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Macrophages Possess Probenecid-inhibitable Organic Anion Transporters that Remove Fluorescent Dyes from the Cytoplasmic Matrix

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Abstract. We introduced several membrane-impermeant fluorescent dyes, including Lucifer Yellow, carboxyfluorescein, and fura-2, into the cytoplasmic matrix of J774 cells and thioglycollate-elicited mouse peritoneal macrophages by ATP permeabilization of the plasma membrane and observed the subsequent fate of these dyes. The dyes did not remain within the cytoplasmic matrix; instead they were sequestered within phase-lucent cytoplasmic vacuoles and released into the extracellular medium. We used Lucifer Yellow to study these processes further. In cells incubated at 37°C, 87% of Lucifer Yellow was released from the cells within 30 min after dye loading. The dye that remained within the cells at this time was predominantly within cytoplasmic vacuoles. Lucifer yellow transport was temperature dependent and occurred against a concentration gradient; therefore it appeared to be an energy-requiring process.

The fluorescent dyes used in these studies are all organic anions. We therefore examined the ability of probenecid (*p*-[dipropylsulfamoyl]benzoic acid), which blocks organic anion transport across many epithelia, to inhibit efflux of Lucifer Yellow, and found that this drug inhibited this process in a dose-dependent and reversible manner. Efflux of Lucifer Yellow from the cells did not require Na^+ co-transport or Cl^- antiport; however, it was inhibited by lowering of the extracellular pH. These experiments indicate that macrophages possess probenecid-inhibitable transporters which are similar in their functional properties to organic anion transporters of epithelial cells. Such organic anion transporters have not been described previously in macrophages; they may mediate the release of naturally occurring organic anions such as prostaglandins, leukotrienes, glutathione, bilirubin, or lactate from macrophages.

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In addition to their role as phagocytes, macrophages are active secretory cells which elaborate at least 100 different substances (15). Many of these substances are enzymes and other proteins that are packaged within vesicles and released upon fusion of these vesicles with the macrophage plasma membrane (27). However, macrophages also elaborate a variety of molecules whose secretory pathways have not been elucidated. Among these are arachidonate derivatives, notably leukotriene C_4 and prostaglandin E_2, which play important roles in the inflammatory and immune responses (18, 19); glutathione (17); bilirubin, which is derived from degraded erythrocytes (16); and lactate produced by glycolysis (12). Soluble cytoplasmic enzymes are involved in the formation of all of these macrophage products.

We report here that several membrane-impermeant fluorescent dyes, which like the above natural products are organic anions, are rapidly secreted after they have been introduced into the cytoplasm of macrophages. We characterized this process using Lucifer Yellow, which is a water-soluble pH-insensitive fluorescent molecule that does not cross cellular membranes (24). This dye has been used as an intracellular marker for neurons; when it is injected into individual neurons, it delineates the processes of the neurons and remains within the cells for long periods of time. Lucifer Yellow also has been used to investigate exchange of cytoplasmic constituents between cells (23). However, in macrophages Lucifer Yellow was rapidly cleared from its original intracellular locus: most of the dye was released into the extracellular medium, and the remainder was sequestered within cytoplasmic vacuoles. We provide evidence that efflux of Lucifer Yellow from J774 cells is mediated by probenecid-inhibitable organic anion transporters, and suggest that these transporters mediate the secretion by macrophages of one or more of the natural products noted above.

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Materials and Methods

Chemicals

ATP (special grade) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Lucifer Yellow CH (tetralithium salt), and fura-2 pentapotassium salt were from Molecular Probes, Inc. (Eugene, OR). 6-Carboxyfluorescein was from Eastman Chemicals (Rochester, NY).

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Probenecid [*p*-(dipropylsulfamoyl) benzoic acid] and sulfinpyrazone were from Sigma Chemical Co. (St. Louis, MO).

Cells and Media

The J774 mouse macrophage-like cell line was grown in spinner cultures in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (56°C, 30 min) FBS (D10F). For microscopy, cells (1–2 × 10^5/coverslip) were plated for 6 h or overnight on 12-mm No. 1 glass coverslips in 16-mm tissue culture wells containing D10F. For quantitative studies, 10^6 cells per well were plated in 16-mm tissue culture wells.

Quantitation of Intracellular Lucifer Yellow

Cell-associated Lucifer Yellow fluorescence was measured as previously described (22, 26). Cells adherent to 16-mm wells in 24-well tissue culture plates were loaded with Lucifer Yellow in the presence of 5 mM ATP. To measure residual intracellular Lucifer Yellow after experiments, medium was removed from wells and the 24-well plates were immersed successively in three beakers of Dulbecco's PBS without divalent cations, the first containing 0.1 mg/ml BSA. Wells were suctioned dry, incubated with 500 μl of 0.05% Triton X-100, and scraped with rubber policemen. 350 μl of the suspension was added to 1.75 ml of a solution of 0.05% Triton X-100 and 0.1 mg/ml BSA. Fluorescence was measured in a fluorescence spectrophotometer (model 650-40; Perkin-Elmer Corp., Norwalk, CT), using an excitation wavelength of 430 nm and an emission wavelength of 540 nm. Standards of known Lucifer Yellow concentration were prepared for each experiment in the same Triton/BSA solution. Protein determinations were performed by a modification of the method of Lowry et al. (13) on all samples, and dye uptake was expressed as nanograms dye per milligrams cell protein. Each condition was performed in triplicate for all experiments. Cell viability was assessed at the end of experiments by trypan blue exclusion (3).

Quantitation of Dye Efflux

Cells plated in 16-mm tissue culture wells were ATP-loaded with Lucifer Yellow as above, immersed successively in three beakers of PD (the first containing an addition of 0.1 mg/ml BSA), and suctioned dry. The wells then were filled with 500 μl of fresh medium. Initially and at the indicated intervals 50-μl samples of the medium were removed and added to fluorimeter cuvettes containing 2 ml of Triton/BSA solution. At the end of experiments wells were processed as above for measurement of residual intracellular dye. Standards of known Lucifer Yellow concentration in Triton/BSA were made for each experiment. 5 mM probenecid did not affect measurement of Lucifer Yellow fluorescence at the wavelengths used in these experiments.

Fluorescence Microscopy

Cells were observed without fixation. Coverslips with adherent cells were washed several times with Dulbecco's PBS without divalent cations, inverted, mounted on microscope slides with interposed glass supports, and

1. *Abbreviation used in this paper*: D10F, Dulbecco's modified Eagle's medium supplemented with 10% FBS.

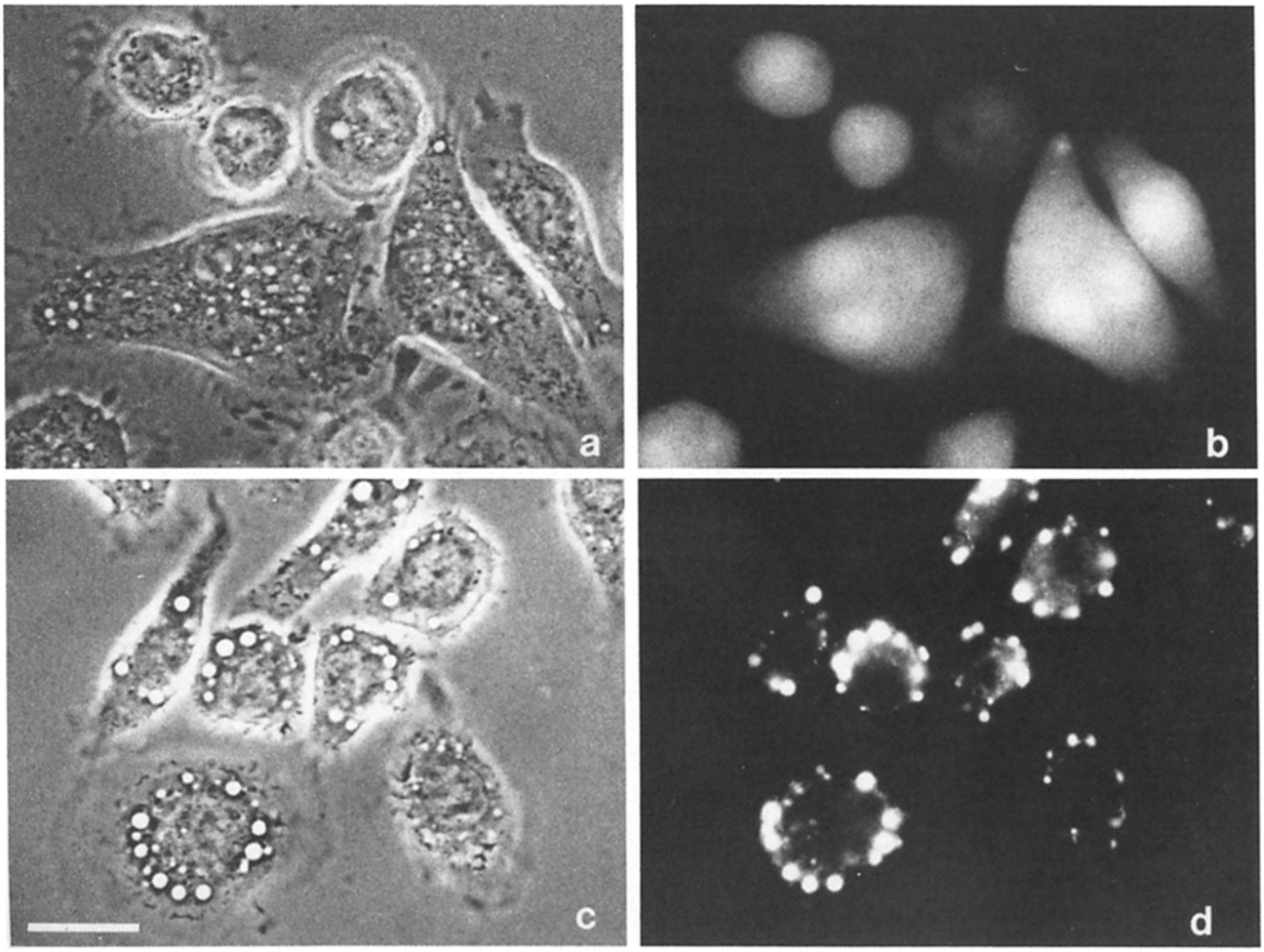

Figure 1. Lucifer Yellow is cleared from the cytoplasmic matrix of J774 cells. Adherent cells were incubated in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow for 5 min, washed, and bathed in D10F at 37°C. Immediately after ATP loading (*a* and *b*), Lucifer Yellow stained the cytoplasmic and nuclear matrices of the cells diffusely. 30 min after ATP loading (*c* and *d*), intracellular Lucifer Yellow was present predominantly within phase-lucent cytoplasmic vacuoles. *a* and *c*, phase; *b* and *d*, fluorescence. Bar, 20 μm.

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then coverslip edges were sealed with liquefied paraffin/vaseline/lanolin gel (1:1:1 mixture). Cells were observed with a Zeiss Photomicroscope III, with fluorescein and ultraviolet filter sets. Photographs were taken with Ektachrome ASA 400 film, exposed at ASA 800, and push-processed, or with Tri-X Pan film, exposed at ASA 1600, and developed with diafine developer.

Scrape Loading

Dyes were loaded into J774 cells without the use of ATP permeabilization by a procedure adapted from McNeil et al. (14). J774 cells were grown to confluence in 35-mm tissue culture plates. After removal of culture medium, 100 μl of a solution containing 150 mM KCl and the dye to be introduced into the cytoplasm was added to the tissue culture plates. Cells were gently scraped from the plates with a rubber policeman, the cell suspension was added to a centrifuge tube with 10 ml fresh D10F at 4°C, and the cells were washed twice at 4°C. The medium was decanted, the cells were resuspended in 2 ml D10F, and 200-μl aliquots of the cell suspension were layered onto 12-mm coverslips resting in 16-mm tissue culture wells. After 10 min at 37°C, 1 ml D10F was added to each well and cells were incubated at 37°C until examined.

Results

ATP Permeabilizes the Plasma Membrane of J774 Cells and Thioglycollate-elicited Mouse Peritoneal Macrophages to Fluorescent Dyes

In the presence of 5 mM extracellular ATP, several membrane-impermeant fluorescent dyes cross the plasma membrane of J774 cells and mouse peritoneal macrophages as assayed by fluorescence microscopy (22). J774 cells exposed to 5 mM ATP for 5 min accumulate carboxyfluorescein (376 D), ethidium bromide (394 D), Lucifer Yellow (457 D), and fura-2 pentapotassium salt (831 D), but not trypan blue (961 D) or Evans blue (961 D). Cells maintained at 37°C again exclude these dyes within 5 min after ATP is removed from the medium (22). Therefore, J774 cells and peritoneal macrophages can be loaded with fluorescent dyes and, presumably, other molecules of ≤831 D, allowed to reseal, and returned to culture for observation by fluorescence microscopy or other experimental manipulations.

Lucifer Yellow is a water-soluble, anionic, pH-insensitive, fluorescent dye that has been characterized as a marker for fluid-phase pinocytosis (26). It does not adsorb to or penetrate cell membranes. We used this dye to investigate the intracellular transport of molecules introduced into the cytoplasm by ATP-loading. Although all of the quantitative measurements reported here were done with Lucifer Yellow, qualitatively similar results were obtained with carboxyfluorescein and fura-2.

Lucifer Yellow introduced into J774 cells in the presence of ATP permeated the cytoplasmic and nuclear matrices of over 95% of the cells (Fig. 1, *a* and *b*). Phase-lucent cytoplasmic vacuoles excluded the dye. This diffuse pattern of intracellular fluorescence also was seen after J774 cells were incubated for 5 min in 5 mM ATP and either 100 μM 6-carboxyfluorescein or 10 μM fura-2. Ethidium bromide, which intercalates into DNA, entered cells during ATP treatment and was concentrated within the nuclei of the cells (not shown). Rat mast cells are permeabilized by ATP in a similar fashion (7).

Lucifer Yellow Is Cleared from the Cytoplasmic Matrix of J774 Cells

J774 cells were loaded with Lucifer Yellow as described, washed, bathed in fresh D10F at 37°C, and the amount of dye remaining within the cells was determined at intervals (Fig. 2). 30 min after dye loading, only 13% of the original quantity of Lucifer Yellow remained within the cells. Thus, Lucifer Yellow does not remain within the cytoplasm of J774 cells as it does within neurons.

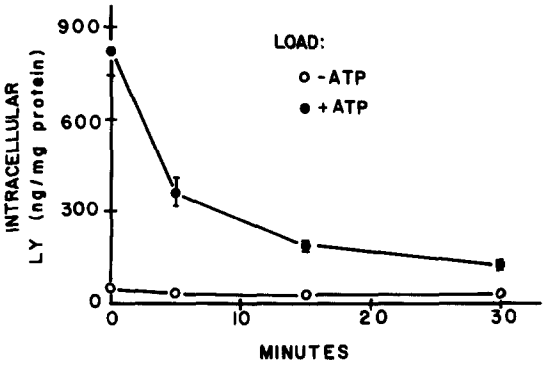

Figure 2. Lucifer Yellow efflux from J774 cells. J774 cells (10^6/well) adherent to 16-mm tissue culture wells were incubated in D10F containing 0.5 mg/ml Lucifer Yellow with or without 5 mM ATP for 5 min, washed, and further incubated in D10F at 37°C. At the indicated times after the removal of Lucifer Yellow and ATP the cells were washed and solubilized in 0.05% Triton X-100. Intracellular Lucifer Yellow and cell protein were measured as described. Results are expressed as residual intracellular Lucifer Yellow per milligram cell protein ± SD, and are the average of three experiments, each performed in triplicate. (*Open circles*) No ATP during exposure to Lucifer Yellow; (*solid circles*) 5 mM ATP during exposure to Lucifer Yellow.

Several lines of evidence show that the rapid efflux of Lucifer Yellow is not due to continued ATP permeabilization, that is, the dye does not efflux from the cells by the same route it entered. First, experiments described below (Figs. 5 and 9) reveal that ATP-induced pores are closed almost instantaneously upon removal of ATP^{4-} from the medium. Second, Lucifer Yellow efflux kinetics are identical both immediately after and 5 min after removal of extracellular ATP from the medium by washing of the cells (data not shown).

Lucifer Yellow Introduced into the Cytoplasm of J774 Cells Is Sequestered in Cytoplasmic Vacuoles

When J774 cells or thioglycollate-elicited mouse peritoneal macrophages were ATP-loaded with Lucifer Yellow, washed, and incubated in D10F without ATP at 37°C for 30 min, most cells exhibited a marked decrease in cytosolic Lucifer Yellow. Dye that remained within the cells was present in phase-lucent cytoplasmic vacuoles (Fig. 1, *c* and *d*). Cytoplasmic clearing and vacuolar sequestration (transfer of dye from the cytoplasmic matrix into vacuoles) were simultaneous processes that became evident ~10 min after the removal of ATP and Lucifer Yellow from the medium: at this time cytoplasmic vacuoles that excluded dye and cytoplasmic vacuoles that concentrated dye appeared within the same cell (Fig. 3). The rate at which dye was removed from the cytoplasm of J774 cells was not uniform; some cells had little residual cytoplasmic dye at 15 min and a few cells retained substantial cytoplasmic Lucifer Yellow at 30 min. Cells loaded with fura-2 pentapotassium salt and 6-carboxyfluorescein similarly cleared these dyes from the cytoplasmic matrix and sequestered them within cytoplasmic vacuoles.

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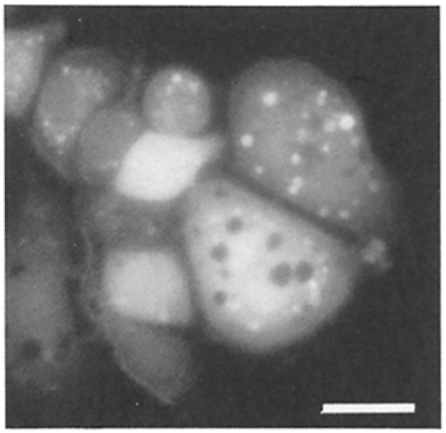

Figure 3. Lucifer Yellow present within the cytoplasm of J774 cells is sequestered within cytoplasmic vacuoles. J774 cells adherent to glass coverslips were exposed to 5 mM ATP and 0.5 mg/ml Lucifer Yellow for 5 min, washed, and incubated in D10F for an additional 10 min. The coverslips were mounted and viewed by fluorescence microscopy using fluorescein optics. At this time the cytoplasmic matrix of the cells still contained Lucifer Yellow. Cytoplasmic vacuoles that excluded the dye and cytoplasmic vacuoles that contained the dye were present within the same cell. Bar, 20 μm.

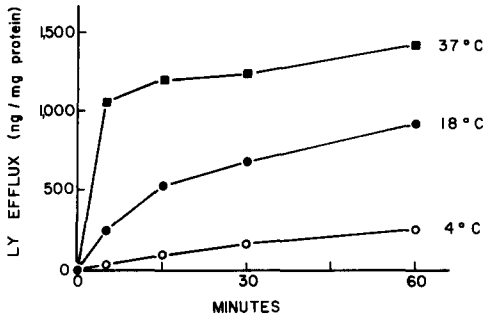

Figure 4. Effect of temperature on Lucifer Yellow efflux from J774 cells. Adherent cells were incubated in D10F containing 5 mM ATP and 1.0 mg/ml Lucifer Yellow at 37°C for 5 min, washed, and bathed in a solution containing 135 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM Hepes, pH 7.4, at 4, 18 or 37°C. Immediately after and at the indicated intervals 50-μl aliquots of medium were removed for quantitation of Lucifer Yellow. Intracellular Lucifer Yellow and cell protein were determined. Results are expressed as nanograms Lucifer Yellow per milligrams cell protein, and are those of a representative experiment (of three) performed in triplicate. (*Open circles*) Cells incubated at 4°C; (*solid circles*) 18°C; (*solid squares*) 37°C.

Clearance and Sequestration of Lucifer Yellow Are Not Artifacts of the ATP-Permeabilization Process

To determine whether exposure of J774 cells to ATP was a prerequisite for the clearance and sequestration of dyes, we also introduced dyes into the cytoplasm of J774 cells by a different procedure, termed "scrape loading" (14). J774 cells were scraped with rubber policemen from 35-mm tissue culture plates into 100 μl of solution containing 150 mM KCl and 0.5 mg/ml Lucifer Yellow, washed at 4°C, and plated. When these cells were observed 15 min later, cells containing Lucifer Yellow (usually ~20% of adherent cells) had a diffuse cytoplasmic pattern of fluorescence. In cells incubated at 37°C for 30 min after plating, Lucifer Yellow was present mostly within cytoplasmic vacuoles that were smaller than the vacuoles seen after ATP loading. 1 h after plating, Lucifer Yellow was cleared completely from the cytoplasmic matrix; the only dye remaining in the cells was contained in vacuoles.

Scrape-loaded cells did regain their normal membrane integrity. Although 4,100-D fluorescein–dextran cannot be introduced into J774 cells by ATP permeabilization, it can be scrape loaded into these cells. Immediately after 4,100-D fluorescein–dextran was scrape loaded into J774 cells it stained the cytoplasmic and nuclear matrices, as did Lucifer Yellow; cytoplasmic vacuoles likewise excluded fluorescein–dextran. However, unlike the situation with Lucifer Yellow, the intracellular pattern of 4,100-D fluorescein–dextran fluorescence remained unchanged for at least 48 h, and cytoplasmic vacuoles continued to exclude the dye. 70,000-D fluorescein–dextran behaved similarly, except that it did not penetrate into the nucleus after scrape loading.

Sequestration and Clearance of Lucifer Yellow Are Active Transport Processes

Lucifer Yellow efflux was temperature dependent; it was slowed at 18°C and nearly stopped at 4°C (Fig. 4). Vacuolar sequestration was also inhibited at 4°C. Cells ATP-loaded with Lucifer Yellow at 37°C, washed, and then maintained at 4°C retained the diffuse pattern of fluorescence for at least 1 h, and vacuolar uptake of Lucifer Yellow did not occur (not shown). Vacuolar sequestration and dye efflux into the medium resumed when these cells were rewarmed to 37°C.

Lucifer Yellow efflux from J774 cells occurred against a concentration gradient. J774 cells were ATP-loaded with Lucifer Yellow for 5 min, washed, and further incubated at 37°C in D10F containing 0.5 mg/ml Lucifer Yellow. Although the intracellular Lucifer Yellow concentration was ~40% of the external Lucifer Yellow concentration (22), dye efflux occurred (data not shown).

To further examine the ability of J774 cells to clear intracellular Lucifer Yellow against a concentration gradient, we took advantage of our observation that ATP^{4-}-induced membrane permeabilization can be rapidly reversed by the addition of sufficient Mg^{2+} to bind extracellular ATP^{4-}. J774 cells incubated in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow continued to take up Lucifer Yellow during a 20-min incubation at 37°C (Fig. 5). When 5 mM $MgSO_4$ was added to parallel samples after the first 5 min, ATP-induced permeabilization was reversed and the cells released Lucifer Yellow over the next 15 min, even though Lucifer Yellow and MgATP were still in the medium. To confirm that the concentration of Lucifer Yellow in the medium was higher than that in the cells, 10 mM EDTA was added 5 min after the addition of $MgSO_4$. This caused the conversion of $MgATP^{2-}$ to ATP^{4-}, which reopened membrane pores and allowed Lucifer Yellow to again move down its concentration gradient from the medium into the cells.

Lucifer Yellow Efflux Occurs in the Absence of a Plasma Membrane Electrical Gradient

ATP permeabilization is accompanied by ion fluxes and a rapid depolarization of the plasma membrane of J774 cells (21, 25). Because Lucifer Yellow is anionic, it was possible that the plasma membrane's electrical potential, or its reestablishment after the removal of ATP, could be important

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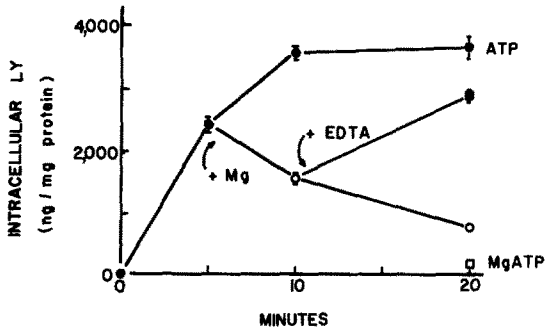

Figure 5. Lucifer Yellow is released from J774 cells after reversal of ATP permeabilization with $MgSO_4$. Adherent cells were incubated at 37°C in D10F containing 0.5 mg/ml Lucifer Yellow and 5 mM ATP. 5 mM $MgSO_4$ was added to some samples initially or after 5 min, and 10 mM EDTA was added to some samples after 10 min. At the indicated intervals, cells were washed and intracellular Lucifer Yellow and cell protein were measured. Results are expressed as nanograms intracellular Lucifer Yellow per milligrams cell protein ± SD, and are those of an experiment representative of six, each done in triplicate. (*Solid circles*) Cells exposed to ATP and Lucifer Yellow; (*open circles*) cells exposed to ATP and Lucifer Yellow, with $MgSO_4$ added after 5 min; (*solid squares*) cells exposed to ATP and Lucifer Yellow, $MgSO_4$ added after 5 min, EDTA added 5 min later; (*open squares*) cells exposed to ATP, Lucifer Yellow, and $MgSO_4$ throughout.

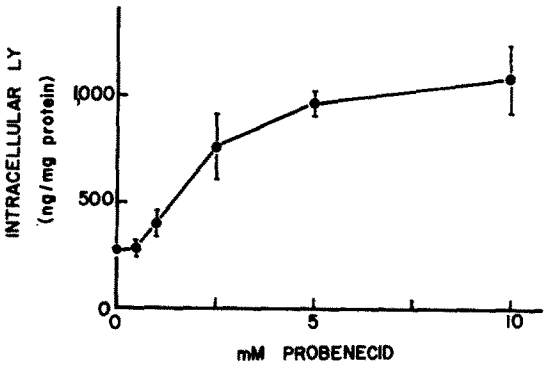

Figure 6. Effect of probenecid on Lucifer Yellow efflux from J774 cells: dose-dependence curve. Adherent cells were incubated at 37°C in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow for 5 min, washed, and bathed in D10F containing the indicated concentration of probenecid for 30 min. Cells were washed and intracellular Lucifer Yellow and cell protein were determined. Total intracellular concentration of Lucifer Yellow immediately after ATP loading was 1480 ng/mg protein. Results are expressed as nanograms intracellular Lucifer Yellow per milligrams cell protein ± SD and are the average of three experiments performed in triplicate.

in promoting Lucifer Yellow efflux from the cytoplasm. We therefore ATP-loaded cells with Lucifer Yellow, washed the cells, and incubated them at 37°C in a buffered salt solution containing either Na^+ (140 mM NaCl, 5.0 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM Hepes) or K^+ (140 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM Hepes) as the predominant extracellular cation. In the presence of high extracellular K^+, the plasma membrane will remain depolarized after the removal of ATP. We found no difference in Lucifer Yellow efflux between cells incubated in high K^+ buffer and cells incubated in high Na^+ buffer (data not shown), indicating that Lucifer Yellow efflux is not dependent on membrane potential.

Lucifer Yellow Efflux from J774 Cells Is Inhibited by Probenecid

Because all of the dyes cleared by J774 cells are organic anions, we examined known inhibitors of organic anion transport to determine whether any of them affected Lucifer Yellow efflux from these cells. 2 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid did not inhibit efflux of Lucifer Yellow. In contrast, probenecid, a drug which is known to inhibit transport of organic anions across many epithelia, inhibited Lucifer Yellow efflux from J774 cells and thioglycollate-elicited mouse peritoneal macrophages. The inhibitory effect of probenecid was dose dependent (Fig. 6): 0.5 mM probenecid had a negligible effect on Lucifer Yellow efflux, and 5.0 mM probenecid caused nearly maximal inhibition. Sulfinpyrazone, another inhibitor of organic anion transport, also inhibited Lucifer Yellow efflux. Sulfinpyrazone was more potent but appeared to be more toxic to J774 cells than probenecid. 2.5 mM sulfinpyrazone caused nearly maximal inhibition of Lucifer Yellow efflux, but after 15 min in 10 mM sulfinpyrazone cell adherence to tissue culture wells was significantly diminished (data not shown).

The effect of probenecid on Lucifer Yellow efflux was reversible (Fig. 7). J774 cells were exposed to ATP and Lucifer Yellow for 5 min, washed, and incubated in D10F at 37°C with or without 5 mM probenecid. After 15 min incubation, cells exposed to probenecid had released only 16% as much Lucifer Yellow as cells not exposed to probenecid. Cells exposed to probenecid released Lucifer Yellow at a constant but markedly reduced rate during a 1-h incubation period. When cells loaded with Lucifer Yellow were exposed to probenecid for 30 min, washed, and further incubated in D10F without probenecid, Lucifer Yellow efflux resumed at a rate similar to that seen in untreated cells. The effect of probenecid on Lucifer Yellow efflux occurred rapidly; incubation of cells

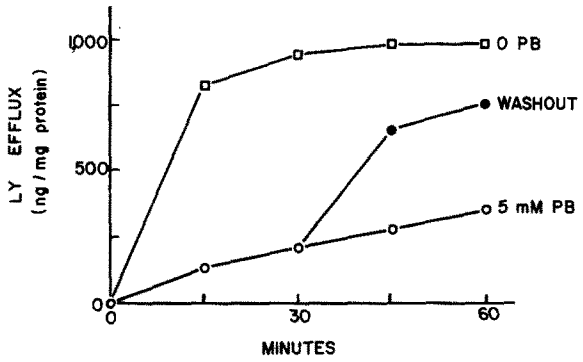

Figure 7. Inhibition of Lucifer Yellow efflux from J774 cells by probenecid is reversed upon removal of probenecid from the medium. Adherent cells were incubated at 37°C in D10F containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow for 5 min, washed, and bathed at 37°C in D10F containing 5 mM probenecid. After 30 min incubation, some cells were washed and incubated in D10F without probenecid. Initially, and at the indicated intervals, 50-μl aliquots of medium were removed and Lucifer Yellow was quantitated. Intracellular Lucifer Yellow and cell protein were measured. Results are expressed as nanograms Lucifer Yellow present in the extracellular medium per milligrams cell protein and are the average of three experiments each done in triplicate. (*Open squares*) Without probenecid; (*open circles*) 5 mM probenecid throughout; (*solid circles*) 5 mM probenecid for 30 min, D10F alone for 30 min.

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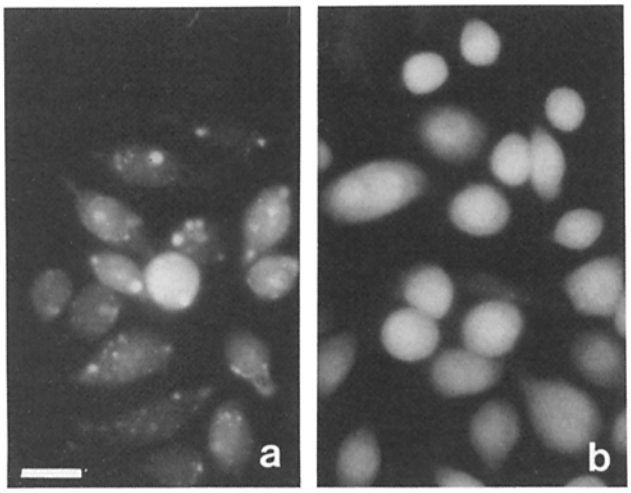

Figure 8. Probenecid inhibits clearance of Lucifer Yellow from the cytoplasmic matrix of J774 cells. Adherent cells were incubated in 0.5 mg Lucifer Yellow and 5 mM ATP for 5 min, washed, and bathed in D10F with 1.0 mM probenecid (*a*) or 2.5 mM probenecid (*b*) for 30 min. Cells were then washed and viewed by fluorescence microscopy. Bar, 20 μm.

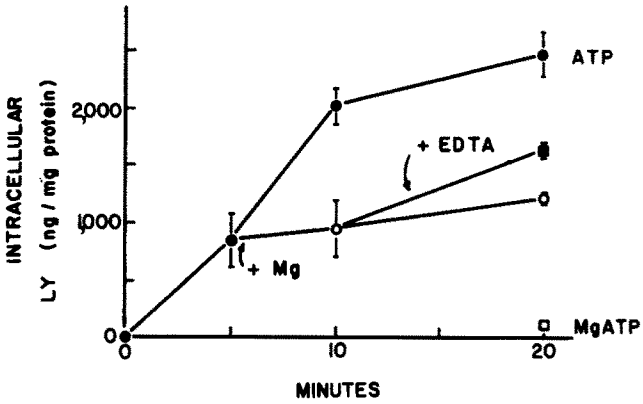

Figure 9. Probenecid blocks efflux of Lucifer Yellow from J774 cells after reversal of ATP permeabilization with $MgSO_4$. Adherent cells were incubated at 37°C in D10F containing 0.5 mg/ml Lucifer Yellow, 5 mM ATP, and 5 mM probenecid. 5 mM $MgSO_4$ was added to some samples initially or after 5 min, and 10 mM EDTA was added to some samples after 10 min. At the indicated times, cells were washed and intracellular Lucifer Yellow and cell protein were measured. Results were expressed as nanograms intracellular Lucifer Yellow per milligrams cell protein, and are the average of three experiments ± SD, each done in triplicate. (*Solid circles*) Cells exposed to ATP, Lucifer Yellow, and probenecid; (*open circles*) cells exposed to ATP, Lucifer Yellow, and probenecid with $MgSO_4$ added after 5 min; (*solid squares*) cells exposed to ATP, Lucifer Yellow, and probenecid with $MgSO_4$ added after 5 min, and EDTA added 5 min later; (*open squares*) cells exposed to ATP, Lucifer Yellow, probenecid, and $MgSO_4$ throughout.

with probenecid before or during ATP permeabilization of the cells was not required to inhibit Lucifer Yellow efflux.

The inhibitory effect of probenecid on Lucifer Yellow efflux was confirmed by microscopy (Fig. 8). J774 cells plated on coverslips were loaded with Lucifer Yellow, washed, incubated in D10F for 30 min at 37°C, and observed by fluorescence microscopy. At this time, in cells treated with 0–0.5 mM probenecid, intracellular Lucifer Yellow was present predominantly within cytoplasmic vacuoles, as described above. Cells exposed to 1.0 mM probenecid retained more dye within the cytoplasmic matrix and sequestered Lucifer Yellow within large cytoplasmic vacuoles (Fig. 8 *a*). Cells exposed to 2.5 or 5.0 mM probenecid maintained diffuse intracellular fluorescence (Fig. 8 *b*).

Probenecid did not inhibit ATP-induced uptake of Lucifer Yellow by J774 cells, nor did it block reversal of ATP-induced permeabilization. We assessed this by repeating the experiments described in Fig. 5 in the presence of 5 mM probenecid (Fig. 9). J774 cells exposed to ATP and Lucifer Yellow in the presence of probenecid continued to accumulate Lucifer Yellow over 20 min, as did cells not exposed to probenecid. When $MgSO_4$ was added after 5 min to cells incubated in ATP, Lucifer Yellow, and probenecid, Lucifer Yellow accumulation was curtailed. As expected, probenecid blocked the efflux of Lucifer Yellow after the ATP-induced pores were closed by the addition of Mg^{2+}. As before, addition of EDTA allowed the pores to reopen and intracellular accumulation of Lucifer Yellow to resume.

Exposure of cells to 5 mM probenecid for 3 h did not affect cell viability (trypan blue exclusion). Exposure of cells to 10 mM probenecid for 1 h also did not decrease cell viability. However, after incubation in 10 mM probenecid for 3 h, many cells were no longer adherent to the tissue culture wells and did not exclude trypan blue.

Probenecid-inhibitable anion transporters have been studied in a variety of polarized epithelia, and have been best characterized in the renal proximal tubule (10). Using apical and basolateral plasma membrane vesicles from these cells, several investigators have concluded that there are different probenecid-sensitive transporters, some of which are anion antiporters, exchanging organic anions either for other organic anions or for inorganic anions such as Cl^- (11), and others which depend on Na^+ co-transport. Several lines of evidence suggest that Lucifer Yellow efflux from J774 cells does not involve Na^+ co-transport. First, an outward Na^+ gradient does not occur under physiologic conditions. Second, Lucifer Yellow efflux occurred at equal rates in the presence and absence of Na^+ in the external medium. Third, J774 cells scrape loaded with Lucifer Yellow in 150 mM KCL cleared the dye. To determine whether replacement of external Cl^- inhibited Lucifer Yellow efflux, J774 cells were loaded with Lucifer Yellow, washed, and incubated in medium containing 150 mM NaCl or 150 mM sodium gluconate for 15 min. Lucifer Yellow efflux was not altered in cells incubated in gluconate (data not shown), suggesting that chloride antiport is not required.

To determine whether a transmembrane pH gradient was necessary for Lucifer Yellow efflux, we loaded cells with Lucifer Yellow and then incubated the cells in a salt solution (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 11 mM glucose) buffered to different pHs with 20 mM MES, Pipes, MOPS, Hepes, or TRIZMA, for 15 min. Residual intracellular Lucifer Yellow was then determined. Lucifer Yellow efflux was maximal at pH >7.0; below pH 7.0 Lucifer Yellow efflux decreased with decreasing external pH (Fig. 10). This result suggests that Lucifer Yellow efflux requires a pH gradient. Organic anion transporters dependent on pH gradients have been described in dog renal tubular plasma

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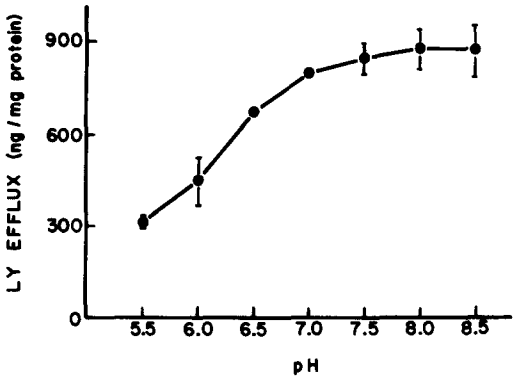

Figure 10. Efflux of Lucifer Yellow from J774 cells is inhibited by lowering of the extracellular pH. Adherent cells were incubated in DlOF containing 5 mM ATP and 0.5 mg/ml Lucifer Yellow for 5 min, washed, and bathed in a solution containing 135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 11 mM glucose, and buffered to the indicated pH with 20 mM concentrations of the following buffers: MES, pH 5.5; MES, pH 6.0;, Pipes, pH 6.5, MOPS, pH 7.0; Hepes, pH 7.5; TRIZMA, pH 8.0; TRIZMA, pH 8.5. Initially and after 15 min incubation at 37°C cells were washed and intracellular Lucifer Yellow and cell protein were measured. Results are expressed as Lucifer Yellow efflux (intracellular Lucifer Yellow initially minus intracellular Lucifer Yellow at 15 min) per milligrams cell protein ± SD, and represent the average of two experiments, each performed in triplicate.

membrane vesicles (1) and rat basolateral liver plasma membrane vesicles (2, 9).

Discussion

Our results indicate that macrophages contain hitherto unrecognized transport system(s) that promote efficient removal of molecules from the cytoplasmic matrix into cytoplasmic vacuoles and the release of these molecules into the extracellular medium. The fluorescent dye Lucifer Yellow is released from J774 cells by a temperature-dependent process that is capable of removing Lucifer Yellow from the cell in the presence of high extracellular concentrations of Lucifer Yellow. Furthermore, this process is inhibited by probenecid, which blocks the transport of urate (11), *p*-amino hippurate (1), penicillin (4), leukotriene C_4 (20) and a host of other organic anions in epithelia, such as the renal proximal tubule, hepatocytes, and the choroid plexus.

These findings suggest that J774 cells have in their membranes organic anion transporters which mediate Lucifer Yellow transport out of the cytoplasm. While organic anion transporters are known to occur in a number of epithelia, we are unaware of previous descriptions of probenecid-inhibitable organic anion transporters in phagocytic leukocytes.

Although the introduction of fluorescent dyes into the cytoplasm of cells was greatly facilitated by the technique of ATP permeabilization, the clearance and sequestration of dyes from the cytoplasmic matrix was not induced by ATP. Scrape-loaded cells also cleared the dyes, as mentioned above. The dyes carboxyfluorescein and fura-2 are also available as membrane-permeant esters which diffuse into the cytoplasmic matrix of cells and are hydrolyzed to their membrane-impermeant forms by cytosolic esterases. When carboxyfluorescein diacetate (Swanson, J., unpublished observation) or fura-2 acetoxymethyl ester (Di Virgilio, F., T. H. Steinberg, J. A. Swanson, and S. C. Silverstein, manuscript submitted for publication) were allowed to diffuse into the cytoplasmic matrix of J774 cells, clearance and vacuolar sequestration of these dyes were observed. Thus, efflux and intracellular sequestration of dye are unrelated to the mechanism of dye loading.

We have been unable to assess directly the relationship between sequestration of Lucifer Yellow within vacuoles and release of Lucifer Yellow from the cells. On the one hand, Lucifer Yellow efflux appears to occur more rapidly than sequestration, suggesting that Lucifer Yellow efflux can occur directly across the plasma membrane and, therefore, that the probenecid-inhibitable organic anion transporter is present within the plasma membrane. On the other hand, our data show that Lucifer Yellow is rapidly sequestered within phase-lucent vacuoles. These vacuoles exhibit several structural and functional properties usually associated with endosomes, and sequestration of Lucifer Yellow within them is inhibited by probenecid (Steinberg, T. H., Swanson, J. A., and S. C. Silverstein, manuscript in preparation). Pinocytosed Lucifer Yellow is rapidly expelled into the medium from the endosomal compartment in these cells (26). Thus it is possible that sequestration of Lucifer Yellow into phase-lucent vacuoles precedes the extrusion of this dye from the cell; the data available do not allow us to determine which of these two efflux pathways predominates. However, it seems reasonable to postulate that the same organic anion transporter is present in both the plasma membrane and in the membrane of the sequestering vacuole, and that this transporter mediates both efflux and sequestration of these dyes.

Organic anion transporters that have been previously characterized involve Na^+ co-transport, or anion antiport, in which case the anion may be Cl^-, OH^-, HCO_3^-, or an organic anion. The organic anion transporter in macrophages is unlikely to require Na^+ co-transport as discussed above, and our evidence that Lucifer Yellow efflux occurs in cells bathed in high K^+ medium excludes the possibility that it is a K^+ co-transporter. Lucifer Yellow efflux is not inhibited by the removal of Cl^- or HCO_3^- from the medium. Our observation that Lucifer Yellow efflux is inhibited by low extracellular pH suggests that this organic anion transporter may involve OH^- antiport or H^+ symport.

What is the biological significance of the presence of an organic anion transporter in macrophages? Organic anion transporters have been characterized and studied predominantly in polarized epithelial cells. However, all cells produce organic acids as byproducts of metabolism. These organic acids must be degraded within the cells or released from the cells into the plasma. Lactic acid is one of the most abundant byproducts of metabolism. It is transported across membranes by some probenecid-sensitive transporters (6). That a specific mechanism for removal of such molecules is necessary is suggested by a recent report of a patient who suffered muscle damage after strenuous exercise (5). The patient's symptoms were traced to an abnormality of lactate release from skeletal muscle leading to an intracellular accumulation of this metabolite. Like muscle cells, macrophages are called upon to perform strenuous work for periods of time, and they may therefore also require mechanisms to rid themselves of organic acids.

Macrophages are very active secretory cells. They elab-

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orate a variety of moderately water-soluble cyclo-oxygenase and lipoxygenase products that are synthesized from plasma membrane arachidonic acid by membrane-associated and cytosolic enzymes (18, 19). These arachidonic acid metabolites must therefore have a means of egress across the plasma membrane. Since these molecules are organic anions that are in the size range of molecules known to be transported by probenecid-sensitive pathways, it is possible that a primary function of the organic anion transporters we have described in J774 cells may be to secrete these arachidonate metabolites. Consistent with this suggestion, Spector and Goetzl have shown that leukotriene C_4 transport in the choroid plexus is blocked by probenecid (20).

Tissue macrophages are the primary cells that remove senescent erythrocytes from the circulation and degrade the erythrocytes' constituents for reutilization. At least 80% of hemoglobin is recycled in this fashion in humans. The conversion of heme to bilirubin occurs in macrophages (16) and the release of unconjugated bilirubin into the plasma involves transport of bilirubin across cellular membranes. It is possible that the organic anion transporter we have described is the pathway by which bilirubin is released from macrophages.

The ability of many antibiotics to eliminate microbial pathogens may depend on the ability of these drugs to achieve adequate concentrations within phagocytes or within the lysosomes of these cells (8). Transport inhibitors like probenecid may block efflux of antibiotics from phagocytes, thereby increasing the effective intracellular concentration of these drugs. Conversely, such transport inhibitors may block the sequestration and delivery of antibiotics into the cells' lysosomes, thereby decreasing their antimicrobial efficacy. The identification of transport systems for organic anions in macrophages will allow exploration of this unresolved issue.

Whatever the function of this probenecid-inhibitable organic anion transporter may be, the ability to block efflux of organic anions from macrophages may have practical value. Many of the fluorescent indicators that are used to measure intracellular [Ca^{2+}] and pH are organic anions, and we demonstrated in the present work that the calcium indicator fura-2 was sequestered within vacuoles in J774 cells. In addition, we have found that intracellular sequestration of fura-2, as well as efflux of fura-2 from J774 cells and mouse peritoneal macrophages, is inhibited by probenecid (Di Virgilio, F., T. H. Steinberg, J. A. Swanson, and S. C. Silverstein, manuscript submitted for publication). Quantitation of changes in intracellular [Ca^{2+}] was facilitated by blocking efflux of fura-2 from the cells' cytoplasm with probenicid, thereby enhancing the reliability of this indicator.

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