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Reduced Temperature Alters *Pseudomonas* Exotoxin A Entry into the Mouse LM Cell

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The movement of *Pseudomonas* exotoxin A (PE) into the cytoplasm of mouse LM fibroblasts was followed by using inhibition of protein synthesis as a biochemical index of toxin activity; biotinyl-PE and avidin-gold colloids were used for electron microscopy. At 37°C both specific antitoxin and pronase-trypsin protected cells against PE toxicity when added within seconds of warming cells, whereas methylamine was protective when added during the first 7 min of endocytosis. Lowering the temperature to 19°C afforded protection when the temperature transition was accomplished within 15 min of the original endocytic event. These data suggest that PE enters an acidic compartment before reaching a step blocked by shifting cells from 37 to 19°C. PE expressed toxicity for LM cells at 19°C, but at a concentration 1 order of magnitude higher than that required at 37°C. At 19°C, antitoxin or trypsin-pronase protection was rapidly ablated. In contrast cells were fully protected by methylamine for 90 min. Using electron microscopy we demonstrated that toxin moved normally (30 s) to coated areas at 19°C, but remained at this site for up to 20 min before being internalized. The majority of the toxin internalized at 19°C remained in endosomes or in Golgi-associated vesicles and was not delivered to lysosomes. The results suggest that, under physiological conditions (37°C), PE rapidly enters cells through coated areas, moves to an acidic compartment (i.e., the endosome), and then probably to the Golgi region en route to lysosomes. The evidence suggests that movement of toxin from endosomes or Golgi vesicles to lysosomes is blocked at 19°C. We hypothesize that the active form of PE enters the cytosol, where it expresses its toxicity during fusion of Golgi-derived, toxin-laden vesicles with lysosomes.

The mechanisms of binding, internalization, and subsequent processing by mammalian cells of ligand-receptor complexes are under intense investigation. A number of ligands which bind to specific membrane receptors first cluster into coated areas on the cell surface. The complexes are then internalized into endosomes and may be recycled to the surface or alternatively are transported via vesicle fusion events, intracellularly. Endosomes are rapidly acidified (18, 39, 42). This acidic environment exerts one of several effects on the internalized receptor-ligand complexes. The acidic environment facilitates the dissociation of receptor and ligand, with the receptor returning to the surface and the ligand being delivered to lysosomes or to another intracellular site (2). Alternatively, the receptor-ligand may not dissociate and the complex may return to the cell surface (e.g., transferrin) or be delivered to lysosomes (for review see references 1 and 2). In the case of certain enveloped viruses, low pH appears to induce membrane fusion and ejection of the viral nucleocapsid into the cytoplasm (17). In the case of diphtheria toxin (DT), low pH is required for expression of biological activity (5, 15, 31).

Several bacterial and plant toxins, including DT, modeccin, abrin, ricin, and probably *Pseudomonas* exotoxin A (PE), possess two functional domains (20). The B region of the toxic proteins is responsible for binding toxin to specific receptors on the target cell surface. The A moiety possesses enzymatic properties and is responsible for blocking protein synthesis in the cell cytoplasm. In the case of DT and PE, the toxins catalyze an NAD-dependent ADP-ribosylation of cytoplasmic elongation factor 2, which is eventually depleted.

The events occurring between toxin binding and the enzyme reaction within the cell cytoplasm have recently been scrutinized for DT and modeccin (4, 16, 33). DT appears to enter the cell cytoplasm by direct passage through a prelysosomal endocytic vesicle. Movement of DT across the vesicle membrane requires an acid pH (4.5 to 5.5), which is thought to facilitate pore formation. In contrast modeccin enters the cytoplasm at a slower rate than DT, and entry may occur after endosomes have fused with other intracellular organelles. We have shown previously that PE binds to specific toxin-binding components on PE-sensitive mouse LM fibroblasts (14) and moves rapidly into coated areas (24). PE is then internalized into endosomes and moves to lysosomes after passage through the Golgi region (24). Here we present evidence which further defines the intracellular trafficking of PE from the cell surface and which tentatively identifies vesicles which permit PE entry into the cytosol.

MATERIALS AND METHODS

Materials. PE was purified and biotinylated as described previously (26). Rabbit antitoxin was prepared against a purified glutaraldehyde-treated lot of PE (13). A pronase (0.025%)-trypsin (2.5%)-EDTA (0.002 M) mixture was used to remove surface-associated PE (14). Horseradish peroxidase (HRP) was used as a marker of fluid-phase endocytosis (36). Mouse LM fibroblasts (ATCC-CCL 1.2 LM), a derivative of L929, were maintained as monolayer cultures in McCoy 5A medium (GIBCO Laboratories) with 5% heat-inactivated fetal calf serum and antibiotics. Vero cells (ATCC CCL81; monkey kidney cells) were maintained in similar medium containing 10% heat-inactivated fetal calf serum. For experimental use, cells were seeded in 24-well culture plates or glass Leighton tubes at a concentration of 2×10^5 to 5×10^5 cells per ml and were incubated overnight before use.

Assay for cytotoxicity. Inhibition of protein synthesis was

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used as a measure of PE biological activity. Protein synthesis was measured by the incorporation of L-[4,5-^{3}H]leucine into trichloroacetic acid-precipitable material (7). The assay medium was 1:1 (vol:vol) McCoy medium and Hanks balanced salts solution with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid). All assays were run in triplicate, and each experiment was repeated a minimum of three times.

Electron microscopy. PE was visualized by using a biotinyl-PE–avidin-gold system as described previously (26). Briefly, cells were exhausted of exogenous biotin by incubation in Hanks balanced salts solution containing 10 mM HEPES, incubated sequentially at 4°C with biotinyl-PE (150 ng/ml) and avidin-gold (5.2 nm), and either fixed immediately (2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer containing 0.05% $CaCl_2$, pH 7.3; 10 min, 4°C) or warmed to the appropriate temperature. In experiments with HRP, monolayers were preincubated with HRP (100 μg/ml) for 18 h at 37°C and were extensively washed before the initiation of the experiment. Monolayers were processed for staining of the HRP reaction product by the method of Graham and Karnovsky (8). Monolayers were washed three times in cold 0.2 M sodium cacodylate buffer and two times with 0.05 M Tris buffer (pH 7.5) at 25°C. They were then incubated for 10 min in 0.5 mg of diaminobenzidine per ml, which was freshly dissolved in 0.05 M Tris buffer (pH 7.5), and H_2O_2 at a final concentration of 0.01% was added. The reaction was allowed to develop for 15 min at 23°C, after which all samples were washed with Tris buffer and cold sodium cacodylate buffer and incubated on ice for 10 min. Subsequently all samples were postfixed with 1% OsO_4 in cacodylate buffer. After fixation for 1 h at 4°C, samples were washed twice (each) with cold cacodylate buffer, distilled water at 23°C, and 70% ethanol at 23°C. Monolayers were stained with 0.5% uranyl acetate in 70% ethanol for 10 min at 23°C and were dehydrated and embedded in TAAB resin (Marivac Ltd., Halifax, Nova Scotia, Canada). After polymerization, ultrathin sections were prepared as described previously (7). All samples were viewed, unstained, in a JEOL-100CX microscope operating at 100 kV. Samples were viewed unstained because the gold colloids and diaminobenzidine precipitate are more easily identified. Cells were scored as to location of gold (toxin) or HRP, number of gold grains per vesicle, and, if possible, type of vesicle. A minimum of 10 cells per time point was viewed, and experiments were repeated at least three times. Siting of a biotinyl-PE–gold complex is designated PE-gold.

RESULTS

Kinetics of toxin entry at 37°C. We have shown previously (14) that PE prebound to the LM cell surface at 4°C exhibits minimal toxicity at 19°C or below, and that this reduced toxicity is not due to a deficit in PE uptake by the cells or to loss of ADP-ribosylation activity of PE. We have suggested that at 37°C PE enters the cytosol during fusion of Golgi-derived vesicles and lysosomes (22). Such membrane fusion events are reduced at 19°C, and thus PE is retained within vesicles. The experiments described here were performed to test this hypothesis further.

Steps involved in the internalization and intracellular movement of toxins have been marked in several ways (4, 16, 33). Failure of PE to be neutralized by specific antitoxin or by trypsin-pronase treatment denotes that toxin has been cleared from the cell surface. The inability to protect cells with basic amines which raise intravesicular pH (18, 27) denotes an acidification event within intracellular vesicles. Reduced toxin activity at temperatures below 19°C delineates a step subsequent to endocytosis which probably involves fusion of vesicles with lysosomes (33, 41). In the experiments described here, PE was prebound to LM cells at 4°C. Cells were then washed and reincubated in medium at 37°C. Antitoxin, trypsin-pronase, or methylamine was added at different times, and protein synthesis was measured 4.5 h later (Fig. 1). All curves were extrapolated to 100% protein synthesis to estimate the earliest time when PE had proceeded beyond the event altered by one of the inhibitory conditions. Endocytosis of PE (insensitivity to antitoxin or to trypsin-pronase) began immediately upon placing cells at 37°C (inset, Fig. 1). Exposure to a low pH environment (insensitivity to methylamine) was evident within 5 to 7 min. Similar experiments were carried out with ammonium chloride (20 mM) or monensin (100 μM) rather than methylamine; in these experiments inhibition of toxin activity was apparent at 9 and 14 min, respectively (data not shown). The

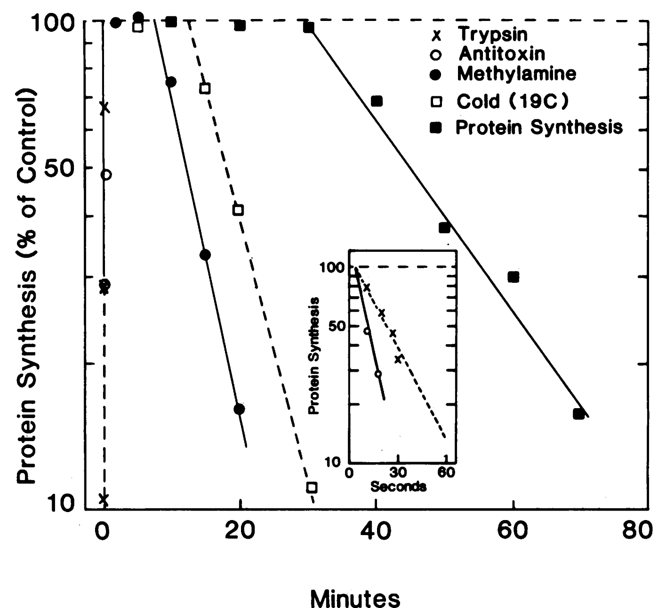

FIG. 1. Kinetics of internalization of PE by LM cells at 37°C. In all cases, monolayers were preincubated with 1 μg of PE per ml for 1 h at 4°C. Monolayers were washed and treated as follows. (○) medium at 37°C was added and aspirated at the indicated times, and cell monolayers were incubated for 30 min at 4°C in medium containing specific antitoxin to neutralize surface PE. Cells were then washed and incubated at 37°C for 4.5 h before measurement of protein synthesis. Protein synthesis is relative to controls that were treated with antitoxin immediately after 4°C incubation with toxin. (×) medium at 37°C was added; when indicated, cells were cooled to 4°C, treated with pronase-trypsin plated, and incubated at 37°C for 4.5 h. Protein synthesis is relative to controls treated with enzyme immediately after 4°C incubation with toxin. (●) medium at 37°C was added; when indicated, medium was replaced with 20 mM methylamine, and cells were incubated at 37°C for 4.5 h until protein synthesis was assayed. Protein synthesis is relative to controls which received methylamine 15 min before warming cells to 37°C. (□) Medium at 37°C was added; when indicated, monolayers were transferred to medium adjusted to 19°C and incubated for 4.5 h at that reduced temperature before measuring protein synthesis. Protein synthesis is relative to controls that were placed at 19°C immediately after the preincubation step. (■) Medium at 37°C was added; when indicated, 10 μCi of [^{3}H]leucine per ml was added for 10 min. Data are relative to cells receiving no toxin.

ability of reduced temperature (19°C) to alter PE toxicity was detectable 14 to 15 min after endocytosis.

It is characteristic of most protein toxins to exhibit a minimum lag time of at least 20 min after binding to the cell surface before toxicity can be detected (20). Under the conditions of our experiments in which a high concentration of PE was prebound to cells at 4°C, the lag time after reincubation at 37°C before the first detectable inhibition of protein synthesis was about 27 min. Our data suggest that the modification of normal toxin routing which is induced by low temperature occurs after binding, internalization, and entry of PE into the low-pH environment of endocytic vesicles.

Inhibitor exchange studies. Experiments in which one inhibitory condition was replaced by another inhibitory condition were performed to show that the event altered by methylamine occurs before the event inhibited at 19°C. A similar approach has been used for tracing the movement of other protein toxins (4, 16) and viruses (17) within mammalian cells. In our experiments, LM cells were incubated with toxin at 19°C and, after a suitable period, were incubated with methylamine and shifted to 37°C. Our previous data predicted that toxin which at 19°C had traversed beyond the amine-sensitive step would at 37°C be processed normally and eventually inhibit cellular protein synthesis. The results of experiments designed to test this prediction are given in Table 1. Monolayers which were incubated with PE at 19°C and then warmed to 37°C (methylamine present throughout) maintained normal protein synthesis (experiment 1). This shows that methylamine protection from PE activity is established at 19°C and expressed at 37°C. Monolayers incubated with PE at 19°C and returned to 37°C in the absence of amine exhibited reduced protein synthesis, a reflection of PE toxicity (experiment 2). Thus, the protective effect of low temperature is reversible after cells are returned to 37°C. Lastly, LM cell monolayers incubated with PE at 19°C and shifted to 37°C in medium containing methylamine exhibited reduced protein synthesis (experiment 3). This confirms that at 19°C PE proceeds beyond the point where methylamine exerts its protective action. Therefore, it can be concluded that the step in toxin trafficking sensitive to neutralization by amines precedes a step in the process blocked at 19°C.

TABLE 1. PE passes the amine sensitive step at 19°C[a]

Expt	Treatment			Protein synthesis	
	19°C (2.5 h)		37°C (2 h), MA	Radioactivity (cpm/μg)	Inhibition (%)
	MA	PE			
1	+	−	+	347.1	0
	+	+	+	336.4	3
2	−	−	−	406.6	0
	−	+	−	4.9	99
3	−	−	+	355.5	0
	−	+	+	35.1	90

[a] In experiment 1, monolayers at 19°C were pretreated with methylamine (20 mM) for 15 min and then incubated with or without PE (10 ng/ml) for 2.5 h at 19°C. Cells were shifted to 37°C medium containing amine, and protein synthesis was assayed after 2 h. In experiment 2, monolayers were incubated at 19°C for 2.5 h with or without PE and then shifted to 37°C for 2 h. In experiment 3, cells were incubated with or without PE for 2.5 h at 19°C; 15 min before the incubation was ended, methylamine was added. Cells were then placed in medium containing amine for 2 h at 37°C. In all cases protein synthesis was assayed by incubating for 10 min at 37°C in presence of 10 μCi of [^{3}H]leucine per ml. Methylamine (MA) concentration was 20 mM; PE concentration was 10 ng/ml.

TABLE 2. Event inhibited by methylamine precedes that inhibited at 19°C[a]

Treatment				Protein synthesis (% inhibition)
37°C (1 h)		Reincubation (3 h)		
MA	PE	Temp (°C)	MA	
+	+	37	+	0
+	+	37	−	64.2
+	+	19	−	4.6

[a] LM cell monolayers were incubated with PE (10 ng/ml) for 1 h in the presence of methylamine (MA; 20 mM), washed, and reincubated at 19°C or 37°C, as indicated, for 3 h. Protein synthesis was then measured. Results are expressed as percent inhibition of protein synthesis when compared with cells treated identically, except no toxin was present.

We also conducted experiments in which the order of the inhibitory conditions imposed was reversed (Table 2). When methylamine was present throughout, PE toxicity was not expressed. The protective effect of methylamine was reversible, i.e., the removal of amine after 1 h at 37°C resulted in the inhibition of protein synthesis in LM cells during a subsequent 3-h incubation. Finally, to demonstrate that the block imposed by reduced temperature (i.e., 19°C) occurs after the methylamine-sensitive step in the intoxication process, LM cells were incubated with toxin and methylamine at 37°C, shifted to 19°C, washed to remove amine, and reincubated at 19°C for 3 h. Under these conditions, protein synthesis remained normal.

Kinetics of PE entry at 19°C. We also estimated the time required at 19°C for PE to pass the steps in toxin processing which are sensitive to antitoxin, to trypsin-pronase, and to methylamine (Fig. 2). All of these events were retarded at 19°C. Toxin became insensitive to trypsin-pronase treatment or to antitoxin neutralization after 30 to 40 s at 19°C (Fig. 2, inset), and the protective effect of methylamine became insignificant when it was added 90 min after PE. Finally at 19°C, there was a lag period of approximately 3 h before a reduction in protein synthesis was first detected. Thus at 19°C there was an interval of 90 min or more of PE residence in acidic vesicles before inhibition of protein synthesis was measurable.

Low-pH treatment of LM cells. The acidic environment of the endosome is believed to be essential for the penetration of certain viruses (17) and toxins (3, 5, 15, 21, 31) across the vesicle membrane into the cytoplasm. The protective effect afforded Chinese hamster ovary (CHO) cells against PE by basic amines or monensin can be overcome if cells are allowed to bind toxin and then briefly exposed to an acidic medium (21). We did not observe a similar reversal of protection in the LM cell system. Monolayers were incubated with high concentrations of PE and then briefly incubated in a pH 4.5 buffer. This exposure of cells to pH 4.5 did not overcome the protective action of ammonium chloride (Table 3) or methylamine (data not given), as indicated by the same level of inhibition of protein synthesis in acid-shocked and untreated cells. In contrast the amine induced resistance of Vero cells, a monkey kidney cell of lesser sensitivity to PE, could be overcome by brief exposure to an acidic environment (Table 3).

Intracellular trafficking of PE monitored by electron microscopy. Low temperatures have been reported by others to block lysosome-endosome fusion (6, 33, 41) and receptor-

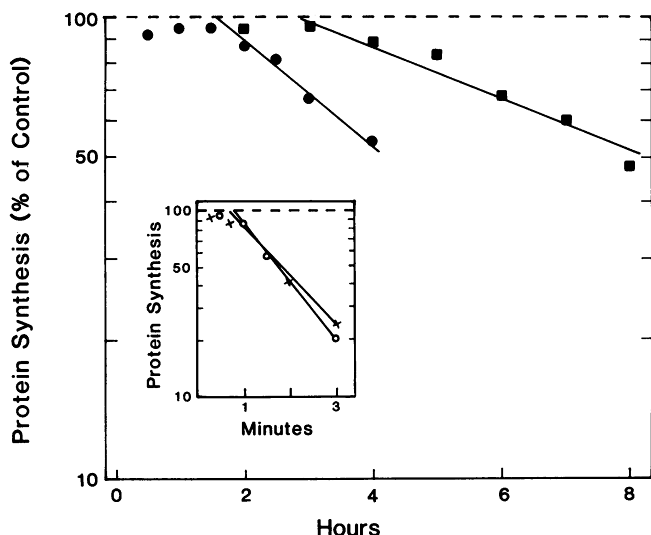

FIG. 2. Kinetics of PE entry in LM cells at 19°C. Monolayers were incubated with 1 μg of PE per ml for 1 h at 4°C and were then washed and treated as described in the legend to Fig. 1, except that reincubation was at 19°C for 4.5 h before measurement of protein synthesis. Symbols: ×, treatment with pronase-trypsin; ○, treatment with antitoxin; ●, treatment with 20 mM methylamine; ■, protein synthesis inhibition. (Inset) Cells were incubated with 4 μg of PE per ml for 1 h at 4°C and washed, and 19°C medium was added. At the indicated times, 10 μCi of [³H]leucine was added for 10 min. Data are relative to cells receiving no toxin.

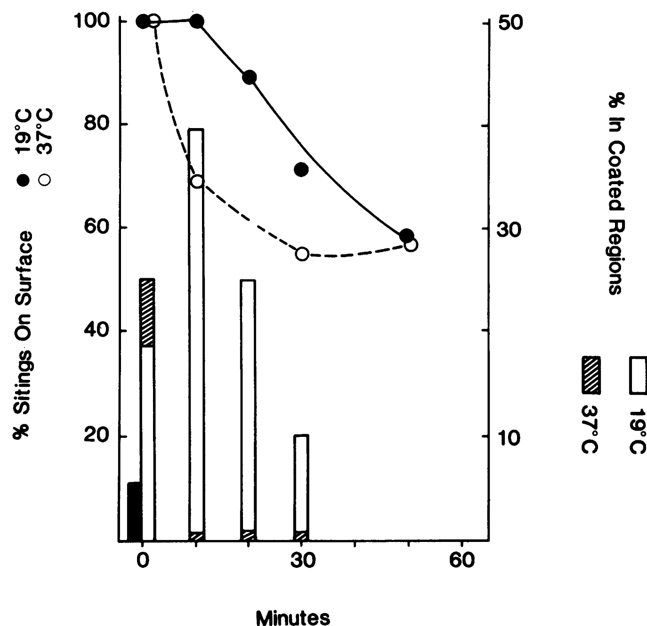

FIG. 3. Entry of PE into LM cells through coated pits at 19°C. LM cell monolayers were exhausted of exogenous biotin, cooled to 4°C, and incubated sequentially with cold biotinyl-PE (150 ng/ml) and avidin-gold. Monolayers were then washed and warmed to 37°C (○, ▨) or 19°C (●, □) for the indicated times, fixed, and processed for electron microscopy. Data are expressed as the percentage of total PE-gold located on the cell surface (●, ○) or the percentage of surface sitings which are located in coated pits or coated vesicles (□, ▨). The solid bar indicates percent sitings in coated areas at 4°C.

ligand dissociation (42). We used direct visualization of toxin by electron microscopy to determine the effects of low temperature on PE entry and routing in LM cells and to corroborate the results obtained with biochemical probes. PE was cleared more slowly from the cell surface at 19°C than at 37°C (Fig. 3). The rapid clustering (30 s) of toxin into coated pits, characteristic of PE entry into sensitive cells at 37°C (7, 24, 26), also occurred at 19°C. However, unlike events occurring at 37°C, most of the PE-gold associated with LM cells at 19°C remained sequestered in coated areas for 20 min before being internalized.

Intracellular movement of toxin was followed in cells which had been preloaded with HRP. HRP is a standard marker of non-receptor-mediated fluid-phase endocytosis (36, 38) and moves rapidly to lysosomes after internalization by L cells (40). Results from a representative experiment are presented in Table 4. LM monolayers were preincubated with HRP for 18 h at 37°C, washed, and incubated for 2 h at 37°C to prelabel lysosomes (37). Biotinyl-toxin and avidin-gold were then bound in the cold before incubation at 19°C or 37°C. The number of internal sitings containing both PE-gold and HRP was determined. Minimal fusion between endosome and lysosome (i.e., coincident siting of PE-gold and HRP) occurred at 19°C, after 60 min of incubation; in fact after 3 h only 10% of all internal sitings contained both HRP and PE-gold. In contrast at 37°C fusion of endosome and lysosome was apparent as early as 10 min and continued to increase for at least 3 h.

Differences in the intracellular distribution of PE-gold in

TABLE 3. Effect of reduced pH on expression of PE cytotoxicity[a]

Cell line	PE concn (μg/ml)	Protein synthesis (% inhibition)	
		pH 7.2	pH 4.5
LM	0.5	59.5	56.5
LM	0.1	18.0	20.1
LM	0.001	6.0	6.7
Vero	10.0	40.5	72.4

[a] LM or Vero cells were seeded in 24-well culture plates and incubated overnight. Cells were preincubated at 37°C for 15 min with ammonium chloride (10 mM), cooled to 4°C, and incubated with PE as indicated for 60 min. Cells were washed, warmed to 37°C, and incubated with buffer at pH 4.5 or 7.2 for 10 min. They were then washed and reincubated for 18 h before protein synthesis was measured. Inhibition is relative to cells treated by an identical protocol but never exposed to toxin. Ammonium chloride was present throughout the entire experiment. Protein synthesis was inhibited > 90% in LM cells and 85% in Vero cells exposed to PE in the absence of ammonium chloride.

TABLE 4. Reduced temperature blocks movement of intracellular PE to lysosomes[a]

Time (min)	Coincident sitings (%)[b]	
	19°C	37°C
5	0	4
10	0	12.8
20	<1	10.3
30	0	11.5
60	1.0	12.0
90	7.4	39.8
180	9.6	55.0

[a] LM cells were preincubated for 18 h at 37°C with HRP (100 μg/ml), washed, and incubated at 37°C for an additional 2 h. Monolayers were then cooled to 4°C and incubated sequentially with biotinyl-PE and avidin-gold. After warming to 19 or 37°C, monolayers were processed as described in Materials and Methods to localize HRP.

[b] Percentage of total HRP sitings which also contain gold (PE).

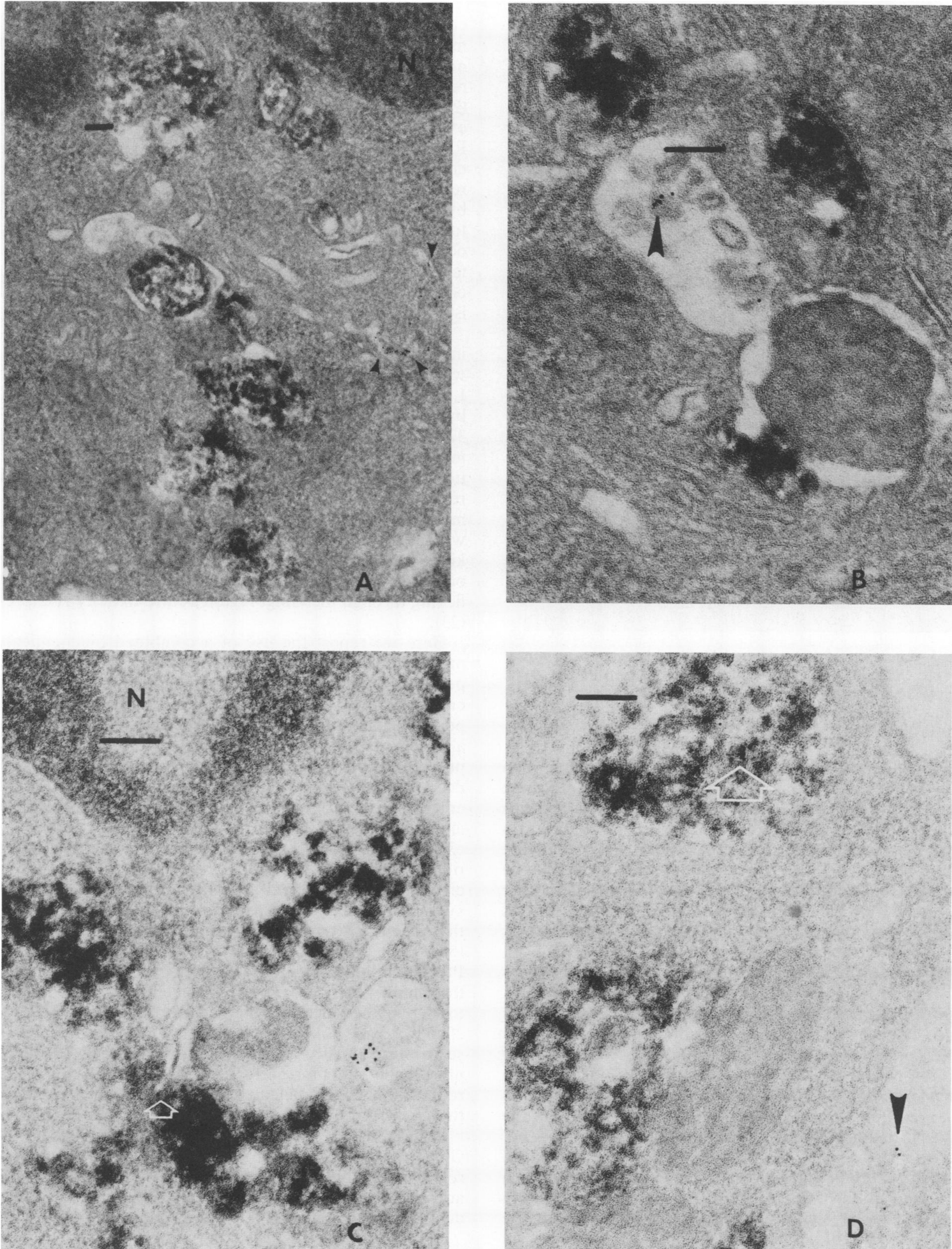

FIG. 4. Progressive movement of PE-gold as a function of warming at 37°C. A and B are 10 min after warming. In A note PE-gold within a tubular structure (small arrowheads), which is possibly an element of the endosomal or Golgi apparatus within the perinuclear region of the cell. In B, note PE-gold (large arrowhead) within a MVB which appears to be fusing with a lysosome. C and D are both 30-min samples. In C note PE-gold within a MVB (black arrowhead) and also within lysosomes (white arrow). In D (underexposed) note lysosome with PE-gold (white arrow) and an HRP-negative vesicle containing PE-gold (black arrowhead). N denotes nucleus in A and C. Bars, 100 nm; A, ×50,000; B, C, and D, ×100,000.

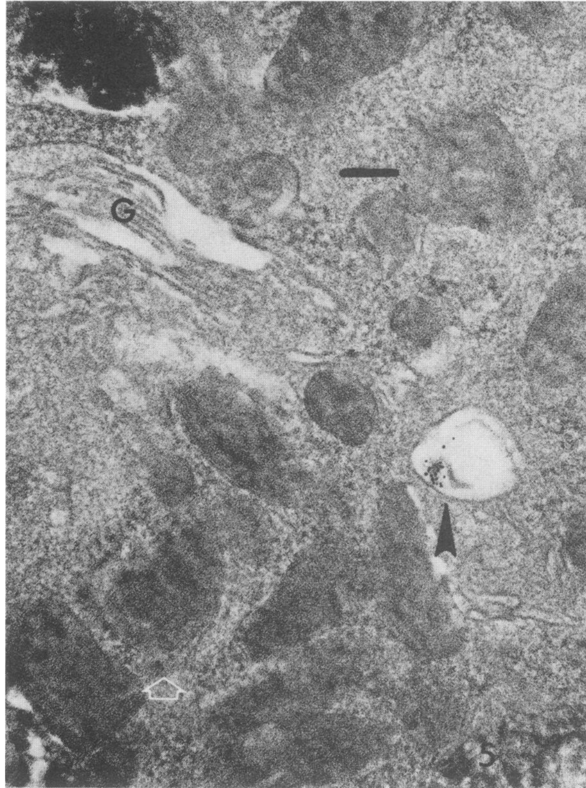

FIG. 5. Location of PE-gold in LM fibroblast after 30 min at 19°C; note PE-gold within MVB (black arrowhead) and in small vesicle (white arrow). All gold sitings are in HRP-negative vesicles. G denotes Golgi cisternae. Bar, 100 nm; ×75,000.

cells incubated at 19 and 37°C are illustrated in Fig. 4 through 6. In a representative experiment, within 10 min of warming cells to 37°C, approximately one-third of the PE-gold sitings were intracellular. Many (20%) of these sitings were in vesicles in the perinuclear region, some of which were tubular in nature (Fig. 4A); these tubular structures could be part of either the endosomal or the Golgi apparatus. Other sitings (28%) were in structures identified morphologically as multivesicular bodies (MVB; Fig. 4B). Other PE-gold sitings were in small electron luscent vesicles, which were considered to be endosomes. In contrast after 10 min at 19°C, less than 2% of all PE-gold was intracellular.

After 30 min at 37°C, over one-half of the total PE-gold was intracellular, with the majority of sitings seen in MVB (Fig. 4C) or in lysosomes (Fig. 4C and D). After a similar incubation period at 19°C, approximately 20% of the toxin was inside the cell. At this time PE-gold was seen in MVB (Fig. 5) and in small vesicles sometimes found in the perinuclear region. In general the number of gold grains per vesicle was less at 19°C than at 37°C. Even after 90 min of incubation at 19°C (Fig. 6) tubular vesicles containing only a few PE-gold grains were regularly observed. This was in contrast to similar samples incubated at 37°C, where the majority of PE-gold was seen in greater numbers in lysosomes.

DISCUSSION

PE is a bacterial protein toxic for mammalian cells by virtue of its ability to inhibit host cell protein synthesis by the enzymatic ADP-ribosylation of elongation factor 2. Because it acts catalytically, only a very small number of PE molecules are required to cause cell death. Native PE does not express enzyme activity and must be activated either by reduction in the presence of urea or by repeated freeze-thaw (20). This implies that cellular processing is required for expression of PE toxicity. We have shown previously that PE enters mouse LM fibroblasts by receptor-mediated endocytosis, i.e., via clathrin-coated pits (7). A subsequent report (24) from this laboratory with biotinyl-PE identified by 5-nm avidin-gold colloids showed that after 15 min at 37°C toxin is consistently seen in the Golgi region of LM cells and occasionally is observed within the Golgi cisternae. After 30 min at 37°C, toxin is uniformly seen within the lysosomal compartment (24, 26). These results have been confirmed by isopycnic centrifugation studies (22).

We also have monitored visually the internalization and intracellular trafficking of biotinyl-DT by LM cells, a cell line which is very resistant to this toxin (25). DT enters mouse LM cells by nonspecific adsorptive pinocytosis rather than by receptor-mediated endocytosis (25). Within 5 to 15 min at 37°C, the toxin has been delivered to lysosomes, and no involvement of the Golgi is apparent. From these observations we concluded that efficient expression of toxicity requires entry by receptor mediated endocytosis and movement through the Golgi apparatus (22, 25). In support of this we have observed that DT-gold internalization by monkey kidney cells (Vero), a cell line extremely sensitive to DT, occurs by receptor-mediated endocytosis and requires trafficking through the Golgi region before toxicity is expressed (23).

Here we report the use of a combined biochemical and morphological approach in conjunction with reduced temperature to more clearly define the site of escape of the enzymatically active form of PE. We considered several possibilities as to how a reduced temperature (19°C) might alter the sensitivity of LM cells to PE. These included (i) preventing toxin internalization, (ii) blocking receptor-ligand dissociation, (iii) preventing toxin activation, (iv) blocking entry of the active form of PE into the cytosol, and (v) altering enzyme activity. Two of these possibilities were excluded in a previous report (14). First, in vitro ADP-ribosylating activity is not reduced at 19°C. Second, sufficient PE is internalized at 19°C to completely halt protein synthesis under the conditions of the assay. Here we report that an early step in the toxin entry process is modified at lower temperatures. Upon warming cells to 19°C, prebound PE rapidly moved to coated areas (Fig. 3). However, rather than immediately entering an intracellular compartment, the toxin remained localized on the cell surface in coated pits for 20 min. Once toxin internalization was initiated, PE was seen intracellularly after 30 min in HRP-negative vesicles, presumably in endosomes. After 30 to 60 min, PE had been routed to the perinuclear region of the cell. Unfortunately the Golgi apparatus is poorly defined in mouse LM fibroblasts. However, given the perinuclear distribution of the Golgi, we feel that some of the sitings of PE-gold in this region are within the Golgi apparatus. Thus, routing of toxin at 19 and 37°C is similar to this point, but the process is delayed considerably at the lower temperature.

A subsequent step critical in expression of toxicity, however, was dramatically altered. As has been described for other ligand systems (6, 12, 33, 34), movement of PE from endosomes or Golgi vesicles to lysosomes was reduced to minimal levels at 19°C. Only rarely was toxin observed in an organelle containing the lysosomal marker HRP. These few sitings could be interpreted (i) to indicate that a small

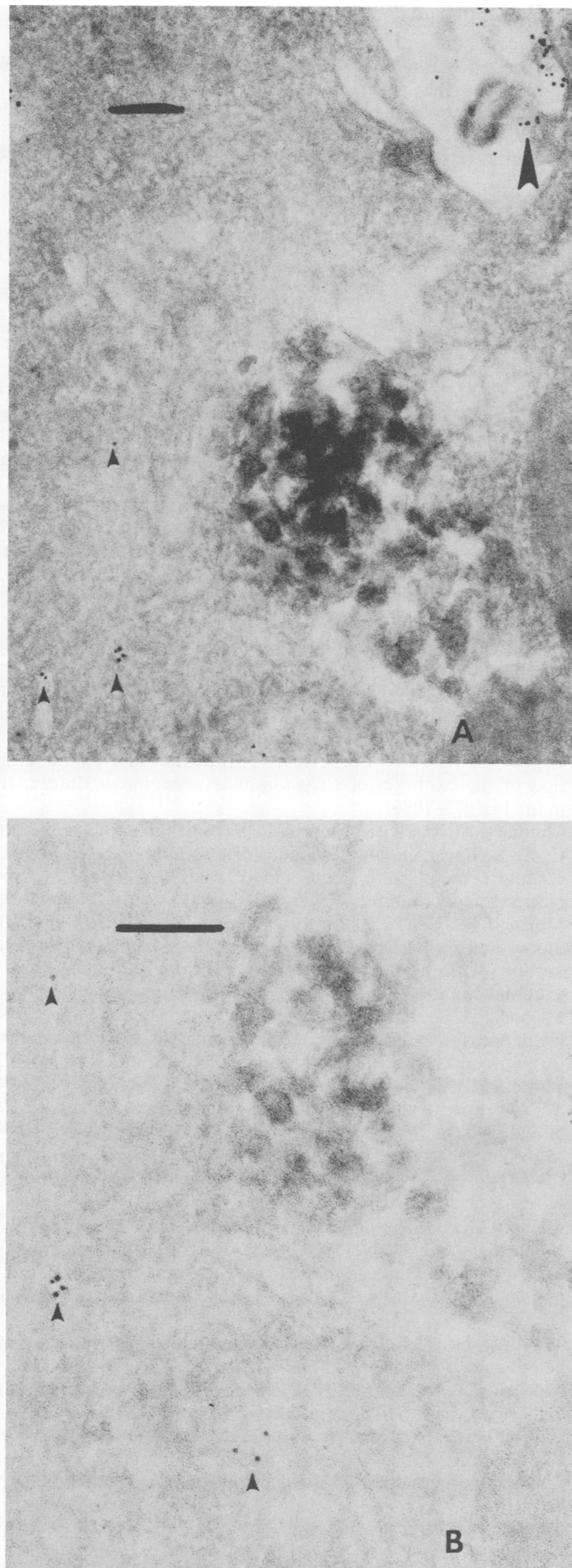

FIG. 6. Location of PE-gold in LM fibroblasts after 90 min at 19°C. In A note large numbers of PE-gold in HRP-negative MVB (large arrowhead) and small tube-shaped vesicles (small arrowheads). B is a higher-magnification, underexposed micrograph of the HRP-positive lysosome showing lack of PE-gold within the vesicle. For comparative purposes, note the gold (arrowheads) within tubular-shaped vesicles. Bars, 100 nm; A, ×100,000; B, ×150,000.

fraction of HRP was localized in endosomes and not delivered to lysosomes or (ii) to represent the rare event when lysosome-endosome fusion occurred, indicating actual movement of PE to the lysosomal compartment.

Kinetic studies also were used to determine the order of events culminating in cell death. These data clearly show that the event altered by reduced temperature occurs after the step altered by methylamine. The experiments with several inhibitory conditions also confirmed that the step in toxin processing blocked by low temperature occurred after the block imposed by methylamine.

The role of acidification in expression of PE activity has not been clearly defined. Moehring and colleagues (3, 19, 21) have isolated CHO cell mutants which are defective in the acidification of endosomes but not of lysosomes. These mutant cells are resistant to both DT and PE. In addition, agents which raise the pH of endosomes, e.g., monensin, methylamine, and ammonium chloride, protect cells from both DT and PE (20, 30). These data suggest that a reduced pH within an intracellular compartment is required for expression of toxicity in both cases.

Another way to determine the requirement for an acidic environment for toxin action is to try to overcome the protective effects of acidotropic agents by exposing cells, with toxin on the surface, to a medium of reduced pH. This procedure is thought to simulate the environment within intracellular acidic compartments. Moehring and Moehring (21) acid shocked cell surface-associated PE into wild-type and mutant CHO cells by reducing the pH of the external medium to 4.5 and thereby reversed monensin protection. Similarly, we have obtained evidence of low pH induced entry of PE into Vero cells in the presence of ammonium chloride or methylamine. However similar acid shock experiments to facilitate entry of PE into mouse LM cells, which are highly sensitive to PE, have been unsuccessful (Table 3) (30). Other investigators also have been unable to overcome the innate resistance of mouse LMTK$^-$ cells (thymidine kinase-deficient mouse LM cells) to DT by low pH treatment (3). Similarly Guillemot et al. (11) reported that low pH induced the entry of concanavalin A-DT conjugates into Vero cells but not into mouse L cells. In contrast O'Keefe and Draper (28) found that acidic medium potentiated the cytotoxicity of a transferrin-DT conjugate for LMTK$^-$ cells. This would suggest that the inability of low pH to potentiate the activity of PE in LM cells (derived from L cells) is not due to a general property of mouse cells. Rather the data indicate that the mechanism of PE entry in LM and in Vero or CHO cells is dissimilar. A step in the processing of PE appears to require a low-pH environment, because agents which raise intracellular pH protect cells against PE and cells defective in vesicle acidification are resistant to PE (15, 19). Several intracellular compartments are known to be acidified; these include coated vesicles, endosomes, CURL vesicles, multivesicular bodies, Golgi vesicles, and lysosomes (34). Which of these are critical in PE processing to express toxicity remains to be determined.

Marnell et al. (16) and Sandvig et al. (33) reported exper-

iments similar to those described here for DT and Vero cells. They interpreted their results to suggest that the enzyme-active fragment of DT enters the cell cytoplasm from a prelysosomal acidic compartment. Results presented here show that the enzymatically active form of PE escapes after the acidification step. This may occur in the endosome, the Golgi, or the lysosomal compartment. Of these three possibilities we consider that escape from the lysosomal compartment is least likely because PE is a nonglycosylated protein (13) and should be rapidly degraded in lysosomes (32). Although escape from endosomes cannot be excluded, we favor escape of toxin into the cytoplasm after Golgi modification.

It may be that an unidentified Golgi enzyme is required for the conversion of protoxin to active toxin by a mechanism analogous to the conversion of proproteins to proteins, e.g., proinsulin to insulin or proalbumin to albumin (35). Under normal conditions the processing of proproteins begins 10 to 20 min after synthesis in the cytoplasm and transfer to the Golgi. Paired basic residues, most frequently Arg-Arg and Lys-Arg, are required for efficient conversion of proteins. PE contains Arg-Arg and Lys-Arg sites at positions 182 to 183 and 185 to 186, respectively (9), and thus toxin may be converted to its active form by normal host cell processes. It may be noteworthy to point out that DT also contains several paired Arg-Arg or Lys-Arg sites positions (103 to 104, 125 to 126, 172 to 173, and 191 to 192) (10).

Orci et al. (29) recently reported that conversion of radiolabeled proinsulin to insulin in pancreatic B cells was inhibited by replacing arginine and lysine with amino acid analogs. This substitution results in the accumulation of radioactive material in Golgi-derived, coated granules. They concluded that the maturation of the coated compartment into a noncoated granule is linked to the effective conversion of the prohormone. To date we have not observed PE-gold in coated vesicles in the Golgi region. However LM fibroblasts (a cell line which has been passaged in vitro for >20 years) have a poorly defined Golgi in which coated Golgi vesicles are extremely rare.

In summary, this study establishes that reduced temperature (i.e., 19°C) retards but does not prevent entry of PE into mouse LM fibroblasts via receptor-mediated endocytosis. We also have established that an event in toxin processing which requires an acidic environment occurs before the event which is blocked at 19°C. Based on both our biochemical and morphological observations we suggest that enzymatically active PE enters the cytoplasm after modification in the Golgi.

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