding of which may occur with hardly any energy consumption.

Therefore, the most probable process to be considered seems to be the first step of phagocytosis in that the adherence of the particles to the surface does not require any energy, and the second step is the engulfing at the local area which would be induced by the lytic process of gelous cytoplasm, or the disturbance of the inter- and intramolecular chemical bonds, the restoration of which will need energy. The third step is the accumulation of the adsorbed colloid particles into the phagocytic vesicles by the aid of the membrane flow, which means the sliding of the outer lipid layer constructing unit membrane on the underlying lipid layer fixed to the cytoplasmic structure.

SUMMARY

For the purpose to clarify the mechanism of phagocytosis or pinocytosis, the observations on the tumor ascites, including the macrophages as well as the tumor cells, were carried out by incubating with the iron colloid with or without pretreatment by several inhibibitors of glycolysis, oxidative phosphorylation and respiration, or under hypotonic or cold environments.

The results have demonstrated that there are three steps in the phagocytosis. The first step is the adhesion of the substance to the cell surface, which is not an energy-requiring process. The second step is the engulfing which proceeds by using the energy supplied by glycolysis. The third is the accumulation of the substance into the vesicles through the canaliculi connecting the cell surface with the vesicles.

The discussion was made on the existence of the active site on the cell surface to which the substance can be adhered, and the accumulation mechanism of the material into the phagocytic vesicles by the membrane flow, the flowing movement of the outer lipid layer of a unit membrane through the canaliculi which connect the cell surface to the phagocytic vesicles.

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