Cerulenin Inhibits the Cytotoxicity of Ricin, Modeccin, *Pseudomonas* Toxin, and Diphtheria Toxin in Brefeldin A-resistant Cell Lines*

(Received for publication, January 12, 1993, and in revised form, March 9, 1993)

Tatsuya Oda and Henry C. Wu

From the Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

We have found that cerulenin, an antibiotic that inhibits de novo fatty acid and cholesterol biosynthesis and fatty acylation of proteins, strongly inhibited the cytotoxicity of ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in a brefeldin A (BFA)-resistant mutant of Vero cells (BER-40). The protective effect of cerulenin against ricin was also observed in two other BFA-resistant cell lines, Madin-Darby canine kidney, and PtK1 cells. In contrast to BER-40 cells, no significant effect of cerulenin was observed in Vero cells. Cerulenin did not affect the binding of ricin to the cell-surface receptors, but reduced significantly the internalization of ricin in BER-40 cells; no effect of cerulenin on the binding or internalization of ricin was observed in Vero, PtK1, and Madin-Darby canine kidney cells. Endocytic uptake of fluid-phase markers such as horseradish peroxidase and lucifer yellow was inhibited by cerulenin in BER-40 cells, but the endocytosis of transferrin via the coated pit/coated vesicle pathway was slightly increased. Cerulenin inhibited the degradation and excretion of ricin in BER-40 cells, and this effect of cerulenin was not observed in Vero cells. Furthermore, cerulenin inhibited the bulk protein secretion in a dose-dependent manner, with BER-40 cells being more susceptible than Vero cells. These results suggest that in addition to its effect on endocytosis, cerulenin interferes with the intracellular trafficking or processing of toxin molecules, and the vesicle transport system in BER-40 cells appears to be cerulenin-sensitive. Since addition of fatty acids and cholesterol did not reverse the effects of cerulenin, the protective effect of cerulenin against protein toxins is not due to an inhibition of de novo fatty acids and cholesterol biosynthesis.

A number of plant (ricin, abrin, and modeccin) and bacterial (diphtheria, *Pseudomonas*, and Shiga) toxins inhibit protein synthesis after gaining entry into the cytosol (1-5). The intoxication pathway of these protein toxins consists of (i) binding to cell-surface receptors, (ii) receptor-mediated endocytosis and intracellular transport through the vesicular system, (iii) translocation of enzymatically active components of the toxins across the vesicle membrane to the cytosol, and (iv) enzymatic inactivation of their intracellular targets, i.e. the 28 S RNA of 60 S ribosomal subunit for ricin, abrin, modeccin, and Shiga toxin, or elongation factor 2 for *Pseudomonas* toxin and diphtheria toxin. In the case of ricin it has been demonstrated that this toxin is transported to the Golgi apparatus, mainly to the trans-Golgi network during intoxication (6-9). This seems to be the case for other toxins except for diphtheria toxin, where translocation of the enzymatically active component occurs from endosomes (10, 11). Youle and Colombatti (12) have shown that a hybridoma cell secreting antibodies against ricin was resistant to the toxic effects of ricin. These results suggest an intersection of the endocytic and the exocytic (secretory) pathways in the target cells during the intoxication process.

The Golgi apparatus has been shown to play a central role in regulating the sorting and trafficking of proteins through the exocytic as well as the endocytic pathways (13, 14). Brefeldin A (BFA), a lipophilic fungal antibiotic, has proved to be a valuable tool in the studies of intracellular protein trafficking. The profound effects of BFA on the structure and function of the Golgi apparatus include an inhibition of protein transport from endoplasmic reticulum (ER) to the Golgi (15, 16), a rapid and reversible disassembly of the Golgi complex (17, 18), and a retrograde transport of resident and itinerant Golgi proteins to the ER (18, 19). Yoshida et al. (20, 21) have shown that BFA blocks the intoxication of Vero and other cell lines by ricin, modeccin, and *Pseudomonas* toxin but has no effect on the cytotoxicity of diphtheria toxin, and these results suggest an involvement of the Golgi apparatus in the intoxication process of these protein toxins except diphtheria toxin. It has been shown that the cytotoxicity of cholera toxin is also inhibited by BFA (22). Recently, a BFA-resistant mutant cell line of Vero cells (BER-40) has been isolated in our laboratory, and BER-40 cells were not protected by BFA against ricin cytotoxicity (23).

The biochemistry of vesicular protein transport is largely unknown. An acylation/decylation cycle may be involved in the vesicular transport of proteins between intracellular compartments based on the requirement of fatty acyl-CoA in the *in vitro* reconstituted system (24). GTP-binding proteins are required for intracellular protein transport (25-27), and guanine nucleotides also modulate the effects of BFA (28). While the precise mechanism of action of BFA is still unclear, evidence is accumulating that GTP-binding proteins are potential targets of BFA action (29-33).

We have investigated the effect of cerulenin on the cytotoxicities of ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in BFA-sensitive and -resistant cell lines. In the present paper, we show that BFA-resistant cell lines, BER-40, PtK1, and MDCK cells, are protected by cerulenin from the cytotoxicities of ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in BFA-sensitive and -resistant cell lines. In the present paper, we show that BFA-resistant cell lines, BER-40, PtK1, and MDCK cells, are protected by cerulenin from the cytotoxicities of ricin, modeccin, *Pseudomonas* toxin, and diphtheria toxin in BFA-sensitive and -resistant cell lines.

*The abbreviations used are: BFA, brefeldin A; MDCK, Madin-Darby canine kidney; ER, endoplasmic reticulum; MEM, minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; endo H, endo-β-N-acetylglucosaminidase; Man-6-PR, mannose 6-phosphate receptor; BFA*, brefeldin A-resistant.

1 The abbreviations used are: BFA, brefeldin A; MDCK, Madin-Darby canine kidney; ER, endoplasmic reticulum; MEM, minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; endo H, endo-β-N-acetylglucosaminidase; Man-6-PR, mannose 6-phosphate receptor; BFA*, brefeldin A-resistant.
Cerulenin Inhibits Ricin Cytotoxicity in BFA\(^{B}\) Cell Lines

diphertheria toxin. In contrast, no significant protection by cerulenin was observed in BFA-sensitive Vero cells against these protein toxins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ricin, transferrin, lucifer yellow, cerulenin, horseradish peroxidase (type II), protein A-Sepharose beads, and fetal bovine serum were obtained from Sigma. Diphertheria toxin and Pseudomonas aeruginosa lipopolysaccharide were purchased from Swiss Serum and Vaccine Institute (Berne, Switzerland). Modeccin and 1,3,4,6-tetrachloro-3α,6α-di-hydroxy-couvrol (IDO-GEN) were obtained from Pierce Chemical Co. [\(^{35}\)S]Methionine (1071 Ci/mmol) was purchased from ICN Biochemicals, Inc. Brefeldin A was obtained from Epicentre Technologies (Madison, WI). Transferrin was saturated with iron just prior to injection as described by Karin and Minz (35), and the reaction was carried out for 30 min at room temperature. The labeled proteins were purified by Sephadex G-25 column chromatography and stored at \(-80^\circ\text{C}\). Endo-\(\beta-N\)-acetylgalactosaminidase (endo H) was obtained from Boehringer Mannheim. Antiserum against human mannose 6-phosphate receptors was the generous gift of Dr. S. Kornfeld and Dr. K. von Figura.

**Cell Culture**—Vero (African green monkey kidney), PtK\(_2\) (potoroo kangaroo kidney), and MDCK (Madin-Darby canine kidney) cells were obtained from the American Type Culture Collection. BFA-resistant mouse fibroblasts were isolated in our lab from cells of the mouse strain C3H/HeJ methanethiosulfonate mutagenesis (23). All these cell lines were grown as monolayers in \(α\)-minimal essential medium (\(α\)-MEM) supplemented with 10% fetal bovine serum, 10 \(μ\)g each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 \(U/ml\)), streptomycin (100 \(μg/ml\)), and 1% bovine serum albumin (BSA). The cells were subcultured 2 days before treatment with 0.1% trypsin, 0.05% EDTA in phosphate-buffered saline (PBS).

**Measurement of Protein Synthesis Inhibition**—Cells were inoculated at a density of 1 \(×\) 10\(^6\) cells/well for Vero and BER-40 cells or 2 \(×\) 10\(^5\) cells/well for PtK\(_2\) and MDCK cells in 0.5 ml of medium using 24-well plates. Two days later, the monolayer culture was pretreated with or without cerulenin in serum-free \(α\)-MEM containing 1% BSA for 1.5 h at 37\(^\circ\text{C}\). Although cerulenin at 200 \(μl/ml\) inhibited \(\text{[\(^{35}\)S]}\text{methionine incorporation, preliminary experiments revealed that this toxic effect of cerulenin was greatly reduced by the addition of 1% BSA to the medium. Accordingly, BSA containing medium was used for the cytotoxicity and binding assays unless otherwise specified. After a 3-h treatment with toxin in the presence or absence of cerulenin, cells were incubated with 0.5 \(μl/ml\) \(\text{[\(^{35}\)S]}\text{methionine for 30 min at 37\(^\circ\text{C}\) in leucine-free medium. The incorporation of \(\text{[\(^{35}\)S]}\text{methionine into phosphatidic acid/phosphatidylethanolamine materials was determined as described previously (20). The results were expressed as percentage of incorporation in control cells incubated without toxin but otherwise treated in the same way.}}\)

**Binding and Internalization of \(\text{[\(^{125}\)I]}\)Labeled Ricin**—The cells were preincubated as described above, treated with or without cerulenin for 1.5 h at 37\(^\circ\text{C}\) in serum-free \( α\)-MEM containing 1% BSA, and then incubated at 4 \(\circ\text{C}\) with \(\text{[\(^{125}\)I]}\) ricin for 1 h. After removal of the radioactive medium by aspiration, the cells were washed twice with ice-cold PBS. Washed cells were solubilized in 1 ml of 0.5 N NaOH and transferred to plastic tubes for counting in a Searle gamma-counter. To measure the internalized ricin, the cells were incubated with \(\text{[\(^{125}\)I]}\) ricin at 37\(^\circ\text{C}\) for 1 h. After removal of the radioactive medium, cells were incubated with 0.1 ml lactose in PBS for 10 min at 4 \(\circ\text{C}\), followed by washing three times with the same solution, and cell-associated radioactivity was measured.

**Measurement of Endocytosis of \(\text{[\(^{125}\)I]}\)Labeled Transferrin, Lucifer Yellow, and Horseradish Peroxidase**—The amount of endocytosed transferrin was determined as described by Karin and Minz (34). Cell monolayers in 6-well plates (1 \(×\) 10\(^6\) cells/well) were pretreated with or without cerulenin for 1.5 h at 37\(^\circ\text{C}\), and then incubated with \(\text{[\(^{125}\)I]}\) transferrin for 1 h at 37\(^\circ\text{C}\) to allow binding and endocytosis. The cells were washed three times with ice-cold PBS, and then cell-surface bound transferrin was released by incubation of the cells with 1 ml of serum-free \( α\)-MEM containing 0.3% (v/v) Pronase for 1 h at 4 \(\circ\text{C}\). A 50-μl aliquot of the cell suspension was then transferred to Eppendorf tubes and centri-
RESULTS

Effect of Cerulenin on the Cytotoxicity of Ricin in Vero, BER-40, PtK, and MDCK Cells—To test the effect of cerulenin on the cytotoxicity of ricin in Vero and BER-40 cells, cells pretreated with cerulenin at 10–30 µg/ml for 1.5 h were incubated with varying concentrations of ricin in the presence or absence of cerulenin for an additional 3 h. After removal of the medium, the cells were labeled with [3H]leucine for 45 min in leucine-free medium, and the amount of [3H]leucine incorporation into proteins was measured. As shown in Fig. 1B, cerulenin inhibited the cytotoxicity of ricin in BER-40 cells in a dose-dependent manner. In contrast to BER-40 cells, cerulenin at 30 µg/ml did not elicit any protective effect on the ricin cytotoxicity in Vero cells (Fig. 1A). The protective effect of cerulenin required preincubation; when ricin and cerulenin were added to cells at the same time, a lesser protective effect was observed (data not shown). Furthermore, the protection by cerulenin from ricin cytotoxicity was reversible, inasmuch as the sensitivity of BER-40 cells toward ricin was partially restored by washing of the cells after a 1.5-h preincubation with cerulenin (data not shown). The protective effect of cerulenin against ricin cytotoxicity was reversed by the addition of a lipid mixture to the culture medium to give final concentrations of cholesterol (20 µg/ml), palmitate (20 µg/ml), and myristate (20 µg/ml) (data not shown). To ascertain whether the uptake of fatty acids and cholesterol was impaired in cerulenin-treated BER-40 cells, the transport of [3H]palmitate or [3H]cholesterol was measured in Vero and BER-40 cells with or without cerulenin treatment. In the absence of cerulenin, uptake of [3H]palmitate or [3H]cholesterol was similar in Vero and BER-40 cells.

After a 1.5-h preincubation with 20 µg/ml cerulenin, the uptake of [3H]palmitate or [3H]cholesterol remained unchanged in Vero and BER-40 cells up to 3 h of further incubation in the presence of cerulenin as compared to the untreated cells (data not shown). These results suggest that the lack of reversal of the protective effect of cerulenin against ricin cytotoxicity by the lipid mixture is not due to a failure in the uptake of fatty acids and cholesterol into cerulenin-treated BER-40 cells.

It has been shown that the action of BFA is species-specific, and two kidney epithelial cell lines, PtK, (potoroo rat kangaroo) and MDCK (canine) cells, are naturally resistant to BFA and cross-resistant to EDIN, a Staphylococcal ADP-ribosyltransferase which inactivates small G protein rho and mimics the action of BFA (33). The morphology of the Golgi apparatus was not altered by BFA in these two cell lines (39–41), and they were not protected from ricin cytotoxicity by BFA (42). These findings prompted us to examine the effect of cerulenin on ricin cytotoxicity in PtK, and MDCK cells. As shown in Fig. 2, cerulenin also protected PtK, and MDCK cells from the ricin cytotoxicity. Thus, there appears to be a correlation between the resistance to BFA and the protection by cerulenin against ricin cytotoxicity. Interestingly, cerulenin stimulated protein synthesis of PtK, and MDCK cells; in the presence of 20 µg/ml cerulenin, protein synthesis in PtK, and MDCK cells was increased by 1.5- and 3.0-fold, respectively (data not shown). Although the mechanism of the stimulation of protein synthesis by cerulenin is unknown, BFA also showed similar stimulative effects on protein synthesis in PtK, cells (data not shown).

Effect of Cerulenin on the Cytotoxicities of Modeccin, Pseudomonas Toxin, and Diphtheria Toxin—To determine the specificity of cerulenin in protecting BFA-resistant cell lines

![Fig. 1. The effect of cerulenin on the cytotoxicity of ricin in Vero (A) and BER-40 (B) cells. Cells grown in 24-well plates were preincubated in α-MEM containing 1% BSA with indicated concentrations of cerulenin (0, 10, 15, 20, and 30 µg/ml) for 1.5 h at 37 °C, followed by the addition of varying concentrations of ricin. Cells were incubated for another 3 h in the presence of cerulenin, and protein synthesis was measured as described in the legend to Fig. 1. Each point represents an average of duplicate measurements.](image)

![Fig. 2. The effect of cerulenin on the cytotoxicity of ricin in PtK, (A) and MDCK (B) cells. Cells grown in 24-well plates were preincubated in α-MEM containing 1% BSA with indicated concentrations of cerulenin (0, 10, 20, and 30 µg/ml) for 1.5 h at 37 °C, followed by the addition of varying concentrations of ricin. Cells were incubated for another 3 h in the presence of cerulenin, and protein synthesis was measured as described in the legend to Fig. 1. Each point represents an average of duplicate measurements.](image)
from ricin cytotoxicity, we examined the effect of cerulenin on the cytotoxicities of other toxins in Vero, BER-40, PtK1, and MDCK cells. The results summarized in Table I clearly indicate that cerulenin inhibited the cytotoxicities of modecin, *Pseudomonas* toxin, and diphtheria toxin to varying extents in each of the BFA-resistant cell lines, whereas no significant effect of cerulenin on the cytotoxicities of these toxins was observed in Vero cells. Diphtheria toxin differs from the other toxins in that its active subunit (A-chain) is translocated to the cytosol from endosomes following acidification (6–9). In accordance with this, intoxication of diphtheria toxin was not inhibited by BFA (21, 43). In contrast to BFA, cerulenin significantly reduced the toxic effect of diphtheria toxin in BFA-resistant cell lines (Table I). Therefore, these results suggest that these toxins including diphtheria toxin share a common step in their intoxication pathways which is affected by cerulenin.

**Effect of Cerulenin on the Binding and Internalization of Ricin**—To ascertain whether or not the protective effect of cerulenin against ricin cytotoxicity in BFA-resistant cell lines is due to an inhibition of ricin binding and/or internalization, we measured the binding and internalization of 125I-ricin in Vero, BER-40, PtK1, and MDCK cells with or without pretreatment with cerulenin. As shown in Fig. 3A, cerulenin did not affect the binding of ricin to cell-surface receptors in the cell lines tested. However, the amounts of internalized ricin decreased with increasing concentrations of cerulenin in BER-40 cells, although no significant change in the endocytic uptake of ricin was observed in Vero, PtK1, and MDCK cells (Fig. 3B). Maximal inhibition of ricin internalization was obtained in BER-40 cells treated with 30 μg/ml cerulenin. This decrease in ricin internalization may contribute to the reduction in the cytotoxic effect of ricin by cerulenin, but probably does not account for the profound inhibition of ricin cytotoxicity in BER-40 cells shown in Fig. 1 and Table I.

**Effect of Cerulenin on the Endocytosis of Fluid Phase Markers and Transferrin**—To further characterize the effect of cerulenin on endocytosis, we examined the effect of cerulenin on the endocytic uptake of fluid-phase markers such as horseradish peroxidase and lucifer yellow, as well as the endocytosis of transferrin which enters cells by the pathway involving clathrin-coated pits/coated vesicles (44–46), in Vero and BER-40 cells. As shown in Table II, cerulenin reduced the uptake of both horseradish peroxidase and lucifer yellow in BER-40 cells, whereas no significant effect of cerulenin on the fluid-phase endocytosis was observed in Vero cells. In contrast to the inhibition of the uptake of fluid-phase markers, cerulenin had a stimulatory effect on the endocytosis of transferrin in both Vero and BER-40 cells. During a 30-min incubation, cerulenin did not affect the binding of 125I-transferrin to cell-surface receptors in both Vero and BER-40 cells (data not shown). The excretion of internalized 125I-transferrin was inhibited about 15 and 30% in cerulenin-treated Vero and BER-40 cells, respectively. These results suggest that the stimulation of transferrin endocytosis by cerulenin is due to an inhibition of the recycling of transferrin receptors in cerulenin-treated cells. Since ricin is considered to enter cells from both clathrin-coated pits and non-clathrin-coated areas of the plasma membrane, these results suggest that cerulenin mainly inhibits the latter pathway of endocytosis of ricin in BER-40 cells.

**Effect of Cerulenin on the Excretion and Degradation of Ricin**—We investigated the effect of cerulenin on ricin excretion and degradation. As shown in Fig. 4, B and D, cerulenin greatly inhibited the release of trichloroacetic acid-soluble radioactivity into the medium in BER-40 cells (Fig. 4D), while the release of total radioactivity excreted into the medium was also decreased by cerulenin treatment in BER-40 cells (Fig. 4B). This effect of cerulenin was not observed in Vero cells (Fig. 4A). These data suggest that cerulenin inhibits the degradation and excretion of ricin in BER-40 cells.

**Effect of Cerulenin on Bulk Protein Secretion**—After a 1.5-h preincubation with or without cerulenin at various concentrations, cells were pulse labeled with [3H]leucine for 20 min, and chased for 1 h in the presence or absence of cerulenin. As shown in Fig. 5, cerulenin inhibited bulk protein secretion in a dose-dependent manner, without affecting the rate of protein synthesis under the same conditions. Inhibition of bulk protein secretion by cerulenin was more pronounced in BER-40 cells than Vero cells, with 20 μg/ml cerulenin causing more than 90% inhibition of protein secretion in BER-40 cells. These results suggest that cerulenin affects bulk protein secretion, especially in BER-40 cells.

**Effect of Cerulenin on the Processing of Mannose 6-Phosphate Receptors**—The effect of cerulenin on the biosynthesis and oligosaccharide processing of mannose 6-phosphate receptors (Man-6-PR) was investigated in Vero and BER-40 cells. Normally, Man-6-PR is glycosylated in the ER, resulting in initial oligosaccharides of the high mannose-type. Subsequently these oligosaccharides are converted to the complex-type during transit through the Golgi apparatus (47, 48). As shown in Fig. 6, after preincubation in the presence of cerulenin, Man-6-PR in both Vero and BER-40 cells became sensitive to endo H treatment as shown by a reduction in molecular size, indicating a lack of endo H-resistant complex-type oligosaccharide. These results suggest that cerulenin inhibits the transport of Man-6-PR from ER to Golgi, although a direct effect of cerulenin on the processing of Man-6-PR oligosaccharide cannot be ruled out. In the case of BER-40 cells, two bands were detected in the presence of cerulenin, with the lower band corresponding to endo H-digested Man-

---

**Table I**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Ricin</th>
<th>Diphtheria toxin</th>
<th>Modeccin</th>
<th><em>Pseudomonas</em> toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>3.4</td>
<td>3.4</td>
<td>0.33</td>
<td>0.39</td>
</tr>
<tr>
<td>BER-40</td>
<td>9.1</td>
<td>1,260</td>
<td>0.41</td>
<td>43</td>
</tr>
<tr>
<td>MDCK</td>
<td>2.9</td>
<td>37.5</td>
<td>29.5</td>
<td>74</td>
</tr>
<tr>
<td>PtK1</td>
<td>8.4</td>
<td>630</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Cerulenin Inhibits Ricin Cytotoxicity in BFA\(^R\) Cell Lines

---

**ID₅₀ (ng/ml)**

Vero     | 3.4            | 3.4              | 0.33     | 0.39               | 6.1 | 8.9 | 285 | 540 |
BER-40   | 9.1            | 1,260            | 0.41     | 43                 | 4.8 | >10,000 | 610 | >10,000 |
MDCK     | 2.9            | 37.5             | 29.5     | 74                 | 19.5 | 1,150  | 355 | 980  |
PtK₁     | 8.4            | 630              | >10,000  | >10,000            | 3,800 | >10,000 | 6,500 | >10,000 |
Cerulenin Inhibits Ricin Cytotoxicity in BFA® Cell Lines

**Fig. 3.** The effect of cerulenin on the binding (A) and endocytosis (B) of ricin in Vero (○), BER-40 (●), Ptk1 (△), and MDCK (□) cells. Cell monolayers in 24-well plates were preincubated in α-MEM containing 1% BSA with varying concentrations of cerulenin for 1.5 h at 37 °C. 125I-Labeled ricin (2 μg/mL, specific activity; 9.6 × 10^6 cpm/μg) was added, and the amounts of cell-surface bound 125I-ricin at 4 °C and endocytosed 125I-ricin at 37 °C after a 1-h incubation were measured as described under “Experimental Procedures.” The amount of bound or endocytosed ricin is expressed in percent of the amount of bound or endocytosed in the absence of cerulenin. Each point represents an average of duplicate measurements.

**Table II**

The effect of cerulenin on the endocytosis of lucifer yellow, horseradish peroxidase, and 125I-transferrin in Vero and BER-40 cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Endocytosed lucifer yellow</th>
<th>% of control</th>
<th>Endocytosed HRP</th>
<th>Endocytosed 125I-transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero</td>
<td>106.3 ± 1.4</td>
<td>85.4 ± 3.1</td>
<td>150.0 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>BER-40</td>
<td>77.6 ± 1.0</td>
<td>72.7 ± 1.1</td>
<td>197.3 ± 5.0</td>
<td></td>
</tr>
</tbody>
</table>

This may indicate that glycosylation of Man-6-PR is also blocked by cerulenin in BER-40 cells.

**Discussion**

Cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienoylamide) has been shown to inhibit the de novo synthesis of fatty acid and sterol by an inhibition of the activities of β-ketoacyl-acyl carrier protein synthetase and β-hydroxy-β-methylglutaryl-CoA synthetase, respectively (49–51). However, it is not clear whether the inhibition of lipid synthesis by cerulenin is directly responsible for a number of biological effects of cerulenin. Indeed, Jacques (52) showed that the inhibition of lipid synthesis and secretion of exoenzymes by cerulenin in bacteria could be differentially relieved by Tween 80. Tween 80 largely eliminated the inhibition of acetate incorporation into lipid by cerulenin, without alleviating the inhibition of the secretion of the exoenzymes by cerulenin. It has been reported that cerulenin inhibits the fatty acylation of viral glycoproteins, resulting in the inhibition of the formation and release of progeny virus particles from chicken embryo fibroblasts infected with Sindbis or vesicular stomatitis virus (53). Furthermore, cerulenin also inhibited the proteolytic processing of the viral glycoprotein in Sindbis virus-infected cells. Similar inhibition of the production of Rous sarcoma virus in transformed chick embryo fibroblasts by cerulenin has been observed, with the inhibition of proteolytic processing of major viral structural proteins (pr76) in cerulenin-treated cells being the earliest detectable effect of cerulenin (54). These effects of cerulenin could not be reversed by the addition of fatty acids, even though the addition of fatty acids could prevent the antibiotic activity of cerulenin in bacteria (49). Since both proteolytic processing and fatty acylation of viral glycoproteins occur in the Golgi apparatus (55, 56), these effects of cerulenin on the proteolytic processing and lipid modification of viral proteins may be indirect consequences of a perturbation of the vesicular transport of proteins through the Golgi complex. A direct effect of cerulenin on specific proteases or fatty acylation enzymes has not been demonstrated.

In the present study we have shown that cerulenin inhibited the cytotoxicities of ricin, modeccin, *Pseudomonas* toxin, and diptheria toxin in a BFA-resistant mutant cell line, BER-40. Such protective effects of cerulenin were not observed in the BFA-sensitive parental Vero cells (Fig. 1, Table I). These results suggest that one of the phenotypes of BER-40 cells is the protection by cerulenin against protein toxins. The pro-
tective effects of cerulenin against toxins were also observed in two naturally BFA-resistant cell lines, PtK and MDCK cells (Fig. 2, Table I). The vesicular transport system is presumably involved in the intracellular trafficking or processing of toxins. In the case of ricin, it has been demonstrated that internalized ricin is transported to the Golgi apparatus (6-9). Several lines of evidence suggest that translocation of ricin A-chain takes place in the trans-Golgi network or a post-trans-Golgi network compartment (42). On the other hand, diphtheria toxin is translocated to the cytosol from acidified endosomes (10, 11). The fact that cerulenin inhibits the cytotoxicities of both diphtheria toxin and other toxins suggests the existence of a common and presumably early step in the intoxication pathways of these toxins which is inhibited by cerulenin. Since fatty acids and cholesterol did not reverse the effects of cerulenin, the protection by cerulenin from the cytotoxicities of toxins is not a consequence of an inhibition of de novo fatty acids and cholesterol biosynthesis.

Although cerulenin did not affect the binding of ricin to the cell-surface receptors, endocytosis of ricin was inhibited by cerulenin in BER-40 cells (Fig. 3). Analysis of the uptake of horseradish peroxidase, lucifer yellow, and transferrin revealed that cerulenin predominantly inhibited the fluid phase endocytosis (Table II). However, no change in the endocytosis of ricin was observed in Vero, PtK, and MDCK cells, even though the latter two cell lines were also protected by cerulenin against ricin cytotoxicity. These results suggest that in addition to its inhibition of fluid phase endocytosis and coated vesicle-independent endocytosis, cerulenin interferes with the intracellular processing or trafficking of toxin molecules beyond endocytosis, and perturbs the intracellular vesicle transport system. This conclusion is supported by the observation that cerulenin inhibited the degradation and excretion of internalized ricin in BER-40 cells (Fig. 4, B and D). In agreement with this hypothesis, cerulenin inhibited bulk protein secretion in Vero and BER-40 cells. Since the processing of oligosaccharide of Man-6-PR from high mannose-type to complex-type was blocked by cerulenin (Fig. 6), vesicle transport from ER to Golgi may also be affected by cerulenin. It has been reported that cerulenin inhibited the secretion of exoenzymes in bacteria (52). In addition, the ability of cerulenin to protect BER-40 cells from ricin cytotoxicity may result from the inhibition of a processing step which activates ricin to allow the delivery of enzymatically active ricin-A chain to the cytosol. Taken together, these results suggest that cerulenin inhibits multiple steps of the vesicle transport system including the budding, vesicle formation, and membrane fusion. The mutation affecting BFA resistance in BER-40 cells results in an alteration of the vesicle membranes in such a way that they become cerulenin-sensitive in the formation or fusion of transport vesicles. A working model of the effects of cerulenin on the intracellular vesicle transport system is shown in Fig. 7.

Because of the multiple biological effects of cerulenin (49, 53), several possibilities can be considered to account for our observations. Post-translational processing of small GTP-binding proteins includes fatty acylation as well as prenylation and proteolysis (57), and palmitoylation may alter the interaction of the target protein with GTP-binding proteins (58). It is conceivable that cerulenin inhibits the fatty acylation of a GTP-binding protein(s) or other proteins which interact with GTP-binding proteins. It has been reported that BFA treatment reduces the palmitate labeling of most of cellular proteins except for p62, which is one of the major palmitoylated proteins with an apparent molecular weight of

**FIG. 5.** The effect of cerulenin on bulk protein secretion (○) and protein synthesis (△) in Vero (○, △) and BER-40 (○, △) cells. Cells grown in 24-well plates were preincubated with varying concentrations of cerulenin for 1.5 h at 37°C in α-MEM containing 1% BSA, pulse labeled with [3H]leucine (20 μCi/ml) for 20 min in leucine-free medium, and chased for 1 h in α-MEM containing 1% BSA with or without cerulenin. Cerulenin, when indicated, was present in the medium throughout the pulse and chase. Bulk protein synthesis and secretion were measured as described under "Experimental Procedures." The amounts of proteins synthesized and secreted in the control cells without cerulenin were taken as 100%. Each point represents an average of duplicate measurements.

**FIG. 6.** The effect of cerulenin on the processing of mannose 6-phosphate receptor in Vero and BER-40 cells. Confluent Vero (lanes 1-4) or BER-40 (lanes 5-8) cells were preincubated with or without cerulenin (20 μg/ml) for 30 min at 37°C in methionine-free medium, pulse labeled with [35S]methionine (300 μCi/ml) for 30 min in methionine-free medium, and chased for 4 h in α-MEM supplemented with 10 mM unlabeled methionine and 1% BSA. Cerulenin, when indicated, was present in the medium throughout the pulse and chase. Immunoprecipitates of Man-6-PR were obtained from Vero and BER-40 cells and incubation in the absence of (-) or presence (+) of endo H was carried out on each sample as described under "Experimental Procedures."
The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear. The recent proposal (60) that BFA may affect vesicle formation or inhibition of ricin cytotoxicity by cerulenin is still unclear. The precise relationship between the BFA resistance and the inhibition of ricin cytotoxicity by cerulenin is still unclear.