A morphologic study of protein ingestion by Ehrlich ascites tumor cells

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Abstract

For the purpose to obtain the information of the mechanism of protein uptake by the tumor cells, some cytochemical and electron microscopic observations were carried out on Ehrlich ascites tumor cells incubated with horseradish peroxidase (basic hemoprotein, molecular weight approximately 40,000) in vitro. In the earlier periods of the incubation peroxidase was found to be adsorbed on some area of surface of the tumor cells forming a thin protein layer, where an active pseudopodia formation was observed. With the lapse of time, the protein was taken in the deep cytoplasm by the infoldings of the cell membrane and accumulated in the cytoplasmic vesicles having limiting membrane. Concerning the accumulation of the protein into the vesicles, small tubular structures in the cytoplasm connecting the cell surface and the vesicles, were considered to participate in the intracellular transportation of peroxidase taken up. In cold environment (2°C), the formation of pseudopodia and deep inward infoldings of the cell membrane was inhibited and simultaneously the uptake of peroxidase stopped. Iodoacetate and sodium fluoride also effected to suppress the pseudopodia and infoldings formation moderately, as well as uptake of peroxidase, though they did not stop completely. These facts have indicated that horseradish peroxidase is taken up by Ehrlich ascites tumor cells through pinocytosis which involves energy-requiring process dependent upon glycolytic metabolism of the tumor cells.
A MORPHOLOGIC STUDY OF PROTEIN INGESTION
BY EHRLICH ASCITES TUMOR CELLS

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All the living cells take up the nutrient selectively from external environment in order to sustain their lives, except the scavenger cells which are functionally differentiated to take up foreign bodies and decompose cell elements only for the purpose to clean up the environment. Recently, many evidences have been accumulated that certain macromolecules may exert biological effects to the cells giving profound effects on the cell metabolism (1—5). These suggest that extracellular macromolecules penetrate into the cell through its membrane by some way. On account of that, the penetration mechanism of macromolecules into the cells is of great interest as the initial step of these phenomena.

The process of penetration of substances into the cells is generally described as dialysis, phagocytosis, pinocytosis and rhopheocytosis as schematically reviewed by Policard and Bessis (6). While a number of cytophysiological, cytochemical and electron microscopic studies have revealed the mechanism of phagocytosis by polymorphonuclear leucocytes, macrophages and cultured fibroblasts in detail, but a few data yet have been obtained about the mechanism of macromolecule uptake as nutrients by the cells such as tumor cells having no phagocytic activity like macrophages (7—19). In recent years, it has been demonstrated that I$^{19}$-labeled human serum albumin penetrates into tumor cells by the endeavouring works of Ryser and his colleagues (10, 15—19). However, as pointed out by themselves, some problems still remain and no definite data are presented on the morphological sequences of the uptake process. And yet, in their quantitative studies incidental adsorption of macromolecule to the cell surface may make the data ambiguous, since macromolecules tend to be adsorbed to the cell surfaces being bound tightly and in some cases the amount of adsorption is far from negligible to that of uptake (10). In addition, the macromolecules invade injured cells and bind more firmly to cytoplasmic structures than to cell surfaces and can be bound to the injured cells in amounts far exceeding the physiological adsorption or
up-take (16). Beside these, it is reported that ferritin and hemoglobin molecules are taken up by tumor cells by membrane vesiculation; namely, pinocytosis (9, 13), but little is known about the route of penetration of the other substances through membrane. This paper deals with the uptake of horseradish peroxidase, a basic plant protein of molecular weight approximately 40,000 by Ehrlich ascites tumor cells and shows that peroxidase is adsorbed to the cell surface and then taken up by pinocytosis.

MATERIALS AND METHODS

Ehrlich ascites tumor cells maintained in ddN mice were used throughout this experiment. The tumor ascites was harvested 7 to 8 days after intraperitoneal inoculation of 0.2 ml of ascites fluid in each mouse. The harvested ascites fluid was poured into tubes containing a large amount of cold Hanks' solution and centrifuged for 5 minutes in cold at 200 g. The supernatant was discarded and the pellet was washed with cold Hanks' solution 5 times with repeated centrifugation, 5 minutes at 200 g. Finally the cells were resuspended in Hanks' solution at the cell population of $10^8$ cells/ml, and 1 ml of the freshly prepared cell suspension served as material for each observation.

The cells were replaced in 1 ml of horseradish peroxidase solution, which contains peroxidase at the concentration of 6 mg/ml in Hanks' solution, and then incubated under various conditions. The incubation was done in polyethylene test tubes at 37°C for 5 to 60 minutes in shaking incubator, shaking frequency 60/minute. Some cells were incubated with Hanks' solution alone under the same conditions and served as controls. After the incubation with peroxidase one series of cell suspension were washed with cold Hanks' solution 5 times in order to remove the excessively adhered peroxidase on the cell surfaces. For the observation of the effects of pH (6.2–8.5) in environment on the protein uptake the newly harvested cells were washed with cold Hanks' solution whose pH was adjusted with phosphate buffer, and then incubated at 37°C for 15 minutes in Hanks' solution containing horseradish peroxidase and having the same pH as the washing media. In all the other series of experiments, incubation was done at pH 7.4.

Incubation with peroxidase in ice cold was done for 15 minutes, after the preincubation of the cells with Hanks' solution at 2°C for 30 minutes. One series of cell suspensions were treated with metabolic inhibitors such as iodoacetate, sodium fluoride, 2,4-dinitrophenol, potassium cyanide and antimycin A. In these series, cell suspensions were previously incubated with Hanks' solution containing each inhibitor for 30 minutes at 37°C, then replaced in the incubation media containing both horseradish peroxidase and each inhibitor, and incubated for 15 minutes at 37°C. To check the effects of metabolic inhibitors on horseradish peroxidase, one series of cell suspension was incubated with peroxidase alone for 15 minutes at first and then treated with each inhibitor for 30 minutes at 37°C. After incubation, the survival test was made with one droplet of cell
suspension by supravital staining with 0.05% eosin solution.

For light microscopic observations, the incubated cells were washed with cold Hanks' solution 3 times and one droplet of cell suspension was mixed with an equal amount of mouse serum and smeared on the slide glass, dried, fixed with ethanol and stained by Sato-Sekiya's method for peroxidase reaction. For electron microscopic observation, the incubated cell suspensions were mixed with excess amount of 2.5% glutaraldehyde in cold Millonig's phosphate buffer, pH 7.2 (20), centrifuged in cold for 5 minutes at 200 g and then the fixative was discarded. The fixed cells were washed with cold Millonig's phosphate buffer 5 times through repeated centrifugation and stored in the same buffer at 0°C overnight. For cytochemical demonstration of peroxidase activity under the electron microscope the method developed by Graham and Karnovsky (21) was employed. The fixed cells were washed twice with 0.05 M Tris-HCl buffer, pH 7.6, and exposed to the reacting media for peroxidase containing 0.02% H2O2, 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer pH 7.6. Immediately after mixing them, the cell suspensions were centrifuged for 5 minutes in cold at 200 g and the supernatant was discarded. After washing twice with Millonig's phosphate buffer, pH 7.2, the cells were postfixed with 1% OsO4 in cold Millonig's phosphate buffer, pH 7.2, and washed with cold distilled water 5 times through repeated centrifugation. Finally, they were packed in small polyethylene tube to make the small blocks (22) and dehydrated through graded ethanol series. These dehydrated blocks were embedded in Epon 812 mixture (23) through propylenoxide (24). Thin sections were made with Porter-Blum MT-1 ultramicrotome, stained with 5% uranyl acetate and/or alkaline lead solution and observed in electron microscope Hitachi HU-11A.

The peroxidase used in this experiment was horseradish peroxidase type II (Sigma Chemical Co.) and horseradish peroxidase (Worthington Biochemical Corp.) and the inhibitors used were iodoacetate (Wako Junyaku Co.), sodium fluoride (Okayama Yakuhin Co.), 2,4-dinitrophenol (Wako Junyaku Co.), potassium cyanide (Wako Junyaku Co.) and antimycin A (Kyowa Hakko Co.)

RESULTS

The horseradish peroxidase used for the present experiment proved to be positively charged in Hanks' solution as revealed by acetate film electrophoresis in barbiturate buffer pH 8.3 and its activity was maintained throughout the treatment with 2.5% glutaraldehyde for 20 minutes in cold. Under light microscope, the ascites tumor cells, which were exposed to peroxidase in vitro for 15 minutes and treated with Sato-Sekiya's method for peroxidase, showed some small granules with peroxidase reaction. The granules were found mainly in the peripheral cytoplasmic area of the tumor cells. The peroxidase positive granules were generally found only in one limited area of the cytoplasmic process. The contaminated macrophages gave an intense reaction filled with the peroxidase positive
droplets diffusely distributed in the whole cytoplasmic area. The tumor cells incubated with the media containing no peroxidase gave no positive reaction in their cytoplasm but the contaminated macrophages gave a marked positive reaction of peroxidase revealing the endogenous peroxidase positive granules. Ehrlich ascites tumor cells seem to have no recognizable endogenous peroxidase as far as light microscopic observation is concerned. No distinct difference was found between the reaction products of endogenous and exogenous peroxidase in macrophages, though some slight difference in reaction intensity among the granules.

By electron microscopy, the ascites tumor cells, which were not exposed to horseradish peroxidase but treated with Graham-Karnovsky’s full medium for peroxidase, showed no peroxidase positive granules indicating the absence of endogenous peroxidase activity. The cells appeared round with short villi-like processes moderate in number and evenly distributed on the whole cell surfaces and large nuclei situated in the central area of the cells. Occasionally, the tumor cells showed protrusion of cytoplasm on one end of the cells toward which nucleus was often concaved. In cytoplasm they had numerous dense free ribosomes and some poorly developed rough surfaced endoplasmic reticula, irregular shaped mitochondria, having the cristae of unusual orientation. Small vesicles were sometimes encountered just under the cytoplasmic membrane and large lipid droplets and cytosomes were also observed in the cytoplasm. Occasionally, virus-like particles which had electron dense core structure were found being diffusely scattered in cytoplasm. Golgi apparatus was of poorly developed. The contaminated leucocytes contained the peroxidase positive

EXPLANATION OF FIGURES

All figures are electron micrographs of Ehrlich ascites tumor cells. With the exception of Fig. 1, all tumor cells are incubated with peroxidase at pH 7.4. For peroxidase-incubated preparations, incubation times are indicated in parentheses. Bar length equals 1 μ in all electron micrographs.

Figure 1. Ehrlich ascites tumor cell incubated with Hanks’ solution alone at 37°C for 10 minutes and treated with Graham-Karnovsky’s full medium. The tumor cell shows round appearance with a few pseudopodia on the cell surface. No staining with reaction products for peroxidase is revealed in the tumor cell. × 5,000

Figures 2—9. Tumor cells incubated with peroxidase at 37°C

Figure 2. Tumor cell incubated with peroxidase (P.O) for 5 minutes. The cell shows a bizarre protrusion (Pr) on one end of the cell, which is composed of grouping pseudopodia. Nucleus is flattened toward the protrusion. Poorly developed Golgi apparatus (G) is seen near the flattened nucleus. Cell membrane increases its density with thin coating of reaction products, but no remarkable staining with the reaction products is seen in cytoplasm. × 7,500
Protein Ingestion by Ehrlich Tumor Cells

granules and erythrocytes showed a marked increase in electron density, while the tumor cells did not show any reaction products in any parts of the cells (Fig. 1).

By a short time incubation (5—10 minutes) with horseradish peroxidase at pH 7.4, many tumor cells showed tongue-like protrusion at one end of the cells, on which numerous cytoplasmic processes or rod-like pseudopodia were vigorously protruded, showing the appearance of dense grouping of microvilli, while other parts of cell surface were less in the pseudopodia in number and rather smooth. In many cases the extrusion having a number of rod-like pseudopodia was formed only on the side toward which the nucleus was concaved or flattened. In addition, irregular infoldings of the cytoplasmic membrane at the root of the crowded pseudopodia were observed forming complex network of irregularly shaped vesicles or tubules. The reaction products for peroxidase were found to be adsorbed thinly on the outer surface of cell membrane increasing in electron density of the coating membrane, but they were more densely accumulated in the area of the protruding cytoplasm having numerous pseudopodia and infoldings (Fig. 2). However, in this area the reaction products were not found in the superficially located larger vesicle and vacuole. Repeated washing of the cells with cold Hanks' solution did not lose the reaction products nor changed the morphologic appearance.

Incubation for 10—20 minutes with peroxidase resulted in an increase in the density of the reaction products in the infoldings and invaginations formed at the root of the grouping pseudopodia on the cytoplasmic extrusion (Figs. 3 and 4). The vesicles with positive peroxidase reaction reached into the deeper cytoplasmic area and some narrow tubular or channel-like canaliculi or semicircular rounded tubules and single or sometimes double circle, where the reaction products appeared. These semicircular tubules or irregular circles with the limiting membrane, and filled with intense reaction products, were found in structureless cytoplasm, and sometimes such structure was surrounded by another circular structure of the same nature. Peroxidase positive vacuole-like structures in diameter of 0.4—0.6μ were frequently observed near the cell surface, but they also often con-

Figure 3. Tumor cell incubated with P.O. for 10 minutes. The cell is slightly elongated in appearance. A conspicuous tongue-like protrusion of cytoplasm having a crowd of pseudopodia is formed. The reaction products (PO) are seen in the roots of the grouping pseudopodia and in the cytoplasmic protrusion. × 12,500

Figure 4. Tumor cell incubated with P.O. for 20 minutes. Nearly vertical section involving cytoplasmic protrusion shows that the cell membrane at the roots of pseudopodia extends inward forming envelop-like infoldings (I) and reaches to deep cytoplasm. The reaction products are seen along the infoldings. × 25,000
tained cytoplasmic process or pseudopodia in the central portion. They may be the tangential cut of the undulating infoldings and processes of the cell surface. Smaller vesicles with diameter of approximately 0.05—0.1 μ and filled with dense reaction products were frequently observed in the superficial to the midportion of the cytoplasm. They were often aligned with the tubules of equal width and sometimes slightly elongated vesicles were situated in the same direction alternatively contiguous with short tubules of the same width (Fig. 6). In addition to the alignment of these smaller vesicles, some tail-like structures of the larger vesicles were sometimes encountered in the deep cytoplasmic area (Fig. 7). Fuzzy coating of reaction products of peroxidase was observed in the inner surface of these vesicles and tubules. In contrast to the tubules or smaller vesicles, the pinocytic vesicles were situated mainly in midportion of cytoplasm and were scarcely encountered just under the cell membrane in the case of Ehrlich ascites tumor cells. Most of these vesicles were found to be situated in the cytoplasm at the side of cytoplasmic protrusion having pseudopodia and infoldings but not in opposite side. Besides amorphous or fuzzy coating of the reaction products on the inner surface of vesicles, they also contained amorphous or fragment-like substances coated with the reaction products which were considered to be desquamated cell membranes as described by Yokomura et al. (22) (Figs. 7 and 8).

By incubating the tumor cells with horseradish peroxidase for 30—60 minutes the reaction products were more densely accumulated in pinocytic vesicles and larger vacuoles, around which many smaller vesicles and tubules were encountered. In addition, these pinocytic vesicles densely filled with reaction products were also observed not only in the cytoplasmic

Figures 5—8. Tumor cells incubated with P. O. for 20 minutes

Figure 5. Besides vacuolar structures containing pseudopodia (V), some circular structures (arrow) having narrow spaces limited by membrane are seen in the transverse section of the cytoplasmic protrusion. Their narrow spaces are filled with the reaction products. × 3,0000

Figure 6. The transverse section of cytoplasmic protrusion shows many straight or slightly winding figures which resemble to canaliculi and contain the reaction products. Small vesicles of the same diameter as the width of canaliculi are also seen. Some of them are aligned with the canicular structure (arrow). × 25,000

Figure 7. The canicular structures extend into deep cytoplasm. Near the nucleus a vesicle appears having a “tail” (arrow). Moderate amount of the reaction products are found to be accumulated in the vesicle. × 25,000

Figure 8. Large amount of the reaction products are found in pinocytic vesicles (Pv). Small vesicles and tubules containing the reaction products are seen near the pinocytic vesicles (arrow). The reaction products are always found in the spaces which are limited by the membrane. × 50,000
Protein Ingestion by Ehrlich Tumor Cells

Sogabe: A morphologic study of protein ingestion by Ehrlich ascites tumor
extrusions having pseudopodia, but even in opposite side, though the polarity of the cells seemed to still be maintained as judged from the morphologic pattern (Fig. 9). The exact interrelationship of localization of these vesicles and Golgi apparatus was not established, though some pinocytic vesicles containing reaction products were often seen near the concaved part of nuclei, around which Golgi apparatus was often encountered. No obvious reaction products deposited in the rough surfaced endoplasmic reticula, mitochondria and cytoplasmic matrix throughout the experiments.

Changes in pH of the incubation media in the range of 6.8 to 8.2 did not show any alteration in the pattern of the deposition of the reaction products in the tumor cells. In the media of pH lower than 6.5 the reaction products showed a tendency to aggregate on cell surface. The tumor cell nuclei appeared translucent with defective staining and the cytoplasmic processes and pseudopodes diminished. Occasionally, the tumor cells engulfed the aggregated reaction products forming small pinocytic vesicles filled with the granular dense reaction products. In the media pH higher than 8.5 the cell membrane was disrupted in many tumor cells and the rough surfaced endoplasmic reticula, nucleoplasm and swollen or ruptured mitochondria dispersed out of the cytoplasm. The reaction products were usually found on these broken cell fragments and membranes of various intracellular organellae but being deposited nonspecifically. The findings indicated that the observation of the pinocytosis of Ehrlich ascites tumor cells can be made in the range of pH 6.8 to 8.2.

The tumor cells incubated with peroxidase at pH 7.4 in ice cold (2°C) for 15 minutes after previous incubation with Hanks' solution alone at 2°C for 30 minutes showed somewhat round appearances with a few short digitation or pseudopodia. The extrusion of cytoplasm on one end of the cell such as seen on the cells incubated at 37°C was not observed. The cell membrane was thinly coated with the reaction products and a few vesicles whose inner surfaces were also coated with reaction products were found situated near the cell membrane but the accumulation of the reaction products were hardly observed. Small invaginations of cell membrane

Figure 9. Tumor cells incubated with P. O. for 60 minutes. Vesicles containing the dense reaction products are distributed in deeper portion of whole cytoplasm. The grouping pseudopodia are still formed on one end of the cells. ×5,000

Figure 10. Tumor cell incubated with P. O. for 15 minutes in ice cold (2°C). The cell has a few pseudopodia but does not form a cytoplasmic protrusion. A few small vesicles are found in the peripheral area of the cell but no remarkable deposition of the reaction products are seen in the cytoplasm. ×12,500
containing the reaction products were rarely encountered. There were no specific morphologic change in cell organelles such as mitochondria, endoplasmic reticula and nuclei (Fig. 10). The results indicated that the tumor cells take up hardly peroxidase into the cells at low temperature.

The tumor cells treated with the glycolytic inhibitors such as iodoacetate and sodium fluoride largely reduced their pinocytic activity for peroxidase but those treated with respiratory inhibitors or uncoupler for oxidative phosphorylation such as 2,4-dinitrophenol, potassium cyanide and antimycin A showed no actual change in pinocytic activity. In the experiment with the metabolic inhibitors, the tumor cells were previously incubated with each inhibitor for 30 minutes at 37°C and then replaced in the media containing both inhibitor and peroxidase and incubated for 15 minutes at 37°C. The pH of incubation media containing inhibitors was 7.4 being adjusted with 0.1N HCl or 0.1N NaOH. The tumor cells incubated with peroxidase for 10 minutes and then treated with each metabolic inhibitor for 30 minutes at 37°C showed an intense deposition of the reaction products as in the cell receiving no treatment with inhibitors. Therefore, individual metabolic inhibitors do not cause any significant decrease in the peroxidase activity at the concentrations used in the present experiment. Treatment with iodoacetate at the concentration of 10^{-3}M, 10^{-4}M and 10^{-5}M resulted in a slight swelling of the tumor cells, which became somewhat round in appearance with poorly distributed pseudopodia on the cell surfaces. Most of nuclei were situated in the central portion of the cells and mitochondria were slightly shrunken with dense matrices. It was not so infrequent to encounter some pseudopodia grouped at one end of the cells and forming membraneous infoldings into cytoplasm at their roots, but not forming prominent tongue-like extrusion. The reaction products thinly coated the cell surfaces and were densely deposited in the membraneous infoldings under the grouped pseudopodia. The reaction products were also deposited in the vesicles, tubules and semicircular spaces having the limiting membrane in the cytoplasm. In addition, these figures of reaction products were frequently found in cytoplasm of the cells which did not show the grouping pseudopodia (Fig. 11). The tumor

Figures 11—15. Tumor cells incubated with P.C. and inhibitors for 15 minutes at 37°C after the preincubations with individual inhibitors for 30 minutes at 37°C.

Figure 11. Tumor cell treated with 10^{-3}M iodoacetate. The cell is moderately swollen in appearance with a small number of swollen pseudopodia. Inward membraneous infoldings are markedly reduced but the reaction products are still observed in the cytoplasm. × 15,000

Figure 12. Tumor cell treated with 10^{-3}M sodium fluoride Observations are the same as in Figure 11. × 30,000
Protein Ingestion by Ehrlich Tumor Cells

cells treated with sodium fluoride at the concentrations of $10^{-3}$M and $10^{-2}$M showed the picture identical with that of the cells treated with iodoacetate, except for mitochondria which were not so shrunken (Fig. 12). In both cases, intracellular deposits of the reaction products were less frequently encountered than in cases of untreated samples, but more frequently than in the cases incubated in ice cold. As the result, it was revealed that both of iodoacetate and sodium fluoride led the tumor cells to decrease in the ability to form the cytoplasmic protrusion having grouped pseudopodia with vigorous infoldings of cell membrane, and diminished the intracellular deposition of the reaction products, though far from complete suppression. The treatment with 2, 4-dinitrophenol at the concentration of $10^{-3}$M, $10^{-4}$M and $10^{-3}$M, and potassium cyanide at $10^{-4}$M, $10^{-2}$M and $10^{-3}$M and antimycin A at 57/ml caused no significant effects on the formation of the pseudopodia and the uptake of peroxidase by the tumor cells (Figs. 13, 14, 15).

DISCUSSION

As demonstrated in this experiment, Ehrlich tumor cells take up horseradish peroxidase by pinocytosis. Horseradish peroxidase used in this experiment is a basic hemoprotein of low molecular weight approximately 40,000 (25, 26). Its enzymic activity is resistant to glutaraldehyde and had the benefits to be demonstrated even on the fixed cells, histochemically with Graham-Karnovsky's method under electron microscope and yet it has been definitely proved that the reaction products deposit accurately at the sites of peroxidase activity (21). The enzyme can easily be demonstrated under light microscope by Sato-Sekiya's method. The reaction seems to be highly specific to peroxidase, because mitochondria were not stained by this method, though erythrocytes increased moderately in electron density by incubation with the medium of Grahan-Karnovsky. Recently, 3, 3'-diaminobenzidine and cytochrome-c was reported to be utilized for the demonstration of activity of cytochrome oxidase (27), but its reaction itself is extremely weak and the possibility that reaction products of 3, 3'-diaminobenzidine and endogenous cytochrome or other

Figures 13—15. Formation of the cytoplasmic protrusion is not suppressed by following respiratory inhibitors and uncoupler for oxidative phosphorylation and the reaction products are found to deposit in the cytoplasm at the roots of grouping pseudopodia.

Figure 13. Tumor cell treated with $10^{-3}$M potassium cyanide $\times 25,000$

Figure 14. Tumor cell treated with antimycin A 57/ml $\times 12,500$

Figure 15. Tumor cell treated with $10^{-3}$M 2, 4-dinitrophenol $\times 20,000$
substrates may interfere with that of peroxidase should be overruled. Endogenous peroxidase also gives positive reaction by this method but the tumor cells have no endogenous peroxidase. Thus the deposition products observed in this experiment were interpreted to represent the sites and the activity of exogenous plant peroxidase.

The question might be raised whether the intracellular deposition of the reaction products indicates the physiological way of protein uptake by Ehrlich ascites tumor cells. It has been claimed that leucocytes form many vacuoles like pinocytic one during degeneration (28) and that tumor cells develop large peripheral vacuoles containing an appreciable number of macromolecules during cell damage (9). It was also contended that the increased albumin fixation by tumor cells is not an expression of any physiological uptake process but rather the results of pathological response to a variety of nonphysiological conditions (16). Considering these reports, the viability of the tumor cells incubated with peroxidase was tested by supravital staining with 0.05% eosin solution and the test revealed that the cells survived through the incubation. The well-maintained ultrastructure of cellular integrity within the range of pH of incubation media 6.8 to 8.2 was also that of living cells. Beyond the range of pH 6.8 to 8.2 cellular damage became prominent showing ambiguous nuclear structure and cell disintegration. The diffuse deposition of the reaction products were seen on the various cell organellae and cytmembranes of the degenerated cell. The process of pinocytosis seems to be initiated with the formation of membraneous infoldings, tubules or smaller vesicles at the area of grouping pseudopodia, and then the pinocytic vesicle will be formed and no peripheral large vacuoles be primarily formed such as observed in degenerative process of the cells. Even with prolonged incubation (60 minutes) the tumor cells did not show any large peripheral vacuoles nor diffuse deposition of the reaction products in the cytoplasm, which can be seen in the degenerated cells. Therefore, it is evident that intracellular deposition of the reaction products indicates the uptake of the exogenous peroxidase by the tumor cells as their physiological response.

The initial stage of uptake of exogenous peroxidase by Ehrlich ascites tumor cells seems to involve active protrusion of one end of the cells. It is of interest that all of the cell surface does not participate in the uptake process at the same time suggesting the intact polarity. It was not clarified, however, with static observation with electron microscope whether it results from mobility of individual tumor cells as the free cells (29) or from the functionally differentiated structures of cell surface for the uptake system (30). Peroxidase which was adsorbed on the cell surface forming thin coat
should be accumulated between the membraneous infoldings or engulfings produced probably by active undulation of cell membrane at the root of pseudopodia and transported into deep cytoplasm through the envelop-like infoldings. The superficially tangential cut surfaces of the cells showed a number of round vacuoles containing some parts of pseudopodia and some cup-shaped vacuole. These vacuoles should be indentation of the cell membrane having the opening on cell surface. In the deeper area of the cytoplasm, peroxidase was found to be densely packed in the structure having the limiting membrane of circular or semicircular form of 0.03 μ—0.1 μ in width. They should not be the rounded tubules, but cut surface of membraneous infoldings which extend into deep cytoplasm taking rolled envelop-like structures. In contrast to them, there are many straight or slightly winding tubular structures of nearly the same width as that of circular figures and peroxidase is also found to be packed densely in these figures. These structures are considered to be genuine tubules, different from cut surfaces of envelop-like structures, since many small round or oval shaped vesicles of the same diameter as the width of the tubules are aligned frequently on the same line of the tubules, showing these vesicles to be the transverse cut surface of the tubules. The pinocytic vesicles seem not to take part primarily in the uptake process, for they are hardly encountered directly beneath the cell surfaces and seem to be not formed by direct pinching off of the engulfed cell membrane in the earlier stage of peroxidase uptake. They appear often later than envelop-like infoldings and tubular structures and accumulate peroxidase with the increase in amount of contents with the progress of incubation time. The pinocytic vesicles located near the center of cells often have a "tail", a tubule at one end. They show fuzzy coating of peroxidase and very often also contain peroxidase coated fragments something like desquamated cytoplasmic membrane. Concerning these tubular system, it has been suggested that smooth surfaced endoplasmic reticula have a close relation to phagocytosis (31). In the uptake of peroxidase by Ehrlich ascites tumor cells, it is evident that the tubular system participates in the intracellular transportation of taken up peroxidase, though they cannot be identified as smooth surfaced endoplasmic reticula. Namely, the tubular system seems to communicate the pinocytic vesicles with the envelop-like membraneous infoldings or directly with cell surfaces and to convey peroxidase which adheres on its membrane. Another mechanism of intracellular transportation of the ingested peroxidase also may be explained as follows: i.e., smaller pinocytic vesicles from direct pinching off of the engulfment of cell surface migrate in the deeper area and fuse together to form larger
vesicles. The ingested peroxidase will be thus transported through the structure having limiting membrane but never by diffusion through the membrane. Morphologic indication of these incidents is generally consistent with the earlier description on pinocytosis by Lewis (32, 33).

Of particular interest is the specificity of macromolecule uptake by the tumor cells in relation to the ingestion of peroxidase. The presence of basic polyamino acids or some basic proteins enhances the uptake of albumin-131 by tumor cells (15, 17), and colloid particles are taken up Ehrlich ascites tumor cells by phagocytosis under the mediation by histone or other basic proteins (34, 35). Furthermore, the presence of negatively charged substance suppresses competitively the uptake (15). Ehrlich ascites tumor cells are reported to be of high negative charge by the sialomucoproteptide coat of the cell surface (36). These facts suggest that interaction of the positively charged particles with the negatively charged cell surface is in a close relation with pinocytosis. On the other hand, it has been reported that acetylated hemoglobin which is less basic than normal hemoglobin shows higher affinity for the uptake system of Ehrlich-Letré ascites carcinoma cells (12) and poly-D-lysine is taken up far greater than poly-L-lysine (19). According to these reports, pinocytosis is controlled not only by interaction of electrical charge but also by molecular configuration of material to be taken up. However, once the pinocytosis is induced by some component, the specificity of uptake for some substance will largely be modified, because if the pinocytosis is enhanced by some effect of basic polyamino acids, then albumin or some colloid particles in the media should be ingested simultaneously. In these cases, albumin or colloid particles may be aspirated incidentally into invaginations of the cell membrane or vesicles formed by the substances having specific affinity to the cell surfaces. Regarding this, the larger the invaginations or the pinocytic vesicles are, the less specific the uptake may become. In this respect, Ehrlich ascites tumor cells should be regarded to take up peroxidase specifically, for peroxidase as a basic protein having an affinity to tumor cell surface adheres primarily on the cell surface and is dragged into the cell through the narrow membranous infolding or tubular system extending deep into cytoplasm. In this process peroxidase adhered to the active site, if any present, of the outer surfaces of the membranous infoldings or tubular system, may be conveyed deep into cytoplasm and accumulated in pinocytic vesicles with inward movement of the cell membrane as postulated by Bennett (37). These processes, the deep infoldings or tubular system, would be noted to offer more benefits to the specificity of uptake than that of direct large pinocytic vesicle formation by engulfment and
pinching off the cell membrane.

Peroxidase uptake by Ehrlich ascites tumor cells was interrupted morphologically at low temperature. This effect of low temperature indicates that uptake involves energy-requiring mechanism as it has long been noted that most of enzymic activities and metabolism of the cells are reduced at low temperature. Iodoacetate and sodium fluoride reduced the activities to form the group of pseudopodia and inward infoldings of the cell membrane, resulting in moderate lowering of peroxidase uptake by the tumor cells, though the uptake could not be stopped. These evidences indicate that peroxidase uptake by Ehrlich ascites tumor cells partially depends upon the energy yielded by glycolysis and also that the uptake of relatively small amounts of peroxidase may undergo independently of the energy yielded by both glycolysis and oxidative phosphorylation. The adherence of substance to the cell membranes as an initial stage of uptake is an energy-independent process (22), but concerning the energy requirement for the uptake process as a whole many reports do not always come in concord. In this respect, it is claimed that phagocytosis and pinocytosis should be distinguished as the penetration process of different characters (19). However, the extent of energy requirement seems to become greater when the dimensions of the substance to be taken up by the same cells is larger. It may be correlated to the excitation of the cells involving movements of both the cell membrane and the cytoplasm. According to the hypothesis proposed by SENO (38), local lytic process of gelous architecture of protein molecules of the cytoplasm just under the cell membrane is elicited in the pinocytic process as a "cooperative phenomenon" by the interaction of cell surface with the substance which adheres on the cell membrane. Such a change in the molecular architecture of the local cytoplasm may result in engulfing or membraneous infolding or pseudopode formation. The process may be concerned with conversion of the helical structure of protein molecules of cytoplasm and cell membrane to coil structure caused by disintegration of hydrogen bonds which act to keep the three dimensional structure of polypeptide chains. The disruption should be repaired and the energy may be consumed to repair the disrupted hydrogen bridges restoring the protein in the helical structure from the coil structure and it seems to be not impossible to form enough ATP by the metabolism of cytoplasm to restore the local intramolecular change of protein. From such a viewpoint, if the cellular energy depot is insufficient for the restoration of the disrupted three dimensional structure of protein of cytoplasm and cell membrane, then the incompleteness or low activity of pinocytosis would be the result. Phagocytosis and pinocytosis
should essentially be the same phenomenon and the study of these phenomena may give an important information for the mechanism of the penetration of macromolecules, as the substance adhered to the cell surface should induce serious molecular changes both in the membrane and cytoplasm which are constructed with the high molecular architecture bound with various weak bonds.

SUMMARY

For the purpose to obtain the information of the mechanism of protein uptake by the tumor cells, some cytochemical and electron microscopic observations were carried out on Ehrlich ascites tumor cells incubated with horseradish peroxidase (basic hemoprotein, molecular weight approximately 40,000) in vitro.

In the earlier periods of the incubation peroxidase was found to be adsorbed on some area of surface of the tumor cells forming a thin protein layer, where an active pseudopodia formation was observed. With the lapse of time, the protein was taken in the deep cytoplasm by the infoldings of the cell membrane and accumulated in the cytoplasmic vesicles having limiting membrane. Concerning the accumulation of the protein into the vesicles, small tubular structures in the cytoplasm connecting the cell surface and the vesicles, were considered to participate in the intracellular transportation of peroxidase taken up. In cold environment (2°C), the formation of pseudopodia and deep inward infoldings of the cell membrane was inhibited and simultaneously the uptake of peroxidase stopped. Iodoacetate and sodium fluoride also effected to suppress the pseudopodia and infoldings formation moderately, as well as uptake of peroxidase, though they did not stop completely. These facts have indicated that horseradish peroxidase is taken up by Ehrlich ascites tumor cells through pinocytosis which involves energy-requiring process dependent upon glycolytic metabolism of the tumor cells.

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REFERENCES

stry by a new technique. *J. Histochem. Cytochem.* 14, 291, 1965


