

# Evidence That Pinocytosis in Lymphoid Cells Has a Low Capacity

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**Abstract.** In contrast to adherent cells, human B and T lymphoblasts, marmoset monkey T lymphoblasts, and mouse T lymphoblasts do not form monolayers and have a poor ability to pinocytose. After a 10-min incubation of lymphoblasts at 37°C, the level of internalized medium reached a plateau. During this time, lymphoblasts pinocytosed 3–4 femtoliters (1 fl = 10<sup>-15</sup> l) of medium per cell as calculated by the quantity of the entrapped pinocytic marker 5(6)-carboxyfluorescein. The levels of pinocytosed liquid did not increase during a subsequent 90-min incubation of cells at 37°C. Adherent HeLa cells took up 27 fl of medium per cell per hour. Other types of adherent cells were reported by others to pinocytose 20 to 90 fl of medium per cell per hour. The process of pinocytosis in lymphoblasts appeared to be reversible since cells which were pre-loaded with carboxyfluorescein and

then incubated at 37°C in fresh medium lost the marker almost completely within 40 min. Similar results were obtained with horseradish peroxidase as the pinocytic marker. Further evidence that lymphoblasts have a low capacity for pinocytic internalization relative to adherent cells was obtained from the observation that Namalwa lymphoblasts were ~100 times more resistant to the cytotoxic action of the protein toxin gelonin than the adherent HeLa cells. Gelonin is a ribosome-inactivating toxin which is not capable of binding to cells, and its only mode for internalization appears to be pinocytosis. Ribosomes in cell lysates of the two lines were equally sensitive to gelonin. It is speculated that the poor pinocytic ability of lymphoid cells may reflect a fundamental difference between adherent and non-adherent cells and that this may impede the targeting of drugs into lymphoid cells.

Pinocytosis is the process by which cells internalize liquid medium and indiscriminately take up substances dissolved in it (38). While pinocytosis has been observed in a variety of cell types (38, 39, 41), quantitative studies of this process have been restricted mainly to macrophages, fibroblasts of varied origin, epithelial cells, and amoeba (1, 31, 33, 39, 41). All the above-mentioned cells are of the adherent type, i.e. they are able to anchor to and spread on solid substrata. Detailed kinetic studies on several types of adherent cells, polymorphonuclear leukocytes (10), pulmonary alveolar macrophages and fetal lung fibroblasts (5), Chinese hamster ovary fibroblasts (1), and mouse peritoneal macrophages (46) indicate that pinocytosis in these cells may consist of at least two steps. In a reversible step, pinosomes are formed and internalized, but then recycled from the cytoplasm to the cell surface where their contents are discharged back into the external medium. However, a fraction of the pinocytosed material does not seem to return to the cell surface, thus constituting an irreversible part of pinocytosis. This partial reversibility of pinocytosis is comparable to the recycling of receptors in receptor-mediated endocytosis (7) and may be related to the process of plasma membrane recycling (24, 39, 49). Pinocytosis of non-adherent lymphoid cells has hitherto not been analyzed quantitatively.

Here we report quantitative studies of pinocytosis in four

lymphoblast cell lines: two human lines, Namalwa and Molt-4, which are a B cell line and a T cell line respectively, the marmoset monkey T cell line 1022, and the mouse T cell line RADA-1. Our results combined with an analysis of the literature provide evidence that lymphoid cells are deficient in the pinocytic function.

## Materials and Methods

### Cells and Cell Culture Maintenance

The human B cell line Namalwa and the human epithelial cell line HeLa were purchased from Flow Laboratories, Inc. (McLean, VA). The human T cell line Molt-4 was purchased from the American Type Culture Collection (Rockville, MD) (ATCC CRL 1582). The mouse T cell line RADA-1 was obtained from Dr. E. Stockert, Memorial Sloan-Kettering Cancer Center. The monkey T cell line 1022 (13) was obtained from Dr. Norman Letvin (New England Regional Primate Research Center). Cells were maintained as asynchronous exponentially growing cultures in RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with 10% heat-treated (30 min at 56°C) fetal calf serum (Flow Laboratories, Inc.) and 2 mM L-glutamine (GIBCO), referred below as "growth medium". HeLa cells were grown as monolayer cultures.

### Purification of 5(6)Carboxyfluorescein (CF)<sup>1</sup>

Carboxyfluorescein, Lot #A12B (Eastman Kodak Co., Rochester, NY), was purified by treatment with activated charcoal followed by precipitation from

<sup>1</sup>Abbreviations used in this paper: CF, 5(6)carboxyfluorescein; HRP, horseradish peroxidase.

ethanol/water (1:2 vol/vol) (36). Our experiments with liposomes (see below) indicated that the purified CF was free of detectable amounts of fluorescent contaminants which can diffuse across artificial phospholipid membranes or contaminants that can facilitate the diffusion of CF across membranes. The ultrasound irradiation method (48) was used in the preparation of CF-loaded liposomes from chromatographically purified egg yolk phosphatidyl choline and cholesterol (molar ratio 8:2). Liposomes were formed in the presence of 0.2 M CF in PBS (pH adjusted to 7.2) and then extensively dialyzed against phosphate-buffered saline (PBS). Fluorescence of liposomes was analyzed on a fluorescence spectrophotometer. The high concentration of CF in the liposomes caused "self-quenching" of the fluorescence (excitation at 469 nm, emission at 510 nm). The release of CF from the liposomes was monitored by the increase of fluorescence. According to these experiments, incubation of CF-loaded liposomes at room temperature or at 37°C for 30 min to PBS did not cause any significant efflux of CF.

### ***Uptake of CF or Horseradish Peroxidase (HRP) by Cells***

For studies of pinocytosis, cells were incubated in suspension in growth medium containing CF or HRP (Sigma Chemical Co., St. Louis, MO) at 37°C. Cells incubated with HRP for different amounts of time were cooled to 4°C, washed twice with cold medium, resuspended in 1 ml of a cold mixture of trypsin (0.5 mg/ml) and versene (0.2 mg/ml) in balanced salt solution (Whittaker M.A. Bioproducts, Walkersville, MD), and incubated at 0°C for 10 min. This treatment with trypsin was performed in order to remove HRP that might be adsorbed onto the surface of cells. Finally, cells were washed twice with cold PBS, and resuspended in 1 ml of PBS. A sample was taken for cell counting on a Coulter Counter (Coulter Electronics Inc., Hialeah, FL). HRP activities were determined by the assay described by Steinman et al. (42) in nondisrupted cells revealing cell surface-associated HRP, as well as in cells lysed by 0.05% Triton X-100 (vol/vol) detergent which exhibited both internalized and cell surface-associated HRP. The amount of HRP taken up by cells was then determined by comparing the difference between HRP activities in disrupted and nondisrupted cells against a standard HRP activity concentration curve. Cells incubated with CF were washed four times with cold PBS and then lysed with 0.05% Triton X-100 in PBS. The concentration of CF in the lysate was determined on a fluorescence spectrophotometer (excitation at 469 nm, emission at 510 nm) and compared to a standard curve.

### ***Fluorescent Microscopy of Namalwa Cells after Exposure to CF***

Cells were incubated with 10 mM CF at 37°C for 20 min, washed four times with cold PBS, resuspended in a 1:1 (vol/vol) mixture of glycerol:PBS, and then examined under a fluorescent microscope using a 100× objective with oil immersion.

### ***Determination of CF Taken Up by Namalwa Cells by Flow Cytometry***

Cells were incubated with 10 mM CF in medium at 37°C and then washed four times with cold PBS. Fluorescence of individual cells was measured on a flow cytometer (FACS-1, Becton-Dickinson, Mountain View, CA). Data accumulated for  $4 \times 10^4$  cells were displayed as a histogram of the fluorescence intensity of single cells versus the number of cells with a given fluorescence intensity.

### ***Release of CF by Namalwa Cells***

Cells were incubated at 37°C or at 0°C in the medium containing 10 mM CF, washed twice with cold growth medium, and incubated in fresh medium at 37°C. After this incubation, cell samples were washed twice with PBS, resuspended in PBS, and disrupted with 0.05% (vol/vol) Triton X-100. Finally, the fluorescence was measured as above.

### ***Intrapinosomal pH Measurements***

**Method a.**  $10^6$  cells that had been incubated with CF were washed four times with PBS at 4°C and then resuspended in 3 ml of PBS and analyzed for their fluorescence. Cells were lysed with 0.05% (vol/vol) Triton X-100 in order to expose the CF to the pH of PBS (pH 7.2), and the fluorescence was measured again. The ratio  $I_{\text{nondisrupted}}/I_{\text{disrupted}}$  was compared to a standard curve of the pH dependence of CF fluorescence intensity (excitation at 469 nm, emission at 510 nm) in relative units ( $I_{\text{pH}7.2} = 1.0$ ). The buffer contained NaCl (145 mM),

$\text{CH}_3\text{COONa}$  (10 mM), and  $\text{NaH}_2\text{PO}_4$  (10 mM), and its pH was adjusted with HCl or NaOH.

**Method b.** pH dependence of the ratio of the emission intensities at 510 nm for two different excitation wavelengths, 450 nm and 491 nm, was measured and compared to a standard curve (30).

Results obtained with methods *a* and *b* were in good agreement.

### ***Cytotoxicity Assays***

**Method a.** Determination of the surviving fraction in Namalwa cells by back-extrapolation of cell proliferation curves. This method has been described elsewhere (16, 47).

**Method b.** Determination of surviving fraction of Namalwa cells by colony formation. Treated and nontreated cells were plated in tissue culture grade multiwell plates containing 96 0.25-ml flat bottom wells per plate. Cells were plated in the presence of  $2 \times 10^4$  feeder cells per well. Gamma-irradiated ( $^{137}\text{Cs}$  Source, 5 k Rad) Namalwa cells were used as feeder cells. Plates with cells were maintained for 20 d at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ , and within this time interval, colonies of  $\sim 10^5$  cells were formed. Colonies were counted and the numbers were used to determine plating efficiency (14) and surviving fractions. The surviving fractions established by the two methods were in good agreement.

**Method c.** Determination of surviving fraction in HeLa cells by colony formation. Cells were plated onto 20-cm<sup>2</sup> polystyrene Petri dishes in 10 ml medium at a density of 5 to 500 cells/cm<sup>2</sup> and left for 24 h at 37°C in a humidified atmosphere containing 5%  $\text{CO}_2$ . Preliminary experiments showed that within this time interval, HeLa cells adhered to substrate and resumed exponential growth. Medium was then replaced with 8–10 ml of fresh medium containing a toxic agent. After 24 h of exposure to the toxin at 37°C, cells were rinsed twice with warm medium and then incubated in 10 ml fresh medium for 8 d. Cells were finally fixed with 1% formaldehyde in PBS (30 min at room temperature), and stained with filtered hematoxylin, Gill No. 3. Colonies of 20 or more cells were then scored and plating efficiencies were calculated.

### ***Preparation of Cell Lysates***

$5 \times 10^8$  Namalwa cells were washed three times in ice-cold 0.145 M NaCl and then resuspended in a final volume of 1.8 ml 0.145 M NaCl. 1.8 ml of ice-cold double-distilled water was then added and the cells were vortexed and incubated at 0°C for 5 min.  $1.1 \times 10^8$  HeLa cells were treated analogously with 1.2 ml of 0.145 M NaCl and 1.2 ml of ice-cold double-distilled water. Finally, cells were disrupted by 50 strokes in an Elvehjem Potter at 4°C. The resulting suspension was immediately centrifuged in an Eppendorf centrifuge (7 min, 4°C) and the supernatant was frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$ .

### ***Assay of Protein Synthesis in a Cell-free System***

The inhibitory activity of gelonin towards protein synthesis was measured in cell lysates. The assay was based on that of Pelham and Jackson (32). 15  $\mu\text{l}$  of the protein synthesis cocktail (32), creatine phosphokinase (0.4  $\mu\text{l}$  of 10 mg/ml solution in a mixture of equal volumes of 20 mM Tris-HCl, pH 7.6, and ethylene glycol), and 36  $\mu\text{l}$  of freshly thawed cell lysate were mixed together at 0°C. 1- $\mu\text{l}$  samples of gelonin, diluted to a desired concentration with PBS containing bovine serum albumin (0.1 mg/ml), were mixed with aliquots (4  $\mu\text{l}$ ) of the cell-free mixture, and the mixtures were incubated at 30°C. Control samples were incubated at 0°C. The incorporation of  $^3\text{H}$ -labeled leucine into protein was quenched by addition of ice-cold solution of leucine in water (0.2 ml of 0.2 mg/ml). Radiolabeled protein was quantified as described by Pelham and Jackson (32).

## **Results**

### ***Uptake of CF and HRP by Lymphoblasts***

CF and HRP were used to detect pinocytosis. CF is highly water soluble and nondegradable by mammalian cells (8, 21, 36, 44, 48). It does not penetrate biological and artificial phospholipid membranes at  $\text{pH} \geq 6$  (8, 21, 35, 36, 44, 48) nor does it bind significantly to cell surfaces (48). HRP has been widely used as a pinocytic marker (38, 40) and was found to be resistant to intracellular degradation in macrophages and fibroblasts of different origin (the half-inactivation time was at least 7 h or longer) (19, 40, 42).

The uptake of HRP and of CF with or without inhibitors of endocytosis, has been measured in the human B lymphoblast cell line Namalwa (Table I). Uptake of HRP and CF by Namalwa cells was inhibited by both incubation at 0°C and by a 1-h pre-treatment of cells with inhibitors of ATP production (10 mM NaN<sub>3</sub> + 50 mM 2-deoxy-D-glucose) which indicates that HRP and CF are internalized via endocytosis (38). However, neither the inhibitors nor the low temperature completely suppressed uptake of HRP or CF by cells. While some HRP associated with cells at 0°C was situated on the cell surface, most of it was inside the cells (Table I). A similar phenomenon has been reported previously for a variety of cells. Pinocytosis was not completely inhibited by metabolic inhibitors, by cytochalasin B, or at low temperature, and this nonsensitive uptake constituted a significant part of the total uptake (10, 11, 18, 25, 28, 31, 42, 50). While the amounts of internalized HRP at 37°C were fourfold higher than those at 0°C, the amounts of cell surface-associated HRP at both temperatures were similar (Table I). Therefore, the greater association of HRP with cells at 37°C should be attributed to endocytosis rather than to an increased binding.

Significant amounts of HRP were associated with the cell surface after incubation of cells with the protein (Table I). This result differed from earlier data reported by Steinman et al. (42), in which no cell surface-associated HRP was detected.

However, it has been recently reported (45, 46) that some HRP was indeed taken up by mouse peritoneal macrophages via adsorptive endocytosis. In addition, Swanson et al. (46) found that HRP was not an inert marker, since it stimulated pinocytosis in macrophages. Thus, HRP does not appear to be an ideal marker for monitoring pinocytosis.

CF acted as a better pinocytic marker, as was indicated by fluorescence microscopy (Fig. 1). Fluorescence was mostly concentrated within intracellular vesicles, which implied internalization of CF via endocytosis. The lack of nonvesicular fluorescence bound to the cell surface and the absence of significant nonvesicular fluorescence in the cytoplasm indicated that CF neither bound to nor penetrated the cell plasma membrane. The absence of binding of CF to the plasma membrane in turn implied pinocytosis rather than adsorptive endocytosis as the pathway of CF internalization. Cells that had not been exposed to CF showed no detectable fluorescence. These fluorescent microscopy observations are in agreement with previously published reports in which exposure of lymphocytes to CF resulted in the appearance of a patchy rim at the margin of the cell with no diffuse fluorescence (48). The amount of CF taken up by Namalwa cells during a 30-min incubation at 37°C was directly proportional to the CF concentration in the medium, and no saturation was evident over the large concentration range tested (0.2 to

Table I. Inhibition of Uptake of HRP and CF by Namalwa Cells

Conditions of incubation	HRP associated with cells		HRP inside cells		CF taken up	
	(Lysed cells) 10 <sup>20</sup> × mol per cell	(Nonlysed cells) 10 <sup>20</sup> × mol per cell	10 <sup>20</sup> × mol per cell	Fraction of control	10 <sup>17</sup> × mol per cell	Fraction of control
Control	6.4	0.8	5.6	1.0	3.8	1.0
10 mM NaN <sub>3</sub> + 50 mM deoxyglucose	2.5	0.7	1.8	0.33	1.1	0.29
0°C	2.4	0.9	1.5	0.27	0.76	0.20

Concentration of HRP in the medium was 50 μM, concentration of CF in the medium was 10 mM. Cells were incubated at 37°C for 1 h without an inhibitor (control); or with NaN<sub>3</sub> and deoxyglucose, a pinocytic marker was added and cells were incubated for 15 min at 37°C, except for the cells that were incubated at 0°C. Cells were then washed and their fluorescence or their HRP activity were measured as described in Materials and Methods. Values are averages of two independent experiments.

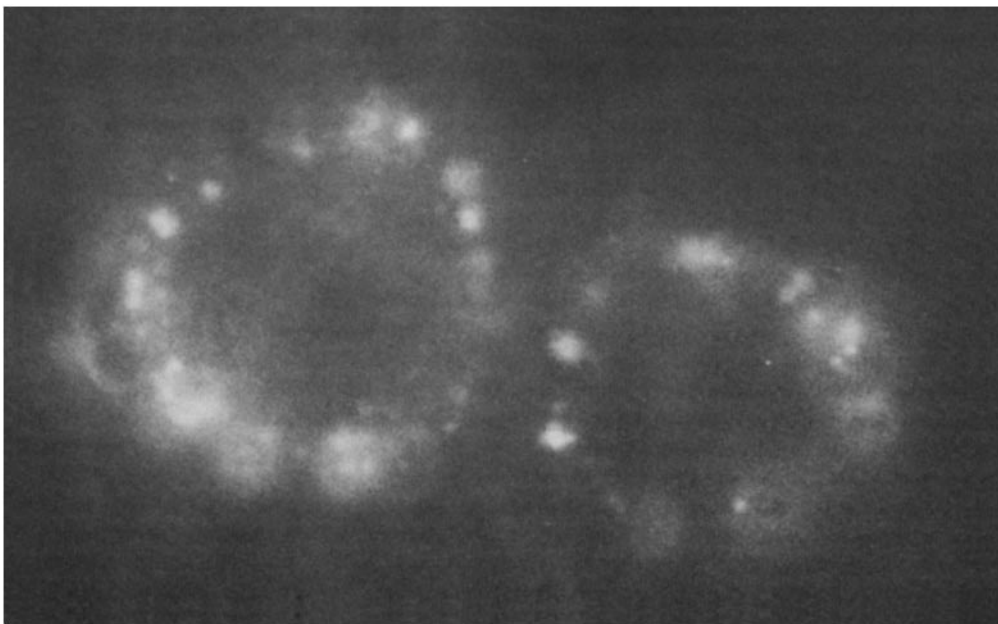


Figure 1. Fluorescent photomicrographs of Namalwa cells after exposure to carboxyfluorescein. Cells were incubated with 10 mM CF at 37°C for 20 min, washed four times with cold PBS, resuspended in a 1:1 (vol/vol) mixture of glycerol/PBS, and examined under a fluorescent microscope using a 100× objective with oil immersion. Upon examination, control cells (cells that had not been exposed to CF) showed no detectable fluorescence.

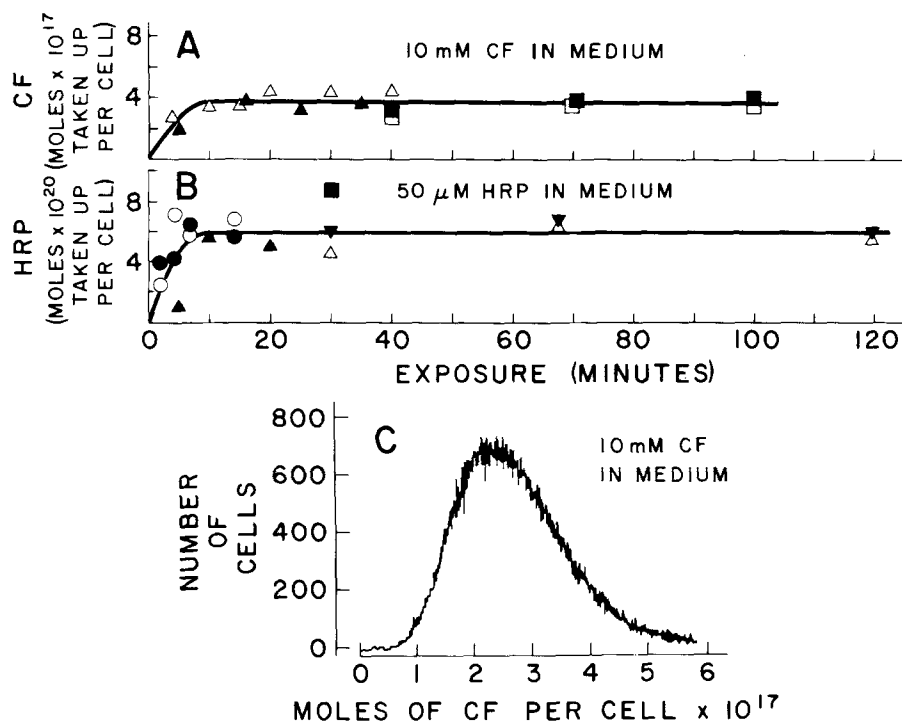


Figure 2. (A) Uptake of CF or (B) HRP by the human lymphoblast cell line Namalwa. Cells were incubated in the growth medium with HRP or CF at 37°C. Cells incubated with HRP were cooled to 4°C, washed as described in Materials and Methods, and then the peroxidase activities were determined in nondisrupted cells and in cells lysed by 0.05% Triton X-100 detergent. The amount of HRP taken up was then determined as the difference between these two values. Cells preincubated with CF were washed four times with cold PBS, lysed with 0.05% Triton X-100 in PBS, and the concentration of CF was determined on a spectrofluorometer (excitation at 469 nm, emission at 510 nm) and compared to a standard curve. Different symbols in the figure represent independent experiments. (C) Determination of CF taken up by Namalwa cells by flow cytometry. Cells were incubated with 10 mM CF in medium at 37°C, and then washed four times with cold PBS. Fluorescence of individual cells was then measured on a flow cytometer and displayed as a histogram of the fluorescence

intensity of single cells versus the number of cells with a given fluorescence intensity. The abscissa was originally expressed in relative units of intensity of fluorescence per cells (linear scale) and then recalibrated using the data of Fig. 2A.

30 mM). This result also indicates that the marker is apparently internalized via liquid pinocytosis.

However, we cannot rule out the possibilities that CF may nonspecifically bind to the cell surface at levels below the limit of detection by fluorescent microscopy, or that CF binds weakly to cells and is washed off the cell membrane during cell preparation for fluorescent microscopy examination. The fast release of CF by lymphoblasts (see below) indicates the absence of tight irreversible binding of CF to the cell surface. The subsequent-to-binding (if it exists) adsorptive nonspecific endocytosis of CF might contribute to the total uptake of the marker. This uptake would still reflect plasma membrane internalization that occurs during pinocytosis, but would also affect the calculated volume of liquid pinocytosed by cells. As for the nature of interaction of CF with cells at 0°C, our data on HRP suggest that at least a fraction of the CF associated with cells at 0°C is internalized. However, we cannot conclude from our experiments that CF does not bind to the cell surface at 0°C.

The kinetics of CF and HRP uptake by Namalwa cells were similar (Fig. 2, A and B). Analogous results were also obtained with the cell lines Molt-4, RADA-1, and 1022 using CF as the marker (data not shown). A plateau in the level of pinocytosis for these cell lines was reached after a 10-min incubation, corresponding to 3–4 fl of medium per cell. The amount of internalized CF by single Namalwa cells was measured by flow cytometry (Fig. 2C), and no major differences were observed among individual cells. The uptake curve can be approximated by a Gaussian distribution with a narrow variance, indicating that the Namalwa cell line behaves as a homogeneous cell population in its ability to take up CF.

Pinocytic capacity of the four lymphoid cell lines of different origin (B and T cells) and different species (human, monkey, and mouse) was very poor (3–4 fl per cell at satura-

tion) compared to that of various adherent cells (20–90 fl per cell per hour) as reported by others (1, 31, 33, 39, 41). To quantitatively compare our data to that previously published on cells with adherence capacity for substrata, we measured pinocytosis in an adherent HeLa cell line. Although HeLa cells can grow in suspension (i.e., they are anchorage independent), they are also able to adhere to substrata. Adherence and anchorage-dependence are not related. Adherence is the ability of cells to anchor and spread on substrata, while anchorage independence and anchorage dependence reflect differences in the regulation of cell cycle progression between some transformed cells and their nontransformed predecessors. HeLa cells possess the machinery that enables them to anchor and spread on substrata, and morphologically these cells are similar to anchorage-dependent epithelial cells. In contrast, lymphoblasts are not able to adhere to or spread on substrata. We compared the levels of pinocytosis by Namalwa and by HeLa cells. The data are presented in Table II. Both cell lines were incubated with CF in suspension (i.e. under similar conditions). The ability of HeLa cells to pinocytose (27 fl per cell per hour) was not only comparable to the previously reported levels of pinocytosis by adherent cells, but also dramatically higher than the levels of pinocytosis by similarly treated Namalwa cells.

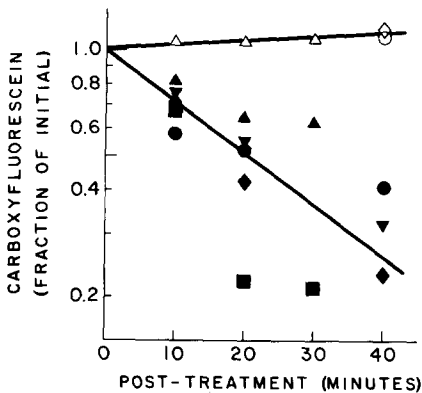
#### Release of CF by Lymphoblasts

CF taken up by lymphoblasts was released during subsequent incubation of the cells in fresh medium at 37°C, but not at 0°C (Fig. 3). Thus, uptake is reversible at 37°C. The fact that cells fully retained their contents at 0°C enabled us to study pinocytosis quantitatively. After incubation with CF at 37°C, cells were immediately cooled down and then freed of the external CF by several washings at 0°C as described in Materials and Methods.

**Table II. Uptake of CF by Suspended Namalwa and by Suspended HeLa Cells**

Conditions of incubation	CF taken up by cells ( $10^{17} \times$ mol per cell)	
	Namalwa cells	HeLa cells
Control	4.3	27
0°C	0.8	7.1

Cells were incubated at 37°C or at 0°C for 1 h in suspension in growth medium containing CF (10 mM). Then cells were washed and their fluorescence was measured as described in Materials and Methods.



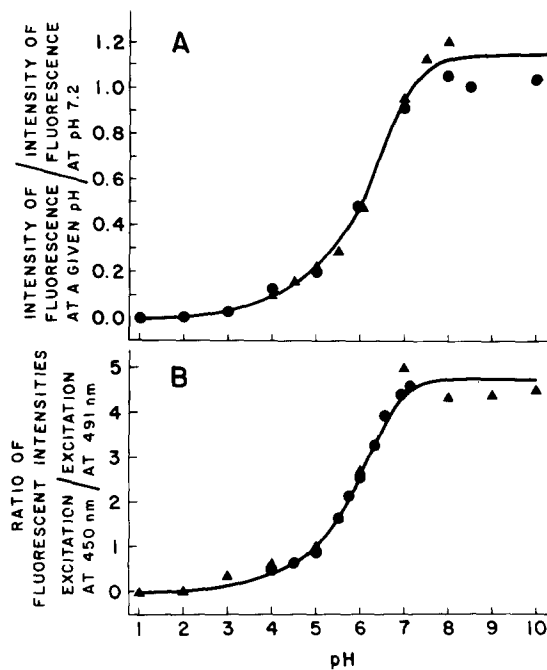
**Figure 3.** Release of CF by Namalwa cells. Cells were incubated at 37°C in the medium containing 10 mM CF for (■, ○, ●) 5 min, (▼) 15 min, (▲, △) 20 min, or (◆, ◇) 30 min, washed twice with cold growth medium, and then incubated in fresh medium at 37°C (filled symbols) or at 0°C (open symbols). Samples of cells were washed twice with PBS, resuspended in PBS, and disrupted with 0.05% Triton X-100. The fluorescence was then measured.

### Measurements of Intrapinosomal pH in Human Lymphoblasts

We have measured the pH in pinosomes of Namalwa and Molt-4 lymphoblasts using CF as a pH-sensitive fluorescent probe. The fluorescence intensity of fluorescein-related compounds as well as the ratio of emission intensities at two excitation wavelengths (450 nm and 491 nm) were reported to depend on pH of the surrounding solution (30). Standard curves for the pH dependence of these two parameters were generated (Fig. 4) for the determination of intrapinosomal pH. According to our observations under the fluorescent microscope, CF was accumulated in pinosomes rather than in the cytoplasm or at the cell surface, and therefore the pH of the pinosomes could be estimated. The level of opalescence caused by the cells in the absence of CF was negligible. Also, we could not detect any leakage of CF from the cells when they were finally suspended in PBS at room temperature since the buffer lost all fluorescence upon cell removal by centrifugation. After a 30-min incubation of the cells at 37°C in medium containing 1 mM CF, the intrapinosomal pH was found to be  $\sim 6.8$ . Thus, the pinosomes were not significantly acidified.

### Cytotoxicity of Gelonin in Namalwa and HeLa Cells

We have tested the cytotoxicity of gelonin, a ribosome-inactivating protein (43), towards Namalwa and HeLa cells. Gelonin is an extremely potent inactivator of protein synthesis in mammalian cell-free translation systems but is not very



**Figure 4.** (A) pH dependence of the fluorescence intensity of CF (excitation at 469 nm, emission at 510 nm) in relative units ( $I_{pH7.2} = 1.0$ ). The buffer contained NaCl (145 mM),  $\text{CH}_3\text{COONa}$  (10 mM), and  $\text{NaH}_2\text{PO}_4$ . For pH measurements,  $10^6$  cells, preincubated with CF, were washed four times with PBS at 4°C, resuspended in 3 ml of PBS, and then the fluorescence was measured. Cells were then lysed with 0.05% Triton X-100 in PBS, pH 7.2 and the fluorescence was measured again. The ratio  $I_{\text{non-disrupted}}/I_{\text{disrupted}}$  was compared with the relationship  $I_{\text{pH}}/I_{\text{pH7.2}}$ . Opalescence by cells in the absence of CF under the conditions used was negligible. (B) pH dependence of the ratio of fluorescence intensity (510 nm emission) at two excitation wavelengths:  $I_{450\text{nm}}/I_{491\text{nm}}$ . The same ratio determined from the fluorescence of CF inside cells was compared to the standard curve. Results obtained with techniques A and B were in good agreement. Different symbols on the figure represent independent experiments.

toxic for intact cells, due to its incapability of binding to cells and penetrating cell membranes (16, 20, 43). Its sole means for cell penetration seems to be the pinocytic pathway. Cytotoxicity data for gelonin are shown in Fig. 5. Namalwa cells appear to be dramatically less sensitive than HeLa cells to the cytotoxic action of gelonin.

### Inhibition of Protein Synthesis by Gelonin in Namalwa and HeLa Cell Lysates

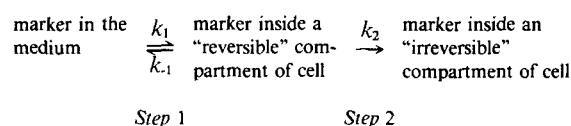
To compare the inhibitory action of gelonin in lysates of Namalwa and HeLa cells, cell-free translation systems from these two cell lines were prepared which showed almost linear incorporations of  $^3\text{H}$ -labeled leucine into TCA-precipitable material over a period of 10 min (Fig. 6a). The effect of gelonin on protein synthesis in these two cell-free translation systems was then analyzed by incubation with a gelonin-containing mixture for 5 min at 30°C. Lysates of the HeLa and Namalwa cells were equally sensitive to the toxic action of gelonin (Fig. 6b).

### Discussion

In our experiments, uptake of the pinocytic markers CF and HRP by lymphoblasts reaches a plateau within 10 min of

incubation at 37°C. Kinetic data suggest that this plateau is due to an equal rate of marker efflux and intake by the cells from the medium after 10 min. The plateau cannot be explained by the toxicity of the pinocytic markers nor can it be attributed to marker depletion from the medium. In the former case, CF and HRP were nontoxic to Namalwa cells since the markers did not inhibit cell division of exponentially growing cultures (data not shown). In the latter, depletion of CF or HRP from the medium was <0.1% of the total amounts present.

Partially reversible pinocytosis has been reported for several adherent types of cells such as polymorphonuclear leukocytes (10), pulmonary alveolar macrophages and mouse peritoneal macrophages (5, 46), fetal lung fibroblasts (5), and Chinese hamster ovary fibroblasts (1) and mouse L-fibroblasts (11). In these cell types, there is an initial "burst" of internalization followed by a slower rate of uptake. It has been proposed that the overall process of pinocytosis can be described by the following two step kinetic scheme (5, 10):



Our results with the lymphoblasts appear to fit the above scheme only when one assumes a very small  $k_2$ , which means

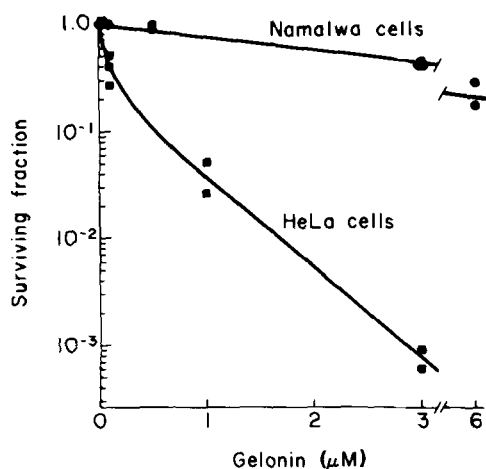


Figure 5. Cytotoxicity of gelonin in Namalwa and HeLa cells. Cells were incubated with gelonin (isolated and purified as described by Stirpe et al. [43]) for 24 h at 37°C in growth medium. After the incubation cells were washed and surviving fractions were determined as described in Materials and Methods.

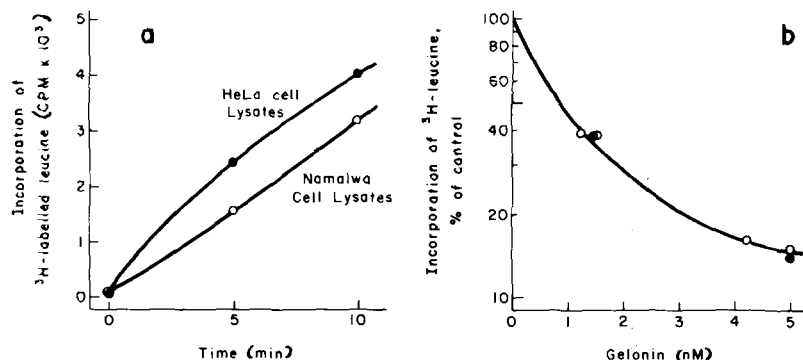


Figure 6. (a) Kinetics of incorporation of <sup>3</sup>H-labeled leucine into trichloroacetic acid-precipitable material by HeLa (●) or Namalwa (○) cell lysates (see Materials and Methods). Each point represents an average of two independent experiments. (b) Inhibition by gelonin of the incorporation of <sup>3</sup>H-labeled leucine in trichloroacetic acid-precipitable material by lysates from HeLa (●) or Namalwa (○) cells. The lysates were incubated with or without gelonin for 5 min. The data represent four independent experiments.

that pinocytosis stops at Step 1. We calculated the kinetic parameters of pinocytosis of CF by lymphoblasts (using the data presented in Fig. 2A and Fig. 3) and compared them with the published parameters for polymorphonuclear leukocytes (10). While the rate constants  $k_{-1}$  for the efflux seem to be similar in lymphoblasts and leukocytes ( $\sim 0.1 \text{ min}^{-1}$ ), the rate of initial internalization (Step 1) was significantly slower in lymphoblasts ( $k_1 = 0.3 \text{ fl} \times \text{cell}^{-1} \text{ min}^{-1}$ ) than in leukocytes ( $k_1 = 80 \text{ fl} \times \text{cell}^{-1} \text{ min}^{-1}$ ) (10). Furthermore, the rate constant  $k_2$  for lymphoblasts, as estimated from the data in Fig. 2, is indeed very small (below our limit of detection, which is  $\sim 10^{-3} \text{ min}^{-1}$ ), while that of leukocytes has been published as being  $\sim 0.1 \text{ min}^{-1}$  (10). Thus, it seems that pinocytosis by lymphoblasts consists only of the first reversible step, and that this first step is rather slow.

Our observations indicate that the four lymphoid cell lines have a significantly lower capacity of liquid pinocytosis (3–4 fl at saturation) than do adherent cells such as macrophages, fibroblasts, and HeLa cells. From experimental results reported by others, we have calculated that rabbit thymocytes pinocytose 1.6 fl of medium per cell in 30 min (37), rat lymphocytes take up 1.3 fl in 3 h (17), and human peripheral blood lymphocytes pinocytose 1.3 fl of medium in 10 min (6). It appears from these data that low levels of pinocytosis may be a common feature of lymphoid cells of varied origin.

Our data on cytotoxicity of gelonin also indicate that lymphoblasts have a low capacity for pinocytic internalization relative to adherent cells. Pinocytotically internalized gelonin is dramatically more toxic in HeLa cells than in Namalwa cells. Since cell-free translation systems of these two types of cells are equally sensitive to gelonin, the different toxicities observed for the intact cells can be attributed to different efficiencies for the delivery of gelonin to the cytoplasm of the cells. Another interesting finding is that pinocytosis by lymphoblasts appears to be fully reversible, in contrast to the partial irreversibility of pinocytosis by macrophages and fibroblasts (6, 10, 11, 46). Pinosomes of lymphoblasts were not significantly acidified and this might also be attributed to the relative defectiveness of the pinocytic function in lymphoblasts. However, endosomes that are formed by lymphoblasts during receptor-mediated endocytosis of various ligands (3, 17, 22, 26, 27) may be either acidified or not, depending on the type of the ligand (22, 26, 27).

Lymphoid cells are well known to differ from many other cell types in their inability to anchor and spread on solid substrata. Our observation that lymphoid cells are also inefficient in pinocytosis may be of major significance and the two phenomena may be linked. There is some evidence that

adsorptive endocytosis may be analogous to spreading on substratum (15, 23, 34). One can speculate that when a cell spreads on a substrate, it makes an attempt to endocytose this infinitely large particle (29). This analogy may help to understand why lymphoblasts are relatively inefficient in pinocytosis: cells which can spread would endocytose better than cells which cannot (such as lymphoblasts). This hypothesis also predicts that cells which can adhere to substrata should be more proficient in adsorptive and receptor-mediated endocytosis. Indeed, the abilities of Chinese hamster ovary cells to endocytose and adhere to substratum seem to correlate (9). We are planning to test this prediction further.

Trophozoites of parasitic amoebae *E. histolytica* are an interesting example of cells that behave intermediately between adherent and non-adherent cells (12). There are striking similarities between pinocytosis by *E. histolytica* (2) and by lymphoid cells: (a) it is fully reversible in both cell types; (b) it reaches a plateau at 3–5 fl of liquid taken up per cell; (c) pinosomes are not acidified.

It was recently reported that the rates of pinocytosis and exocytosis in macrophages, as well as the size of the irreversible part of pinocytosis, can be regulated by external signals such as the presence or absence of amino acids, phorbol myristate acetate, or HRP in the medium (4, 46). It would be of interest to investigate whether certain substances could induce the irreversible component of pinocytosis in lymphoblasts which is usually absent.

The poor pinocytic capacity of lymphoid cells may restrict the efficiency of targeting drugs or drug-carriers inside these cells. Indeed, our data on gelonin cytotoxicity, as well as data of Straubinger et al. (44) on poor internalization of liposomes by T lymphoid cells when compared to fibroblasts and macrophages, seem to support this suggestion.

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## References

- Adams, C. J., K. M. Maurey, and B. Storrie. 1982. Exocytosis of pinocytic contents by Chinese hamster ovary cells. *J. Cell Biol.* 93:632–637.
- Aley, S. B., Z. A. Cohn, and W. A. Scott. 1984. Endocytosis in *Entamoeba histolytica*. *J. Exp. Med.* 160:724–737.
- Antoine, J.-C., S. Avrameas, N. K. Gonatas, A. Stieber, and J. O. Gonatas. 1974. Plasma membrane and internalized immunoglobulins of lymph node cells studied with conjugates of antibody or its Fab fragments with horseradish peroxidase. *J. Cell Biol.* 63:12–23.
- Besterman, J. M., J. A. Airhart, R. B. Low, and D. E. Rannels. 1983. Pinocytosis and intracellular degradation of exogenous protein: modulation by amino acids. *J. Cell Biol.* 96:1586–1591.
- Besterman, J. M., J. A. Airhart, R. C. Woodworth, and R. B. Low. 1981. Exocytosis of pinocytosed fluid in cultured cells: kinetic evidence for rapid turnover and compartmentation. *J. Cell Biol.* 91:716–727.
- Blumenthal, R., E. Ralston, P. Dragstein, L. D. Leserman, and J. N. Weinstein. 1982. Lipid vesicle-cell interactions: analysis of a model for transfer of contents from adsorbed vesicles to cells. *Membr. Biochem.* 4:283–303.
- Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of migrant membrane proteins. *Cell.* 32:663–667.
- Bruning, J. W., M. J. Kardol, and R. Arentzen. 1980. Carboxyfluorescein

fluorochromasia assays. I. Non-radioactively labeled cell mediated lymphoysis. *J. Immunol. Methods* 33:33–44.

- Cheung, E., and R. L. Juliano. 1985. cAMP-induced phenotypic reversion of adhesion, aggregation, and endocytosis in adhesion-defective CHO cell variants. *J. Cell. Physiol.* 124:337–343.
- Daukas, G., D. A. Lauffenburger, and S. Zigmond. 1983. Reversible pinocytosis in polymorphonuclear leukocytes. *J. Cell Biol.* 96:1642–1650.
- Deurs, B., C. Röpke, and N. Thorball. 1984. Kinetics of pinocytosis studied by flow cytometry. *Eur. J. Cell Biol.* 34:96–102.
- Diamond, L. S. 1968. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amoebae. *J. Parasitol.* 54:1047–1056.
- Falk, L., J. Wright, L. Wolfe, and F. Deinhardt. 1974. *Herpesvirus Ateles*: transformation *in vitro* of marmoset splenic lymphocytes. *Int. J. Cancer.* 14:244–251.
- Furth, E. E., W. G. Thilly, B. W. Penman, H. L. Liber, and W. M. Rand. 1981. Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates. *Anal. Biochem.* 110:1–8.
- Goldmacher, V. S. 1984. Macrophages: inhibition of endocytosis by anchorage to substrate. *Exp. Cell Res.* 154:632–635.
- Goldmacher, V. S., J. Anderson, W. A. Blättler, J. M. Lambert, and P. D. Senter. 1985. Antibody complement-mediated cytotoxicity is enhanced by ribosome inactivating proteins. *J. Immunol.* 135:3648–3651.
- Goud, B., J.-C. Antoine, N. K. Gonatas, A. Stieber, and S. Avrameas. 1981. A comparative study of fluid-phase and adsorptive endocytosis of horseradish peroxidase in lymphoid cells. *Exp. Cell Res.* 132:375–386.
- Jennings, M. A., and L. Florey. 1967. An investigation of some properties of endothelium related to capillary permeability. *Proc. R. Soc. Lond. B Biol. Sci.* 167:39–63.
- Kaplan, J., and M. Nielsen. 1978. Pinocytic activity of rabbit alveolar macrophages *in vitro*. *J. Reticuloendothel. Soc.* 24:673–685.
- Lambert, J. M., A. Yau-Young, P. D. Senter, W. A. Blättler, and V. S. Goldmacher. 1985. Purified immunotoxins that are reactive with human lymphoid cells: monoclonal antibodies conjugated to the ribosome-inactivating proteins gelonin and the pokeweed antiviral proteins. *J. Biol. Chem.* 260:12035–12041.
- Lelkes, P. I., and H. B. Tandeter. 1982. Studies on the methodology of the carboxyfluorescein assay and on the mechanism of liposome stabilization by red blood cells *in vitro*. *Biochim. Biophys. Acta.* 716:410–419.
- Metzezeau, P., I. Elguindi, and M. E. Goldberg. 1984. Endocytosis of the membrane immunoglobulins of mouse spleen B-cells: a quantitative study of its rate, amount and sensitivity to physiological, physical and cross-linking agents. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2235–2242.
- Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc and complement-receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
- Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1983. Membrane proteins of the vacuolar system. III. Further studies on the composition and recycling of endocytic vacuole membrane in cultured macrophages. *J. Cell Biol.* 96:29–36.
- Munthe-Kaas, A. C. 1977. Uptake of macromolecules by rat Kupffer cells *in vitro*. *Exp. Cell Res.* 107:55–62.
- Murphy, R. F., E. Bisaccia, C. R. Cantor, C. Berger, and R. L. Edelson. 1984. Internalization and acidification of insulin by activated human lymphocytes. *J. Cell. Physiol.* 121:351–356.
- Murphy, R. F., D. B. Tse, C. R. Cantor, and B. Pernis. 1984. Acidification of internalized class I major histocompatibility complex antigen by T lymphoblasts. *Cell. Immunol.* 88:336–342.
- Nagura, H., and J. Asai. 1976. Pinocytosis by macrophages. Kinetics and morphology. *J. Cell Biol.* 70(2, Pt. 2):89a. (Abstr.)
- North, R. J. 1970. Endocytosis. *Semin. Hematol.* 7:161–171.
- Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.
- Ose, L., T. Ose, R. Reinertsen, and T. Berg. 1980. Fluid endocytosis in isolated rat parenchymal and non-parenchymal liver cells. *Exp. Cell Res.* 126:109–119.
- Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247–256.
- Pratten, M. K., K. E. Williams, and J. B. Lloyd. 1977. A quantitative study of pinocytosis and intracellular proteolysis in rat peritoneal macrophages. *Biochem. J.* 168:365–372.
- Rabinovitch, M., R. E. Manejias, and V. Nussenzweig. 1975. Selective phagocytic paralysis induced by immobilized immune complexes. *J. Exp. Med.* 142:827–838.
- Ralston, E., R. Blumenthal, J. N. Weinstein, S. O. Sharrow, and P. Henkart. 1980. Lysophosphatidylcholine in liposomal membranes. Enhanced permeability but little effect on transfer of a water-soluble fluorescent marker into human lymphocytes. *Biochim. Biophys. Acta.* 597:543–551.
- Ralston, E., L. M. Hjelmeland, R. D. Klausner, J. N. Weinstein, and R. Blumenthal. 1981. Carboxyfluorescein as a probe for liposome-cell interactions. Effect of impurities and purification of the dye. *Biochim. Biophys. Acta.* 649:133–137.
- Roosmond, R. C., and D. C. Urii. 1982. Peculiar behaviour of rabbit

- thymocytes in interaction with liposomes of different compositions shown by fluorescence polarization studies, lipid analysis, and uptake of vesicle-entrapped carboxyfluorescein. *Biochim. Biophys. Acta.* 689:499-512.
38. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669-722.
39. Steinman, R. M., S. E. Brodie, and Z. A. Cohn. 1976. Membrane flow during pinocytosis. *J. Cell Biol.* 68:665-687.
40. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrophages in vitro. *J. Cell Biol.* 55:186-204.
41. Steinman, R. M., I. S. Mellman, W. A. Muller, and Z. A. Cohn. 1983. Endocytosis and the recycling of plasma membrane. *J. Cell Biol.* 96:1-27.
42. Steinman, R. M., J. M. Silver, and Z. A. Cohn. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. *J. Cell Biol.* 63:949-969.
43. Stürpe, F., S. Olsnes, and A. Pihl. 1980. Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells. *J. Biol. Chem.* 255:6947-6953.
44. Straubinger, R. M., K. Hong, D. S. Friend, and D. Papahadjopoulos. 1983. Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles. *Cell.* 32:1069-1079.
45. Sung, S.-S. J., R. S. Nelson, and S. C. Silverstein. 1983. The role of the mannose/*N*-acetylglucosamine receptor in the pinocytosis of horseradish peroxidase by mouse peritoneal macrophages. *J. Cell. Physiol.* 116:21-25.
46. Swanson, J. A., B. D. Yirinec, and S. C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851-859.
47. Thilly, W. G., J. G. DeLuca, H. Hoppe, and B. W. Penman. 1978. Phenotypic lag and mutation to 6-thioguanine resistance in diploid human lymphoblasts. *Mutat. Res.* 50:137-144.
48. Weinstein, J. N., R. Blumenthal, S. O. Sharrow, and P. A. Henkart. 1978. Antibody-mediated targeting of liposomes. Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells. *Biochim. Biophys. Acta.* 509:272-288.
49. Widnell, C. C., Y.-J. Schneider, B. Pierre, P. Baudhuin, and A. Trouet. 1982. Evidence for a continual exchange of 5'-nucleotidase between the cell surface and cytoplasmic membranes in cultured rat fibroblasts. *Cell.* 28:61-70.
50. Wills, E. J., P. Davies, A. S. Allison, and A. D. Haswell. 1972. Cytochalasin B fails to inhibit pinocytosis by macrophages. *Nat. New Biol.* 240:58-60.