Animal cell mutants unable to take up biologically active glycerophospholipids

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Abstract We have isolated two mutant strains from the murine, macrophage-like cell line, RAW 264.7, that are resistant to the cytotoxic effects of the antineoplastic, platelet activating factor analogue, 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine (ET-18-OMe). The mutants were isolated using a single round of selection to ensure that resistance was due to a single gene defect. These mutants, RAW.R1 and RAW.R23, are approximately 20 times more resistant to ET-18-OMe (ID_{50} = 15–17 μM) than the parent strain (ID_{50} = 0.7–1.0 μM). Resistance to ET-18-OMe was due to a 90–95% reduction in the ability to take up and accumulate this compound. The uptake of other choline glycerophospholipids (e.g., platelet activating factor and 1-acyl-2-lyso-*sn*-glycero-3-phosphocholine) was also severely affected. This defect was not limited to choline glycerophospholipids; the uptake of an ethanolamine glycerophospholipid (1-alkyl-2-lyso-*sn*-glycero-3-phosphoethanolamine) was reduced by 80%. The uptake of palmitic acid, an amphipathic molecule bearing no phosphate-containing head group, was unaffected in the mutants. There was little metabolism of ET-18-OMe by either the wild-type or mutant cells. Binding of ET-18-OMe appeared to be normal in the mutants, but internalization of pre-bound ET-18-OMe was reduced. Uptake of non-lipid ligands such as horseradish peroxidase, lucifer yellow, and transferrin was normal in the mutants demonstrating that fluid-phase and receptor-mediated endocytosis is functional. The ability to generate mutants displaying a lesion that affects the uptake of both choline and ethanolamine phospholipids demonstrates that these species are internalized by RAW cells through one common primary route or through pathways that share a common factor. These mutants, and this approach to their isolation, offer a system with which to study and define the mechanisms of glycerophospholipid uptake into macrophages as well as other cell types.—**Zoeller, R. A., M. D. Layne, and E. J. Modest.** Animal cell mutants unable to take up biologically active glycerophospholipids. *J. Lipid Res.* 1995. **36:** 1866–1875.

Supplementary key words somatic cell mutants • antineoplastic phospholipids • glycerophospholipid uptake • platelet activating factor

All animal cells can take up and utilize phospholipids. The mode of internalization of a phospholipid can be dictated by its head group (1–3), the cell type (4), and the way in which the phospholipid is presented to the cell (e.g., as monomers, aggregates such as liposomes and micelles, or associated with serum components such as lipoproteins or albumin). Phospholipids can be internalized, after insertion into the plasma membrane, during endocytosis or phagocytosis (2, 5). Alternatively, phospholipids can be taken up as a soluble component of the medium during these processes. In certain cell types the uptake of amino-phospholipids such as phosphatidylethanolamine appears to involve a protein-mediated translocation of the phospholipid across the plasma membrane (6).

Platelet activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) and lysophosphatidylcholine (1-acyl-2-lyso-*sn*-glycero-3-phosphocholine; lysoGPC) are two phospholipids that can be found in the extracellular space. PAF, a potent hormone, is formed and released by cells, causing aggregation of platelets and activating a variety of cell types (7, 8). LysoGPC, generated during oxidation of low density lipoprotein (9), as well as during ischemia (10–12), has been reported to be a potent chemotactic factor for monocytes and may be instrumental in the formation of atherosclerotic plaque (9). Although a receptor has been described for PAF, the nature of the chemotactic effect of lysoGPC is not understood. It has been suggested that metabolism of lysoGPC, once inside the cell, is part of the stimulation process (13). Lipid activators such as PAF and lysoGPC not only interact with cells to cause an effect, they must

Abbreviations: ET-18-OMe, 1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine; ET-16S-OEt, 1-S-hexadecyl-2-ethyl-*rac*-glycero-3-phosphocholine; PAF, platelet activating factor or 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoPAF, 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; DNA, deoxyribonucleic acid; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; HPRT, hypoxanthinephosphoribosyltransferase; O, ouabain; HRP, horseradish peroxidase.

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be taken up, modified, and inactivated by cells. How these phospholipids enter cells and the importance of this process for cell activation and the metabolism of these compounds are not well understood.

ET-18-OMe (1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine) is one of a class of novel, membrane interactive anticancer compounds (for comprehensive reviews, see refs. 14 and 15). Structurally, ET-18-OMe is similar to choline phospholipids such as PAF and lysoGPC, but it bears ether-linked alkyl chains at both the *sn*-1 and *sn*-2 positions on the glycerol backbone. The sensitivity of a cell to ET-18-OMe and similar compounds can generally, but not universally, be correlated with a cell's ability to accumulate the compound (16). These compounds are not metabolized by animal cells to any great extent and it has been proposed that ET-18-OMe accumulates rapidly enough within sensitive cells to cause membrane disruption and cell death (15).

We have isolated clonal strains from the macrophage-like tumor cell line RAW 264.7 (17) that are resistant to the cytotoxic effects of ET-18-OMe. We hypothesized that ET-18-OMe resistance in some of the mutants would be a result of their inability to take up this compound as well as naturally occurring phospholipid species such as PAF and lysoGPC. In this report we describe the isolation and initial characterization of such mutants. Studies using these resistant strains suggest a single route for uptake of ET-18-OMe and similar glycerophospholipids in the RAW cells.

MATERIALS AND METHODS

Materials

[Octadecyl-9,10-^{3}H]1-octadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF) (5.2 TBq/mmol), [choline-methyl-^{3}H]L-α-dipalmitoyl phosphatidylcholine (1.5 TBq/mmol), [9,10-^{3}H]palmitic acid (1.11 TBq/mmol), [1-^{3}H]ethanolamine (1.0 TBq/mmol), [methyl-^{3}H]thymidine (74 Gbq/mmol), and [^{125}I-labeled] differic human transferrin were purchased from DuPont-New England Nuclear. [Alkyl-1′,2′-^{3}H]1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPAF) (2 TBq/mmol) was purchased from Amersham (Arlington Heights, IL). [Choline-methyl-^{3}H]1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine was produced by phospholipase A_2 digestion of the purchased tritium-labeled dipalmitoyl phosphatidylcholine. [1-^{3}H-ethanolamine]1-alkyl-2-lyso-*sn*-glycero-3-phosphoethanolamine (approximately 15 GBq/mmol) was isolated as described (18) after labeling of the plasmalogen-deficient mutant strain RAW.12 with [1-^{3}H]ethanolamine. [Octadecyl-9,10-^{3}H]1-octa-decyl-2-methyl-*sn*-glycero-3-phosphocholine (2.1 TBq/ mmol) and 1-S-hexadecyl-2-ethyl-*rac*-glycero-3-phosphocholine were kindly supplied by Dr. Claude Piantadosi and colleagues, University of North Carolina (Chapel Hill, NC). 1-Octadecyl-2-methyl-*rac*-glycero-3-phosphocholine was the generous gift of Dr. R. Nordstrom, Medmark pharma GmbH (Munich, Germany). Non-radioactive lysoPAF was purchased from Avanti Polar Lipids (Alabaster, AL). Lucifer yellow was purchased from Molecular Probes (Eugene, OR). Other non-radioactive lipids and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cells and culture conditions

RAW 264.7 cells (ATCC TIB71) were obtained from the American Type Culture Collection and maintained in suspension culture at 37°C in a 5% CO_2/95% air atmosphere in Ham's F12 medium containing 10% fetal calf serum (Hazelton Laboratories) supplemented with 1 mM glutamine, penicillin G (100 units/ml), and streptomycin (75 units/ml). This medium is designated F12c. For experiments, selections and mutageneses, RAW cells were plated onto tissue culture plastic or in glass scintillation vials (to which they adhered). Mutageneses were performed using ethyl methanesulfonate (19). RAW.R1.OA2 is a derivative of the RAW.R1 mutant strain which bears the selection markers hypoxanthinephosphoribosyltransferase deficiency (HPRT$^-$) and ouabain resistance (O^r) and RAW.108.OA4 is an ether lipid-deficient derivative of RAW 264.7 which also bears these secondary markers, HPRT$^-$/O^r. RAW.108 is similar to wild-type cells with regard to ET-18-OMe sensitivity and uptake. The cell lines bearing these selection markers were generated essentially as described elsewhere (20).

Hybridization analyses

Hybrids were generated by fusing a HPRT$^-$/O^r cell line with cells not bearing these markers. RAW/RAW fusions using polyethyleneglycol and selection of hybrids were performed as described for CHO/CHO fusions (21) with the only exception that the RAW cells were incubated in the presence of polyethyleneglycol for 10 min instead of 1 min.

Growth inhibition

Cells were plated in 96-well tissue culture dishes in 0.1 ml at 10^4 cells/well and allowed to adhere overnight. Cytotoxic compounds were then added at various concentrations in 0.1 ml F12c and the cells were maintained for 46 h. All compounds were added to the growth medium from concentrated ethanol stock solutions with the final ethanol concentration not exceeding 0.25%. The cells were then labeled by the addition of 1.0 μCi [^{3}H-methyl]thymidine in 50 μl F12c. After 2 h at 37°C medium was removed, the cells were washed 3 times

with 0.2 ml phosphate-buffered saline (PBS), then lysed with 0.2 ml H_2O at 37°C for 15 min. Thymidine-labeled DNA was harvested with water, collected on Whatman grade 934-AH glass microfiber filter paper (MASH II, Whittaker MA Bioproducts, Walkersville, MD), and dried overnight. Radioactivity associated with the filter discs was assessed by liquid scintillation.

Lipid uptake assays

In most phospholipid uptake studies, cells were plated in 96-well plates in 0.2 ml F12c medium at 2.5×10^4 cells/well and allowed to adhere overnight. The compound to be tested was added, as an ethanol solution, to F12c medium in a sterile glass vial to achieve a concentration of 5 μM and sonicated for 10 min in a sonicating bath. The final ethanol concentration did not exceed 0.25%. To each well was added 0.05 ml of this solution to achieve a final concentration of 1 μM and the cells were incubated at 37°C. After the desired incubation time labeled medium was removed, the cells were washed 3 times with PBS containing 5% fetal bovine serum, the plates were allowed to dry, and the cellular material was solubilized in 0.2 ml of 0.5 N NaOH overnight at 4°C. Aliquots were removed for liquid scintillation counting. Wells containing no cells were used as controls for nonspecific binding of compound to the plastic. Cells were also plated in 24-well plates at similar density and treated with similar levels of non-radioactive lipid. These were used for protein determinations using the method of Lowry et al. (22).

ET-18-OMe binding and internalization

Cells were plated into 24-well plates at 2×10^5 cells/well and allowed to attach overnight at 37°C. The plates containing the cells were placed on ice for 30 min, then the medium was removed and replaced with 0.35 ml of ice-cold HEPES-buffered RPMI medium (RPMI/HEPES; pH 7.4) containing 0.25 mg/ml fatty acid-free bovine serum albumin and 8×10^5 cpm tritiated ET-18-OMe (53.4 nM) plus 1 μM carrier ET-18-OMe (binding medium). After 30 min on ice, binding medium was removed, and the cells were washed twice with ice-cold RPMI/HEPES medium. RPMI/HEPES (0.5 ml) was added and cells were incubated at room temperature (23°C) to allow for internalization to occur. The cells were placed back on ice, medium was removed and replaced with 0.8 ml ice-cold RPMI/HEPES medium containing 10 mg/ml fatty acid-free BSA, and incubated on ice for 1 h to remove ET-18-OMe remaining on the cell surface. Medium was removed, the cells were washed twice with ice-cold RPMI/HEPES, and solubilized in 1% Triton X-100. Cell-associated radioactivity was determined by counting an aliquot using LSC. Wells containing no cells were used as controls in all cases. Separate wells, containing cells treated in a similar fashion, were used for protein determinations.

Metabolism of ET-18-OMe by RAW cells

Cells were plated into sterile glass scintillation vials at 5×10^5 cells/vial and allowed to attach overnight. Medium was removed and replaced with 0.8 ml growth medium containing 1 μM tritiated ET-18-OMe (8×10^5 cpm). After 6 h at 37°C, 2 ml methanol, 1 ml chloroform, 100 μg carrier lipid (total mouse liver lipids), and 30 μl concentrated HCl were added and this single phase mixture was allowed to sit at room temperature for 30 min prior to transfer to a test tube. One ml chloroform and 1 ml PBS were added to form two phases. Phases were separated by centrifugation and the organic phase was recovered. After removal of solvent using a stream of nitrogen, samples were spotted on thin-layer chromatography (silica gel 60, E. Merck) and the plates were developed in chloroform–methanol–acetic acid–H_2O 75:75:16:8. The plates were sprayed with EN^{3}HANCE (New England Nuclear/DuPont) and exposed to X-ray film at -70°C.

Measurements of endocytosis

Transferrin uptake was measured using a modification of a method described earlier (23, 24). Cells were plated in a 24-well dish at 5×10^5 cells/well in 0.5 ml growth medium (F12c). After allowing the cells to attach for 4 h at 37°C, the medium was removed, cells were washed once with 1 ml binding buffer (Ham's F12 medium supplemented with 20 mM HEPES, pH 7.25, and 1 mg/ml BSA) and 0.25 ml ^{125}I-labeled diferric human transferrin (16.85 nM) in binding buffer was added. This later solution was prewarmed to 37°C prior to addition. At the indicated times 0.75 ml ice-cold binding buffer was added and the cells were placed on ice. The cells were washed 4 times with ice-cold binding buffer and treated with 1 ml of an ice-cold 0.25% pronase solution in Ham's F12/20 mM HEPES, pH 7.25, to remove bound but uninternalized transferrin. After 2 h the pronase was removed and the cells were solubilized in 0.5 ml of 0.5 M NaOH for 1 h at 37°C. Cell-associated radioactivity was determined by counting an aliquot in a LKB, Model 1275, Minigamma gamma counter. Values from cells incubated in the presence of ^{125}I-labeled transferrin at 0°C were used as controls to correct for unremoved surface-bound transferrin.

The uptake of lucifer yellow was monitored as described previously (25). Cells were plated in 24-well dishes at 2.5×10^5 cells/well and allowed to attach overnight at 37°C. Medium was removed and 0.45 ml of F12c containing 0.5 mg/ml lucifer yellow was added. After the indicated times at 37°C, the medium was removed, the cells were washed twice with 1 ml PBS

containing 1 mg/ml BSA followed by two washes with PBS. The cells were then solubilized using 1.5 ml 0.05% Triton X-100 containing 0.1 mg/ml BSA. Fluorescence was measured using a Perkin-Elmer, model 650-10S, spectrofluorimeter. Values obtained from samples were compared with those obtained from a standard curve for quantitation of cell-associated lucifer yellow. Wells containing no cells were used as controls.

Uptake of horseradish peroxidase (HRP) was monitored as described by Steinman, Silver, and Cohn (26). Briefly, cells were plated out as for lucifer yellow uptake assays. Medium was replaced with 0.3 ml F12c containing 1 mg/ml HRP (type II). After incubation at 37°C, HRP-containing medium was removed, the cells were washed 6 times with ice-cold F12c followed by one washing with ice-cold PBS. Cells and internalized HRP were solubilized in 1.0 ml 0.05% Triton X-100 (4°C for 2 h). Aliquots were assayed for HRP activity (26) and compared to standard curves generated using known amounts of HRP for quantitation.

RESULTS

Isolation of ET-18-OMe-resistant mutants

RAW 264.7 cells are extremely sensitive to the anticancer, diether phospholipid, ET-18-OMe, displaying an ID_{50} of approximately 1 μM (**Fig. 1**). The cytotoxic effects of this compound are apparent within 12–48 h after addition depending upon the concentration (data not shown). When a mutagenized population of RAW 264.7 cells (5×10^5 cells) was exposed to medium containing 20 μM ET-18-OMe for 10 days, approximately 20 macroscopic colonies resulted. Similar trials with unmutagenized cells generated 0–2 colonies (not shown). After initial selection, two ET-18-OMe resistant populations from separate mutageneses (RA and RB) were grown for several generations in the absence of ET-18-OMe and retested for resistance. Both RA and RB remained resistant to much higher concentrations of ET-18-OMe (not shown).

One clonal strain from each resistant population was isolated and examined in more detail. Both RAW.R1 (from the RA population) and RAW.R23 (from the RB population) were similar in their resistance to ET-18-

TABLE 1. Cytotoxicity of choline phospholipids

	ID_{50}		
	ET-18-OMe	ET-16S-OEt	Lyso-PAF
	μM		
RAW 264.7	0.7	0.7	8.5
RAW.R1	20.0	20.7	91.2
RAW.R23	17.6	17.3	56.6

Cells were plated out and treated with varying concentrations of each compound. Approximately 46 h after the addition of the compound, cells were pulsed with [methyl-^{3}H]thymidine as described in Materials and Methods. Curves similar to those in Fig. 1 were generated and used to determine the ID_{50}. ID_{50} is defined as the concentration required to reduce cellular thymidine incorporation by 50%.

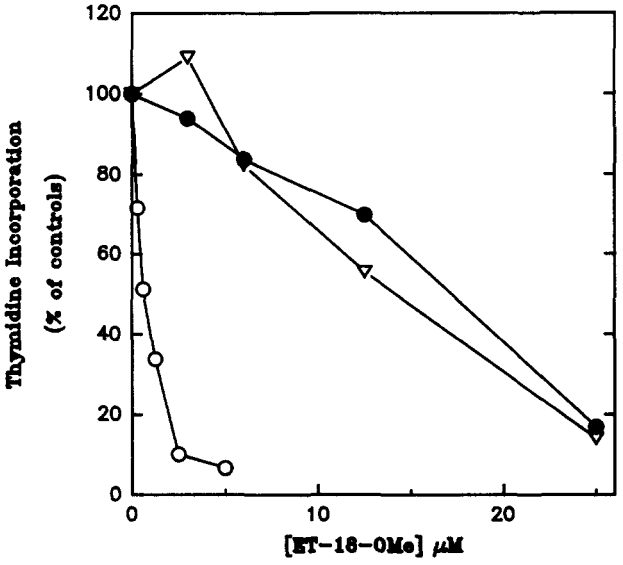


Fig. 1. Sensitivity of RAW strains to growth inhibition by ET-18-OMe. Cells were plated and treated with varying concentrations of ET-18-OMe as described in Materials and Methods. Control wells contained cells that were treated only with 0.25% ethanol. Values from wells containing only medium (no cells) were subtracted from all other values. All values represent the average of three samples and varied by less than 10%; (○) RAW 264.7; (●) RAW.R1; and (▽) RAW.R23.

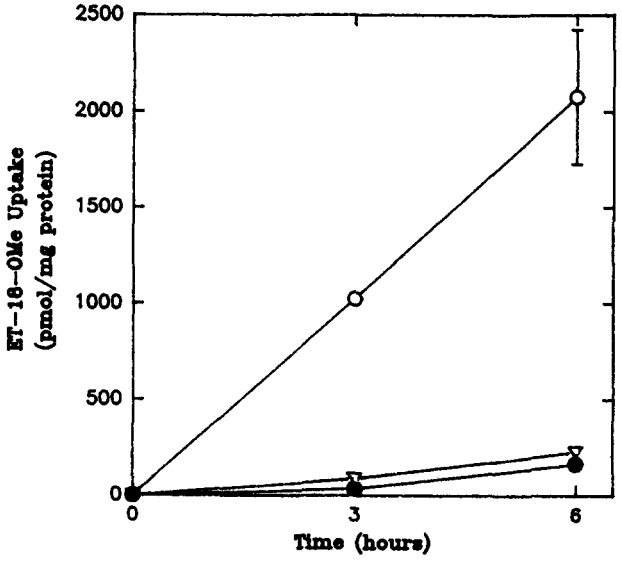
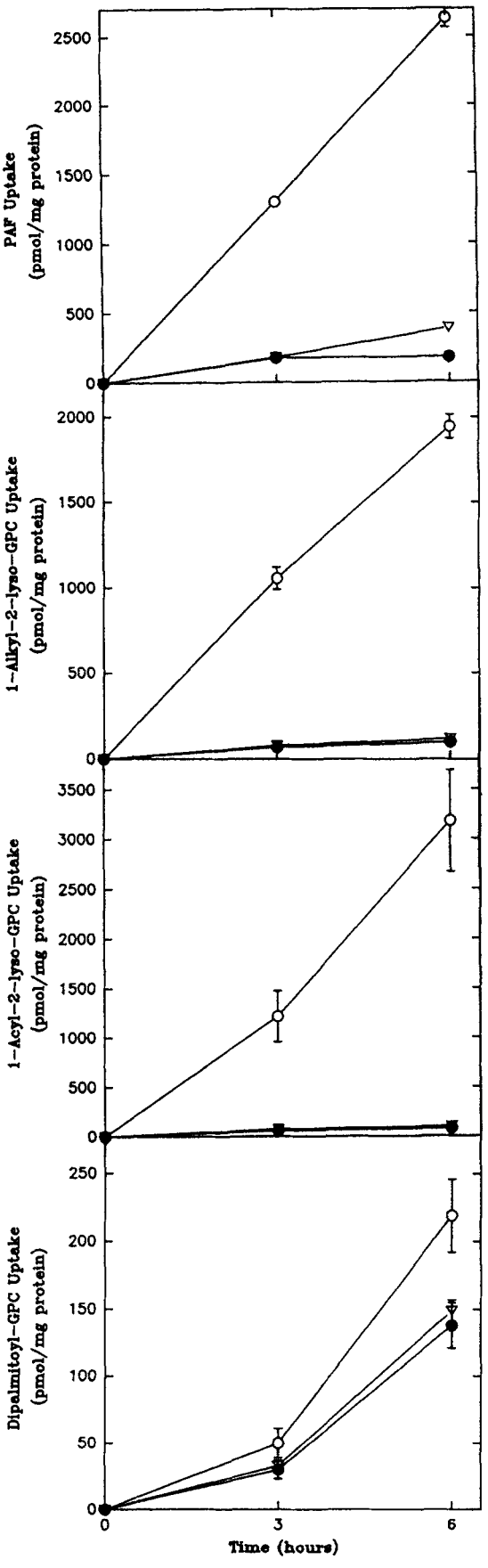


Fig. 2. Uptake of ET-18-OMe by RAW 264.7 and resistant strains R1 and R23. Labeling was performed as described in Materials and Methods. All values represent the average of three determinations ± standard deviation; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.



OMe (Fig. 1). There was no obvious change in the growth rate of either RAW.R1 or RAW.R23 when compared to wild-type cells, when they were grown in normal medium (no ET-18-OMe). After initial selection, these mutants were not exposed to selective pressure, yet they maintained their resistance to ET-18-OMe after 40 passages, demonstrating that the phenotype is stable.

The mutant strains were also very resistant to a sulfur analogue of ET-18-OMe, ET-16S-OEt (27, 28), as well as to a naturally occurring, choline phospholipid (1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; lysoPAF) (**Table 1**). The latter compound was much less toxic than ET-18-OMe or ET-16S-OEt, but the increased resistance of RAW.R1 and RAW.R23 to lysoPAF toxicity was consistent with their resistance to the diether phospholipids.

Uptake of lipids

The resistance of RAW.R1 and RAW.R23 to ET-18-OMe was due to a 90–95% reduction in the ability of these mutant cells to take up this compound from the medium (**Fig. 2**). Rates of uptake of ET-18-OMe were not dependent upon concentration. Approximately 5% of the labeled compound was taken up within 6 h using ET-18-OMe concentrations of 50 nM to 2 μM under these conditions (concentrations that left the wild-type cells viable for at least 12 h as judged by trypan blue dye exclusion).

The defect in RAW.R1 and RAW.R23 was not specific to the diether phospholipids. The uptake of other choline glycerophospholipids was also dramatically reduced. These included platelet activating factor (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), lysoPAF and lysoGPC (1-acyl-2-lyso-*sn*-glycero-3-phosphocholine) (**Fig. 3**). Dipalmitoyl phosphatidylcholine was taken up at a much slower rate than the other compounds tested (only 7% of the rate of lysoGPC) probably due to insolubility of the phospholipid which bears two long-chain fatty acyl groups (29). The decrease in uptake of the diacyl GPC by the mutant cells was less dramatic (35–40% reduction).

RAW.R1 and RAW.R23 were also less able to take up an ethanolamine phospholipid, 1-alkyl-2-lyso-*sn*-glycero-3-phosphoethanolamine (an ethanolamine analogue of lysoPAF). This phospholipid was taken up by wild-type cells at a rate similar to the choline phospholipids (**Fig. 4**), and uptake was greatly reduced in the mutants, although the reduction of the uptake of this phos-

Fig. 3. Uptake of different choline phospholipids by RAW cells. Labeling was performed as described in Materials and Methods. All values represent the average of three determinations ± standard deviation; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

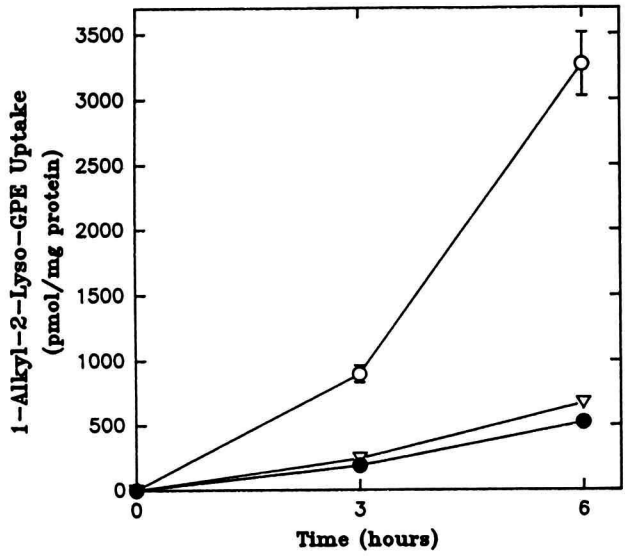


Fig. 4. Uptake of an ethanolamine phospholipid by RAW cells. Labeling was performed as described in Materials and Methods. All values represent the average ± standard deviations of three determinations; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

pholipid was slightly less severe than observed for its choline-bearing counterpart, 1-alkyl-2-lyso-*sn*-GPC (80% vs. ≥ 90% respectively).

The uptake of a fatty acid, an amphipathic molecule which lacks a phosphate-containing head group, was also examined. Uptake of palmitic acid was identical in wild-type and mutant cells (**Fig. 5**). We observed no differences in the uptake of arachidonic acid either (data not shown).

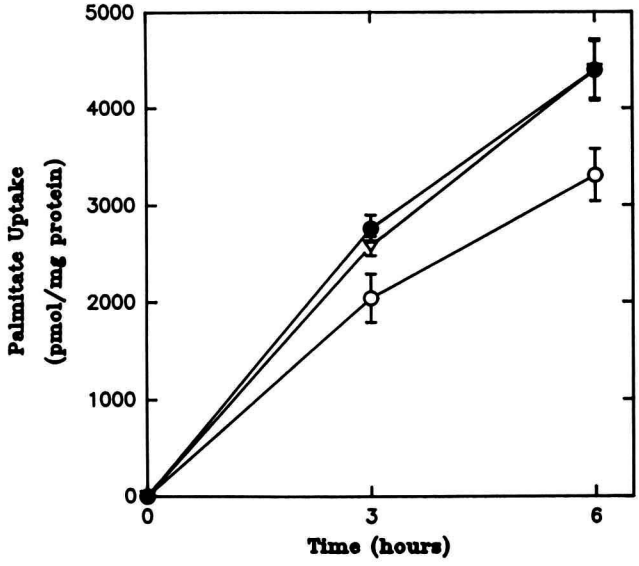


Fig. 5. Uptake of palmitate by wild-type and mutant RAW cells. Labeling was performed as described in Materials and Methods. Similar experiments, using F12 medium containing 1 μM fatty acid-free BSA (no serum) as the labeling medium, gave very similar results. All values represent the average of four determinations ± standard deviations; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

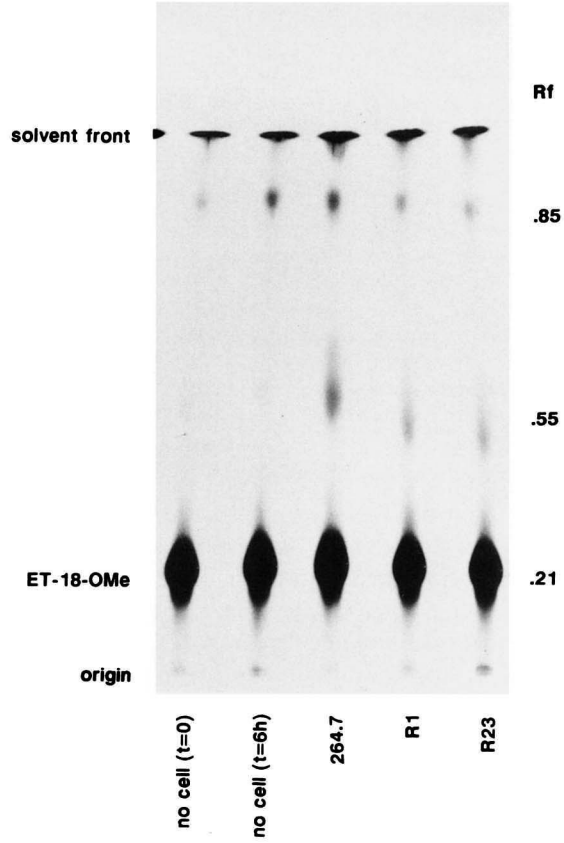


Fig. 6. Lack of metabolism of ET-18-OMe by RAW cells. Cells were incubated for 6 h at 37°C in the presence of [^{3}H]ET-18-OMe; both cells and medium were recovered together and extracted for analysis by thin-layer chromatography as described in Materials and Methods.

Metabolism of ET-18-OMe by RAW cells

Although previous studies have demonstrated that ET-18-OMe is only very slowly metabolized by cultured cells (30), the possibility still existed that increased breakdown or modification of ET-18-OMe could explain the resistance of the mutants to this compound. We therefore examined the ability of the three RAW strains to metabolize radioactive ET-18-OMe.

Figure 6 demonstrates the lack of metabolism of this compound by either wild-type or mutant RAW cells. The cells were incubated in the presence of 1 μM tritiated ET-18-OMe for 6 h. Under conditions used for the metabolism study (a larger number of cells; see Materials and Methods), 54% and 5% of the label was cell-associated in wild-type and mutant cells, respectively (data not shown). Cells and medium were harvested together in order to recover any ET-18-OMe metabolites that may have been released back into the medium. Metabolism of ET-18-OMe, labeled in the octadecyl chain, could result in radioactive 1-octadecyl-2-lyso-GPC (removal of the *sn*-2 methyl group), 1-octadecyl-2-methyl-glycero-3-phosphate (phospholipase D activity), 1-alkyl-2-methoxy-

TABLE 2. Binding of ET-18-OMe to RAW cells at 0°C

	ET-18-OMe Bound
	pmol/mg protein
RAW 264.7	66.7 ± 1.0
RAW.R1	75.5 ± 2.8
RAW.R23	76.2 ± 0.3

Cells were plated out and binding was performed as for the binding and internalization experiment described in Materials and Methods. Values represent the average ± standard deviation of three determinations.

glycerol (phospholipase C activity), or other neutral species (fatty alcohol, fatty aldehyde, fatty acid).

After 6 h, there was very little metabolism of any kind. Approximately 6% of the label was found with an unidentified neutral fraction traveling with the solvent front prior to addition to the cells and this did not change when incubated with the cells (data not shown). The major metabolite, which appeared primarily in the presence of the wild-type cells (R_f = 0.55), represented less than 1% of the total label and was visualized only when the X-ray film was heavily exposed. This minor level of metabolism could not account for difference in labeling found in the mutants.

Binding and internalization of ET-18-OMe

We measured the rates at which the cells were able to internalize ET-18-OMe that had already been bound to the cells. This was done by allowing the tritium-labeled ligand to bind to the outer leaflet of the plasma membrane at 0–4°C (on ice). At 0°C, cellular processes, such as membrane internalization and possible protein-facilitated transbilayer movement, are greatly slowed or stopped. Using our conditions (1 μM ET-18-OMe in the presence of 0.25 mg/ml BSA), maximal binding was reached fairly rapidly (within 30 min) and further incubation (up to 3 h) at 0°C did not change the amount of ET-18-OMe associated with the cells. Also, under these conditions, the binding of ET-18-OMe to both mutant and wild-type cells was similar (**Table 2**). This represented 0.75% of the label that was added to the cells. Extraction and analysis of the bound label by TLC showed this to be identical (94% ET-18-OMe) to that which was added (not shown). After maximal binding had been achieved (30 min), unbound ET-18-OMe was removed, and the cells were warmed to room temperature to allow internalization processes to become active. After the appropriate amount of time, the cells were returned to 0°C and the ligand remaining on the outer cell surface was removed using BSA. Internalized ET-18-OMe is unavailable to BSA and could therefore not be removed. This type of experiment has been used to monitor internalization of phospholipids by erythrocytes (31, 32).

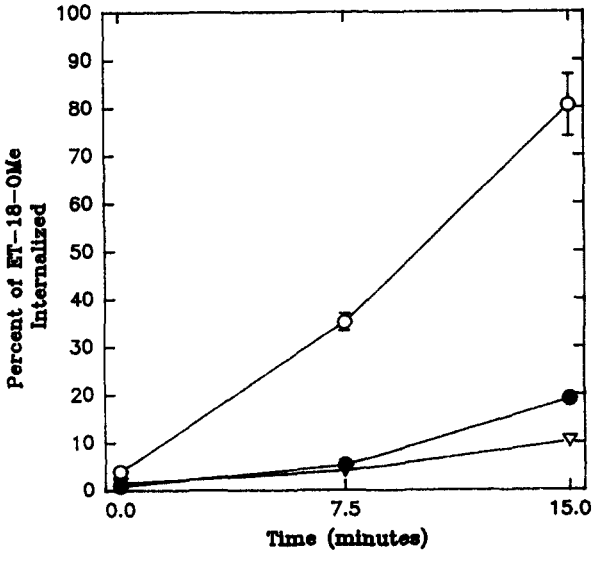


Fig. 7. Internalization of pre-bound ET-18-OMe into RAW cells. ET-18-OMe was allowed to bind to the outer surface of the cells at 0°C for 30 min. After removal of the unbound label, the cells were incubated at room temperature (23°C) to allow internalization to occur. Label still remaining on the cell surface was removed by re-incubation at 0°C in BSA-containing medium. Internalized label is considered to be that label that could not be removed in this way. All values represent the average ± standard deviation of three samples; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

As **Figure 7** shows, internalization of ET-18-OMe was very rapid in wild-type cells. Within 15 min at room temperature, 80% of the pre-bound label could not be removed by treatment with BSA. Internalization did not occur at 0°C; after incubations for up to 90 min (maximum time tested) greater that 95% of the label could be recovered from the cells with BSA. Internalization was greatly reduced in both of the mutants with reductions of 80% and 90% in RAW.R1 and RAW.R23, respectively. The difference in internalization rates could not be explained by loss of the ligand back into the medium. Measurements of the medium taken during the internalization phase of the experiment showed that approximately 4% of the bound ligand is released into the medium during the 15-min incubation at 23°C by all of the strains (a range of 3.8–4.6% between strains).

Hybridization analyses

Hybrids resulting from the fusion of uptake competent cells (RAW 264.7; experiment 1 and RAW.108.OA4; experiment 2) with either RAW.R1 or RAW.R23 were able to take up ET-18-OMe as well, or nearly as well, as the uptake competent strain, demonstrating that the mutations associated with these mutants are recessive (**Table 3**). Fusion between the two mutant strains (experiment 1) resulted in hybrids that remained unable to accumulate ET-18-OMe, demonstrating that the same gene had been affected.

TABLE 3. ET-18-OMe uptake by RAW cells and RAW/RAW hybrids

	ET-18-OMe Uptake during 6 H
	cpm/μg protein
Experiment 1	
RAW 264.7	666 ± 69
RAW.R1	81 ± 3
RAW.R23	115 ± 8
RAW.R1.OA2	91 ± 6
264.7 × R1.OA2 hybrid	875 ± 33
R1 × R1.OA2 hybrid	83 ± 8
R23 × R1.OA2 hybrid	115 ± 8
Experiment 2	
RAW.108.OA4	1302 ± 229
RAW.R1	137 ± 20
RAW.R23	176 ± 3
R1 × 108.OA4 hybrid	575 ± 101
R23 × 108.OA4 hybrid	807 ± 82

ET-18-OMe uptake assays were performed for 6 h at 37°C as described in Material and Methods. All values represent the average ± standard deviations of three samples.

Endocytosis

Measurements of both receptor-mediated and fluid-phase endocytosis revealed no differences between the mutants and the parent cells. Transferrin, which is internalized after binding to its receptor (23), was internalized to similar extents in all cell lines (**Fig. 8**). As transferrin is returned to the cell surface and released by cells, the uptake assays were performed for short periods of time, while uptake is still the dominant process. Cell-associated radioactivity during these early time points represents both binding and internalization and a decrease in either would result in a decrease in total accumulation of transferrin.

Both lucifer yellow and horseradish peroxidase (HRP) are taken into cells through fluid-phase endocytosis (25, 26). They were both taken up at similar rates in all cell lines (**Fig. 9**). In fact, RAW.R23 appeared to take up these ligands at an slightly enhanced rate compared to the parent strain.

DISCUSSION

We have described the isolation of clonal strains from a macrophage-like cell line that are highly resistant to the cytotoxic effects of the antineoplastic phospholipid, ET-18-OMe. Resistance was due to a severe (≥ 90%) reduction in ET-18-OMe uptake. Another ET-18-OMe-resistant cell line has previously been described, displaying a 50% reduction in ET-18-OMe uptake (33). The reason for our ability to isolate mutants displaying such a stringent phenotype is uncertain. It may be due to the different cell type used in our study (a macrophage-like cell line). The fact that we used clonal mutant strains obtained from a single selection event may also explain our results. The previous resistant "population" was generated by continuous growth of the cells in increasing concentrations of ET-18-OMe (33). Clonal isolates were not generated. This population is probably not genetically homogeneous and represents a mixture of variants that are resistant due to lesions in different genes. Uptake deficient mutants may represent only a fraction of the resistant population.

At least three steps can be involved in the uptake of a lipid: *1)* association with the plasma membrane; *2)* internalization; and *3)* metabolism. It is unlikely that a change in metabolism (step 3) is causing the uptake deficiency because ET-18-OMe was not appreciably metabolized by either the wild-type or mutant cells (Fig. 6). Also, the uptake of a variety of different glycerophospholipids (which should be metabolized through separate pathways) was similarly affected.

Association with the plasma membrane (step 1) is also unlikely to be a factor. During our studies involving internalization of prebound ET-18-OMe, we found all strains to bind identical amounts of ligand at 4°C (Table 2). The inability of the mutant cells to take up compounds that differed with respect to headgroup and substitutions at the *sn*-1 and *sn*-2 positions of the glycerol backbone and our observation that ET-18-OMe uptake was independent of the concentration used (not shown) supports the notion that these compounds associate with the plasma membrane in a nonspecific manner. Studies using other cell lines (15) show no evidence to suggest that uptake of ET-18-OMe is a specific, saturable

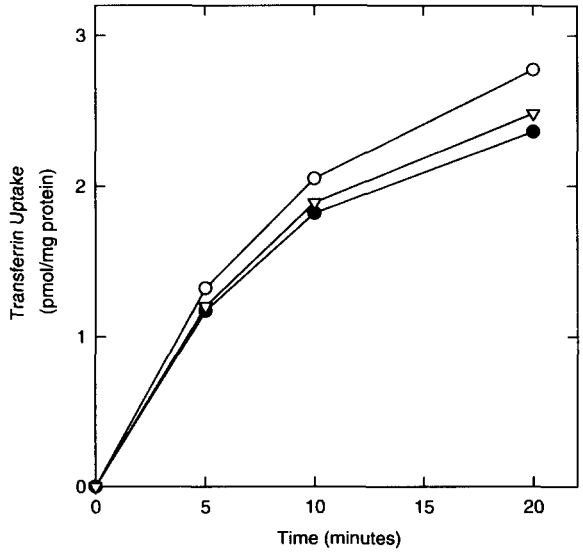


Fig. 8. Uptake of transferrin. Measurements of ^{125}I-labeled human transferrin was measured at 37°C using conditions outlined in Materials and Methods. Values represent the averages of duplicate determinations and did not differ by greater that 10%; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

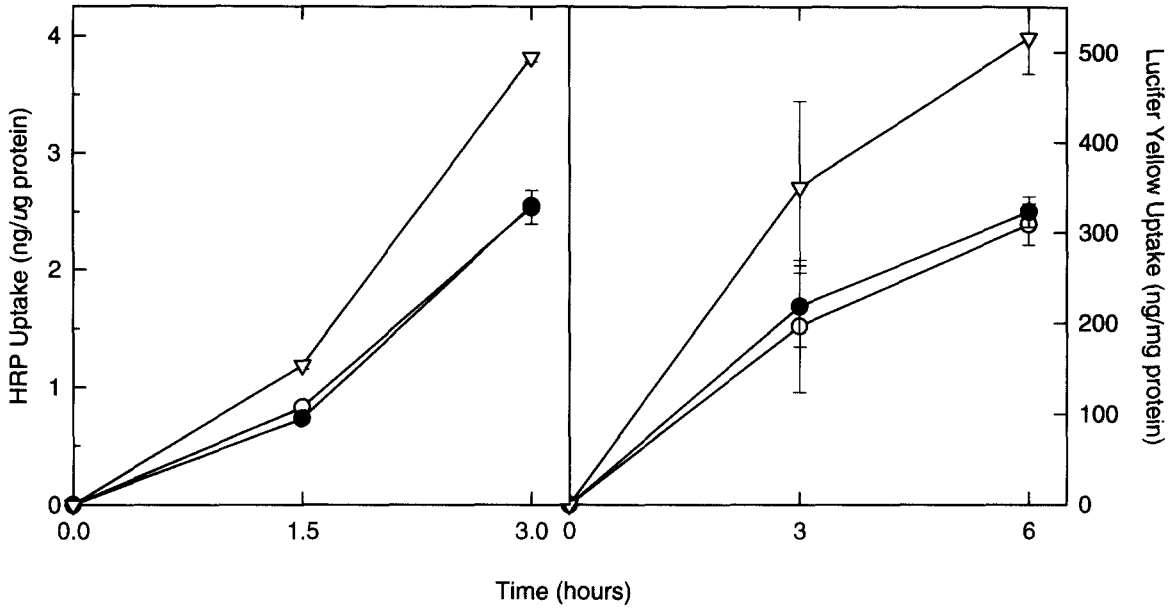


Fig. 9. Uptake of fluid-phase markers lucifer yellow and horseradish peroxidase. Uptake measurements were performed as described in Materials and Methods. All values represent the average ± standard deviation of three samples; (○) RAW 264.7; (●) RAW.R1; (▽) RAW.R23.

process and these compounds do not compete with platelet activating factor for binding to its receptor (15). Lysophospholipids have a high monomer concentration (e.g., the critical micelle concentration of 1-palmitoyl GPC = 7×10^{-6} M; ref. 29) and are known to insert spontaneously into synthetic bilayers (34, 35). This is probably the mechanism of ET-18-OMe membrane association.

Finally, the data presented in Fig. 7 strongly suggest that an internalization process has been disrupted in the mutants (step 2). In these experiments, we measured the internalization of pre-bound ligand. The binding of ET-18-OMe was therefore not a factor. The defect in internalization was similar in severity to the uptake defect measured over a 3- to 6-h period (Fig. 2).

The fact that the uptake of both choline and ethanolamine phospholipids was greatly reduced in these cells was somewhat surprising in light of studies using fluorescent phospholipids (2, 6) which suggest that these phospholipids enter cells through different pathways. A translocator system, specific for aminophospholipids, has been described (6). Such a transporter for choline phospholipids has not been identified in cultured cells, although a protein-mediated transfer of lysoGPC has been described for the brush border of rabbit intestine (36).

Previous studies using other cell lines suggest that choline glycerophospholipids enter those cells through endocytosis after binding (2, 6). It is possible that a membrane internalization process such as endocytosis or the processing of internalized membranes may be defective. Yeast mutants, defective in membrane trafficking and secretion, are much less able to internalize fluorescent phosphatidylcholine (37). Our early examinations of endocytosis, however, show that this process is normal in the mutants. These glycerophospholipids appear to be transported through a more specific process than general endocytosis.

Regardless of the process involved, we have shown that, in macrophages, these varied glycerophospholipid species are taken up through one common process or through processes that require at least one common factor. Two of the phospholipids that we have examined, PAF and lysoGPC, induce known biological responses in target cells. These activators must be removed from the milieu once released. Macrophages, which appear to be able to take these compounds up rapidly, are likely to be important in their clearance. These mutants should help us to define how such cells do this. It also will be interesting to see whether any of these phospholipids are able to stimulate the RAW cells and whether internalization is required for responsiveness. This seems unlikely to be the case for platelet activating factor, as a cell surface receptor has been described (38). Part of lysophosphatidylcholine's activity, however, is thought to be dependent upon metabolism within the target cell (13). The RAW mutants, or similar mutants in responsive cell lines, should help to examine this possibility.

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