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Visualization of Intracellular Routing of Immunotoxin by Immunogold Double-labelling

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ABSTRACT

Using immunogold double-labelling the intracellular routing of immunotoxin molecules has been studied in cultured Molt-4 cells by electron microscopy.

Molt-4 cells were incubated with anti-T cell immunotoxin consisting of monoclonal antibodies against CD2, CD5, CD8, and CD27 and ricin and colloidal gold labelled rabbit anti-ricin IgG and goat anti-mouse IgG antibodies. Cells were taken, after various intervals, and processed for electron microscope observation. The results indicate that immunotoxin conjugate molecules are internalized via coated pits by receptor-mediated endocytosis and endocytosed from the invaginations of the noncoated areas of the plasma membrane into target cells. The molecules of ricin do not disassociate from the monoclonal antibodies routing in the cells during certain period of time. They are delivered synchronously to the endosomes, vacuolar and tubulo-vesicular portions of the endosomal system. The coated pits that contained gold particles or were gold particle free were seen at the surface of the plasma membrane of Molt-4 cells. The observation may suggest the presence of multiple receptor sites at the surface membrane of target cells

KEY WORDS

immunotoxin, receptor - mediated endocytosis, immunogold labelling, electron microscopy.

INTRODUCTION

Ricin, a potent cytotoxin, consists of two functionally distinct polypeptide chains linked by a disulfide bond. The A-chain is an enzyme which, following translocation into the cytosol inactivates the ribosomal 60s subunit and interferes with protein synthesis. The B-chain is a lectin that binds to cell surface receptors (the glycoproteins and glycolipids with terminal galactose residues) [1] [2]. The mechanism of action of native ricin entry into cells involves receptor-mediated endocytosis (RME), which has been well established for a large number of ligands, such as low density lipoprotein (LDL), asialoglycoprotein, epidermal growth factor (EGF) and transferrin [3-9].

Immunotoxins are hybrid molecules formed by an antibody chemically linked to a polypeptide toxin. Monoclonal antibodies against cell surface antigens are used to direct toxins or their enzymatically active A-chains towards specific target cells [10] [11]. Only a few data are available as to the intracellular trafficking of immunotoxin. In the present study using anti-T cell immunotoxin, colloidal gold labelled anti-ricin IgG and anti-mouse IgG antibodies by immunogold double-label electron microscopic technique, we have visualized the intracellular movement of the whole ricin carried by monoclonal antibodies.

MATERIALS AND METHODS

Cell line

Molt-4 cells (human T lymphoblast line) were cultured in RPMI 1640, supplemented with 10% fetal calf serum and antibiotics at 37°C in an atmosphere containing 5% CO_2

Monoclonal antibodies

Murine anti-human T cell MoAbs: DS27, anti-CD5; JN205; anti-CD2; P218, anti-CD8; and P81, anti CD27.

Immunotoxins

MoAbs were coupled to ricin by N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) as described previously [12].

Immunogold reagents

5 nm and 15 nm colloidal gold particles were prepared according to the tannic acid method of Slot and Geuze [13]. The concentration of protein of rabbit anti-ricin IgG and goat anti-mouse IgG necessary to stabilize the gold was determined according to Wang et al. [14]. In the studies reported here, dilutions of 1:20 or 1:40 in PBS pH8.2 of the stock solutions were used.

Electron microscopy

Immunotoxins, at a final concentration of 1 X 10^{-9}M were added to the cells followed by adding both colloidal gold labelled second antibodies in the presence of 0.1M galactose. In some experiments immunotoxin was omitted, but cells were incubated with both colloidal gold labelled second antibodies. After incubating at 37°C for various intervals (5, 15, 30, 60 min and 2.5, 3.5 h) cells were washed with PBS, pH 8.2.

Following the washing procedure, cells were fixed with 3.1% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2-7.4 at 4°C for 1 h and centrifugated in cacodylate buffer for 15 min at 1500g. Thereafter the pellets were treated with 1% OsO_4 in cacodylate buffer, pH 7.2-7.4, at 4°C for 1 h, followed by ethanol dehydration and embedding in Epon 812. sixty nm-thick sections were cut with a diamond knife and collected on Formvar-coated grids and stained with uranyl acetate and lead citrate. They were examined and photographed directly in a Philips EM 400 T electron microscope operated at 80 KV.

RESULTS

After cells were incubated with immunotoxins and colloidal gold labelled anti-ricin and anti-mouse IgG antibodies for 5-30 min at 37°C, both 5 nm and 15 nm gold particles were found on the surface of the plasma membrane and the digitations of the cell surface, and in the clathrin coated pits (Fig. 1,2). Gold particles were not uniformly distributed along the plasma membrane, neither were grouped in clusters on the cell surface or appeared as accumulations in the coated pits, whereas large areas of the membrane were free of tracer.

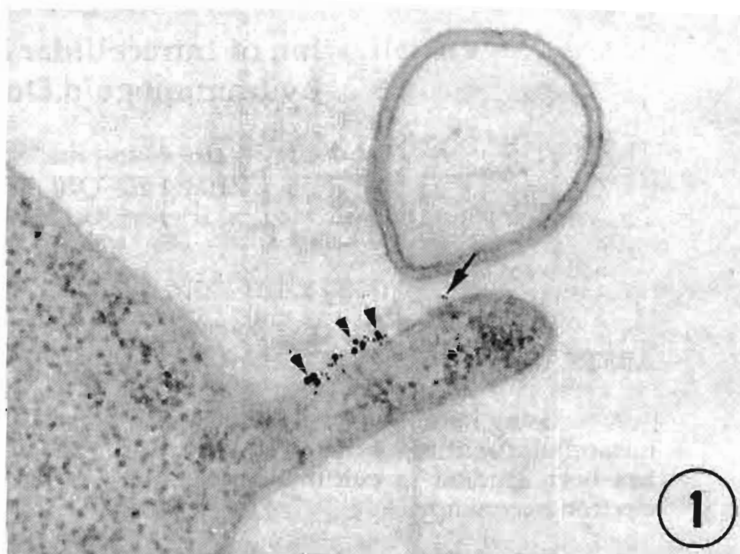

Fig. 1 Cells were incubated with immunotoxin and immunogold labelled second antibodies at 37°C for 30 min. Both antiricin IgG (15 nm) (arrow heads) and anti-mouse IgG (5 nm0 (arrows) were seen on a digitation of the cell surface.

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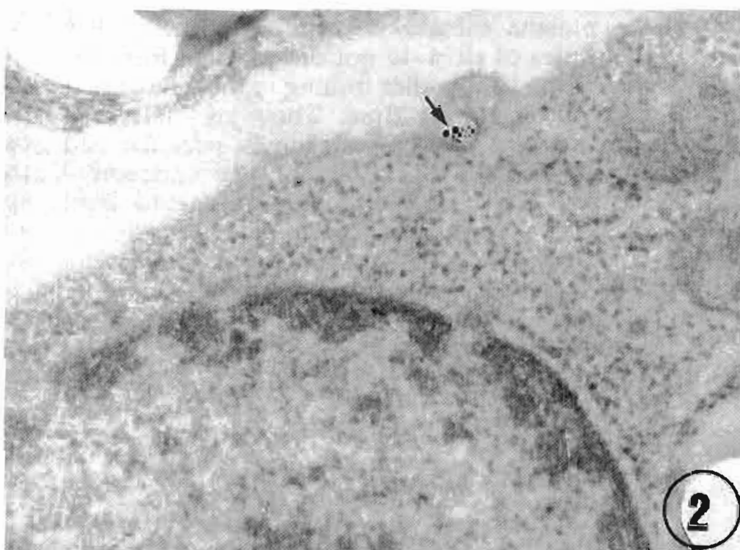

Fig. 2 Cells incubated as in Fig. 1. Immunotoxin conjugates was internalized into a cell via a coated pit (arrow).

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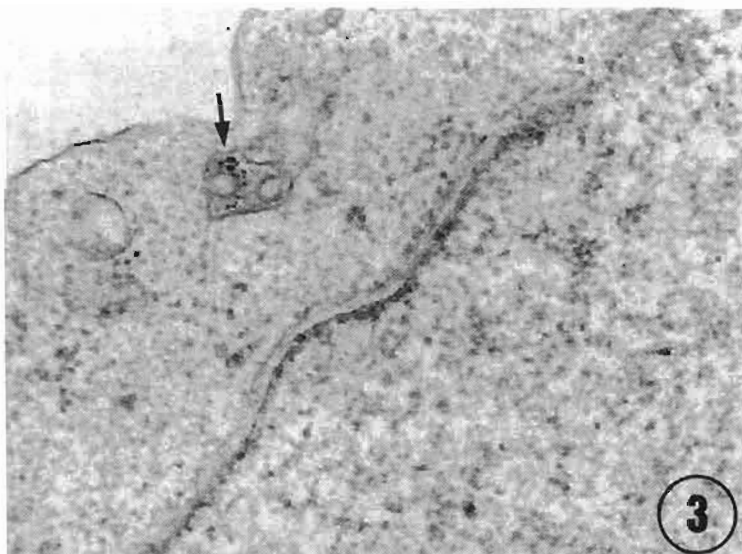

Fig. 3 Cells following incubation at 37°C for 60 min. A multivesicularbody (arrow) was labelled with 5 nm and 15 nm gold particles.
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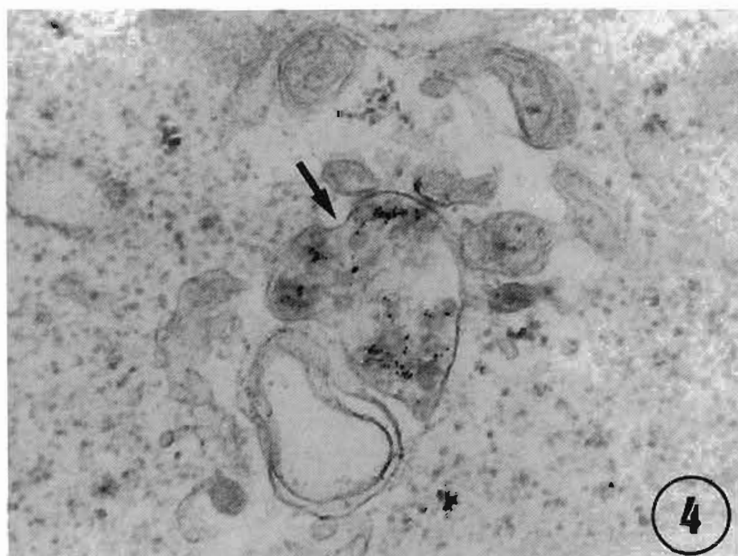

Fig. 4 Cells incubated as in Fig. 1. An endosome labelled with both 5 nm and 15 nm gold particles was found just inside a invagination of the plasma membrane (arrow).
X 68400

After 30-60 min of incubation at 37°C, both internalizaed gold particles were clearly visible in coated vesicular profiles, and in some tubular and uncoated vesicular profiles, and in large vacuolar profiles. The large vacuolar endosomes, where two sizes of gold particles typically adhered to the inner aspect of the vacuolar membrane, sometimes showed a few internal vesicular profiles, representing "multivesicular bodies" (Fig. 3). Some of endosomes containing gold particles were very close to the plasma membrane or just inside the microinvaginations of the uncoated plasma membrane (Fig. 4). Some of the double-labelled multivesicular bodies were seen near the Golgi apparatus (Fig. 5). However distinct stacked Golgi cisterns were not labelled.

After 30 min of incubation at 37°C, the lysosomes became labelled with both 5nm and 15nm gold particles (Fig. 6). Some large lysosomes contained internal vesicles and flocculent materials and others were small and dark-stained.

After 2.5 h of incubation at 37°C, the labelling of the cell surface was present, but there was less tubular and vesicular labelling. The multivesicular bodies were very seldom identified. Nevertheless two sizes of gold particles were clusterd in the lysosomes in deeper cell interior (Fig. 7). Furthermore some gold particles were seen free in the cytoplasma in small cluster or mixed with intracellular membrane fragments (Fig. 8).

After 3.5 h, some gold particles (both 5 nm and 15 nm) were still seen on the surface of the plasma membrane and coated pits in patches (Fig. 9), but the labelling was greatly decreased. Small tubular and vesicular structures were mostly unlabelled, and so were most endosomes. Many coated pits and vesicles containing none gold particles were noted at the surface of the plasma membrane (Fig. 10). Some of them were alongside coated pits and vesicles that contained both gold particles of both sizes.

For the control cells, which were not incubated with immunotoxin but colloidal gold labelled anti-ricin IgG and anti-mouse IgG antibodies, no labelling was found either at the cell surface or in the endosomal vacuolar, tubulo-vesicular structures and lysosomes.

DISCUSSION

Internalization of native ricin has been

elusidated ultrastructurally by usin ricin conjugated to markers such as ferritin [15] [16] [17], horseradish perioxidase [18], and colloidal gold [19], and in the form of a ricin-immunotoxin adsorbed to colloidal gold [20]. These studies provide the pictures of the native ricin pathway in the various cells. The intracellular routing of ricin that has been described is in agreement with many models for RME of physiological ligands. After binding to cell surface receptors, ricin conjugate molecules are internalized to reach the vacuolar and tubulo-vesicular portions of the endosomal system. From there, some molecules may be delivered to lysosomes [21-23]. Moreover some molecules (about 5%) are locate in the Golgi complex. Current evidence suggests that most of the immunotoxin must enter the trans-Golgi network, which may be of crucial importance for the inhibition of the protein synthesis, befor translocation to the cytosol occurs [24].

For immunotoxin the intrinsic binding specificity of the toxin is replaced by that of an antibody against a cell surface antigen. There is few information which demonstrates the mechanism of the action of immunotoxin in the cells. Using immunogold double-labelling to detect both antibodies and toxins we observed that immunotoxin is internalized rapidly via the coated pits and uncoated microinvaginations of the plasma membrane following binding to the cell surface antigens. Subsequently it enters into the endosomal vacular, tubulo-vesicular structures and multivesicular bodies as well as lysosomes. The results allow us to suggest that anti-T cell immunotoxin enters cells by different pathways, at least including (1) entry by a receptor-mediated event through coated pits (2) entry by the process of adsorptive micropinocytosis.

Irregular distribution of the tracers at the surface of the plasma membrane may reveal the distributive characteristics of T lymphocytic differentiation antigens at the cell surface. The possible explanation for the clusters of gold particles may be considered as the formation of the patch of immunotoxin conjugate molecules induced by divalent antibodies [20]. In addition, gold particles carrying antibody molecules also constitute a multivalent ligand capable of causing movement and clusterring of immunotoxin complexes on the plasma membrane.

In the present study, we have visualized the multivesivular bodies double-labelled around the Golgi regions, but we could not detect the localization of immunotoxin in the Golgi

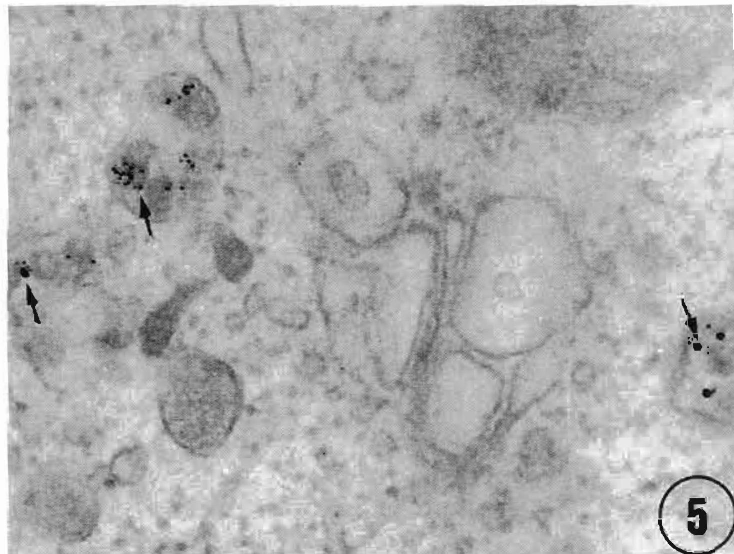

Fig. 5 Cells incubated as in Fig. 3. Multivesicular bodies labelled with 5 nm and 15 nm gold particles (arrows) were shown near the Golgi complex (G).

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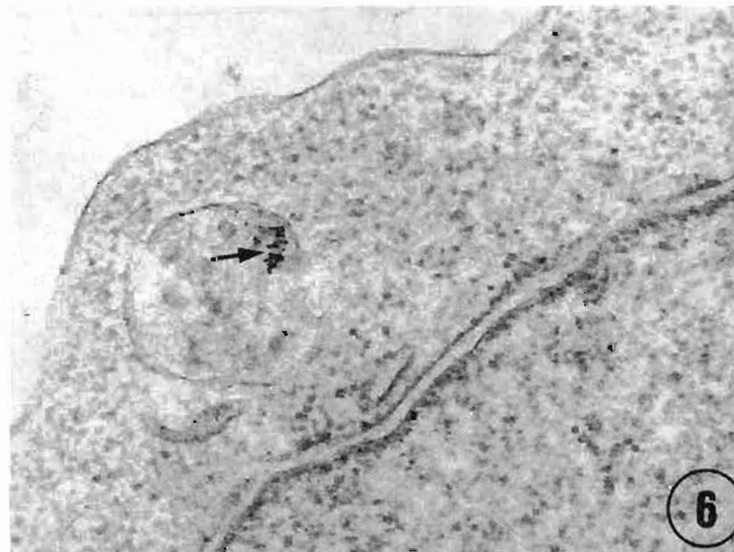

Fig. 6 Cells incubated as in Fig. 1. A lysosome was labelled with both gold particles (arrow).

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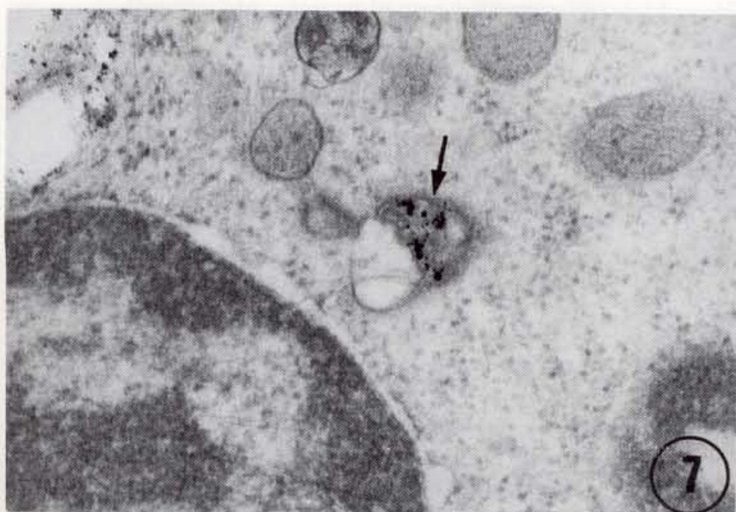

Fig. 7 Cells following incubation at 37°C for 2.5 h. A lysosome labelled with 5 nm and 15 nm gold particles (arrow) was in the proximity of a nuclear.

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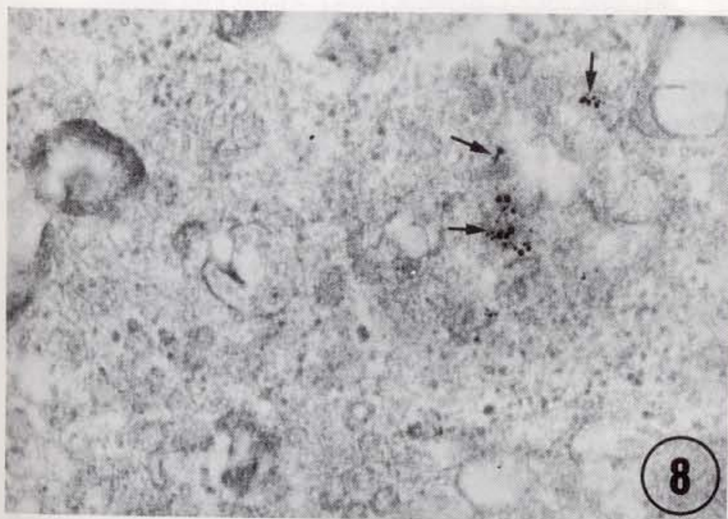

Fig. 8 Cells incubated as in Fig. 7. The gold particles were free in the cytoplasma in small cluster (arrows).

X 53200

complex. It has been suggested that an efficient binding site for immunotoxin could be one that is efficiently endocytosed and the transported to the trans-Golgi network. Moreover the toxic effect of ricin is coupled to the delivery to the Golgi complex where ricin enters the biosynthetic/exocytotic route. The conclusion drawn by Deurs et al. [24] shows that native ricin and monovalent conjugates of ricin were routed to the trans-Golgi network, whereas polyvalent conjugates containing several ricin molecules were routed to the lysosomes. Mellmen et al. [25] and Mellman and Plutner [26] found that macrophage Fc-receptors tagged by polyvalent IgG were directed to lysosomes only, whereas monovalent Fab-FcR complexes are rapidly returned to the cell surface. Therefore it can be postulated that the valency of the conjugates may be important for the determination of the intracellular fate of immunotoxin conjugates. It have also been noted that aggregation of immunotoxins by a second antibody to induce capping is likely to route the conjugates to the lysosomes.

For immunotoxin, it must remain intact in the circulation for a considerable period of time and be able to penetrate through capillary walls and into tumors. Our study indicates that ricin did not disassociate from monoclonal antibodies. The antibodies and ricin were internalized and delivered to the endosomal system synchronously. Much less is known about the translocation of the natural toxins and immunotoxins. How immunotoxin acrosses intracellular membrane barriers to reach the cytoplasma is not clearly defined, although we have observed the gold particles were free in the cytoplasma. It has been estimated that the entry of a single molecule of toxin into the cytosol may be sufficient to kill a cell because of its high enzymatic activity. Therefore it may be technically impossible to trace a small number of immunotoxin molecules which has successfully reached their targets.

After incubation for 2.5 - 3.5 h at 37°C it was found that coated pits and vesicles that contained gold particles or were gold particle free were in close proximity to one other. This observation suggests the presence of membrane or pits with different populations of receptors. However we cannot exclude the possibility that the loss of the labelling by all treatment (fixation, washing, immunoincubations).

RESUMEN

Por medio de microscopia electrónica y técnicas de doble marcaje con oro coloidal se ha estudiado la ruta de las moléculas de inmunotoxina células cultivadas Molt-4

Las células Molt-4 fueron incubadas con una inmunotoxina anti-células T, consistente de anticuerpos monoclonales contra CD2, CD5, CD8, y CD27 conjugados con ricin. Las células también fueron incubadas, al mismo tiempo, con anticuerpos (IgG) de conejo anti-ricin marcados con oro coloidal y con anticuerpos (IgG) de cabra anti-ratón marcados con oro coloidal. Las células se incubaron a diferentes periodos de tiempo y se procesaron para su observación en el microscopio electrónico.

Los resultados indican que las moléculas de inmunotoxina conjugada son internalizadas vía cavidades cubiertas, por endocitosis mediada por receptores y endocitadas desde las invaginaciones de áreas no cubiertas de la membrana citoplasmática en las celulas blanco. Por un cierto periodo de tiempo, las moléculas de ricin no se disocian de los anticuerpos monoclonales que están en ruta hacia el interior celular. Ellas son liberadas sincrónicamente a los endosomas y a las porciones vacuolares y tubulo-vesiculares del sistema endosomal. Las cavidades cubiertas, conteniendo partículas de oro coloidal o libres de ellas fueron vistas en la superficie de la membrana citoplasmática de las células Molt-4. Las observaciones parecen sugerir la presencia de múltiples sitios receptores en la superficie de la membrana citoplasmática de las células blanco

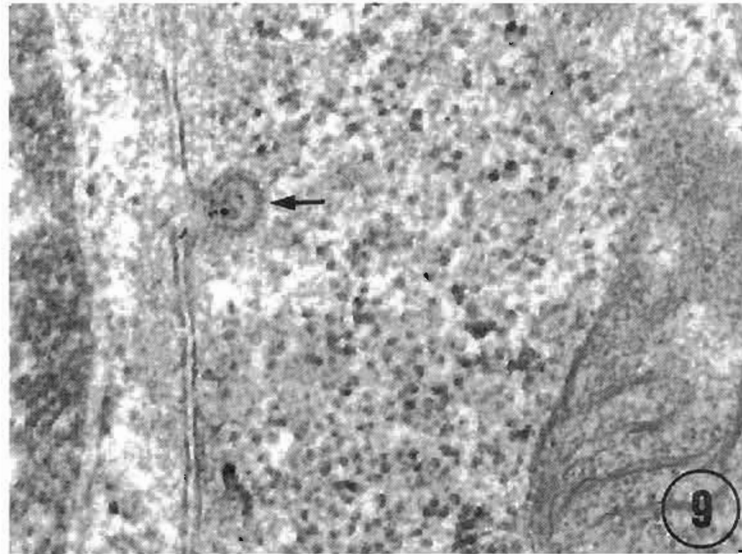

Fig. 9 Cells following incubation at 37°C for 3.5 h. Distinct double-lablling was seen in a coated pit (arrow).
X 70000

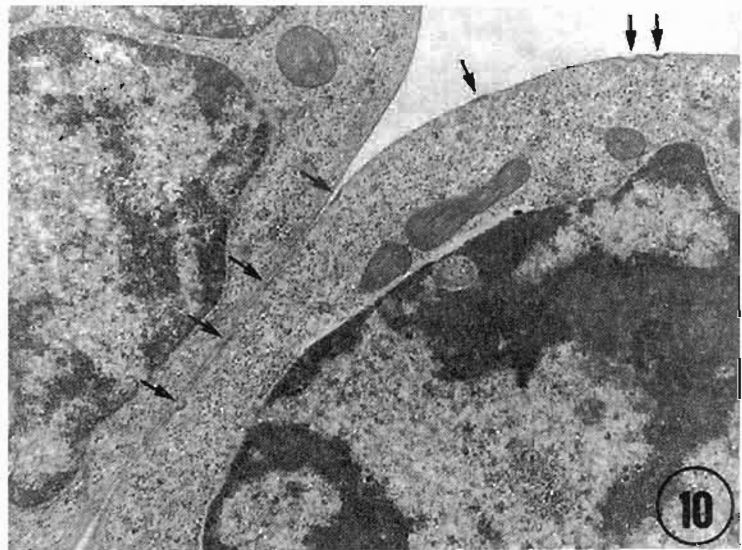

Fig. 10. Cells incubated as in Fig. 9. Coated pits unlabelled (arrows) were observed along the surface of plasma membrane.
X 18200

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