

Brefeldin A Protects Ricin-Induced Cytotoxicity in Human Cancer KB Cell Line, but not in its Resistant Counterpart with Altered Golgi Structures

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ABSTRACT. Brefeldin A (BFA), an isoprenoid fungal metabolite, dramatically disrupts intracellular protein transport and protein secretion. BFA protects cells from the cytotoxicity of a plant toxin, ricin or pseudomonas toxin, but not that of diphtheria toxin (YOSHIDA *et al.*, 1991. *Expt. Cell Res.*, 192: 389–395.). In this study, we examined whether BFA could differentially change the cytotoxicity of ricin between BFA-sensitive cells and BFA-resistant cells. As a BFA-resistant cell line, we used a resistant cell line, KB/BF2-2, derived from BFA-sensitive human cancer KB cells. BFA treatment caused the disappearance of typical Golgi cisternae and the concomitant appearance of dilated vesicles in the cytoplasm in KB cells. By contrast, KB/BF2-2 cells had already altered Golgi structures with poor development of cisternae and also many vesicles in the absence of BFA, and BFA treatment did not further induce the morphological changes. Although a plasma membrane-specific marker protein, α -adaptin, was localized similarly in KB/BF2-2 as KB, Golgi specific markers such as β -cop and γ -adaptin were distributed in the cytoplasmic small vesicles as well as Golgi compartments in KB/BF2-2 cells in the absence of BFA, and the mutant cells showed no apparent changes in the distribution even when exposed to BFA. Ricin inhibited protein synthesis in KB and KB/BF2-2 to similar levels while pretreatment of KB cells with BFA at 0.1 μ g/ml almost completely reversed the inhibitory effect of ricin. By contrast, the pre-exposure of KB/BF2-2 cells to 1.0 μ g/ml BFA only partially rescued the ricin-induced inhibition of protein synthesis. Exposure to BFA at 30 min before ricin addition or at 0 min with ricin rescued the protein synthesis inhibition, but no rescue occurred when BFA was added 30 min after ricin addition. BFA could not rescue the protein synthesis inhibition by another toxin, diphtheria toxin. Our results suggest that BFA-resistant mutation causes a specific change in the endocytic membrane traffic of ricin in human cells, and also that cytotoxicity of diphtheria toxin does not share a common pathway of the intracellular transport with that of ricin.

Brefeldin A (BFA), an isoprenoid fungal metabolite, blocks intracellular protein transport and protein secretion (12, 23, 26, 42). BFA dramatically disintegrates the Golgi apparatus and induces the redistribution of the Golgi proteins into the endoplasmic reticulum (ER) (5, 12, 13, 22, 23, 41, 50) possibly through its specific inhibition of the anterograde transport from the ER to the Golgi (20, 22, 23). We also reported that BFA inhibits degradation of low density lipoprotein (LDL) receptor in macrophages (40) and also O-linked sialylation of the LDL receptor (39). As an early event, BFA dissociates a 110 kDa coat protein subunit, β -cop, of non-clathrin-coated vesicles from the Golgi apparatus (6, 9,

45). Thus, BFA appears to prevent the assembly of non-clathrin-coated vesicles onto the Golgi apparatus, as confirmed by blocking the vesicular transport in a cell-free system (28). Further studies have demonstrated the involvement of GTP-binding proteins in vesicular transport and Golgi function (2, 3) and BFA thus modulates the β -cop redistribution through such GTP-binding proteins (7, 8). Some models have been proposed by Klausner *et al.* (20) concerning pleiotropic effects of BFA on membrane traffic within the central vacuolar system, although its primary target site is not yet known.

Ricin, a plant protein toxin, is internalized into mammalian cells by receptor-mediated endocytosis, and it modifies its target, 28S ribosomal RNA in the cytosol (10, 11). Following endocytosis of the plant toxin, ricin

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molecules are observed in Golgi elements (43). The cytotoxicity of ricin in mammalian cells is modulated by monensin, a Golgi-targeting agent (29, 34), and also by a monensin-resistant mutation (47). Yoshida *et al.* (48) have further demonstrated that disruption of the Golgi function by BFA inhibits the cytotoxicity of ricin or modeccin, another plant toxin, but not that of bacterial toxin, diphtheria toxin. These findings suggest that Golgi elements are involved in the cytotoxic mechanism of ricin. We have recently isolated and characterized BFA-resistant mutants from the human epidermoid cancer KB cells, which have altered Golgi structures and function (37). In the present study, we examined whether ricin cytotoxicity or intracellular transport of ricin was modified in the BFA-resistant mutant in the presence of BFA. We discussed if the intracellular pathway of ricin was altered in the BFA-resistant cells.

MATERIALS AND METHODS

Cells lines and cell culture. Human epidermoid carcinoma KB cells and their BFA-resistant cell lines, were cultured at 37°C in minimal essential medium (MEM) containing 10% newborn calf serum (Flow Laboratories) (37).

Isolation of higher BFA-resistant mutants from BFA-resistant KB/BF2 cells. Since KB/BF2 cells showed only about 10-fold higher resistance to BFA (37), and the BFA-resistant phenotype had not been maintained stably during serial cell culture in the absence of BFA, we decided to further isolate a higher resistant mutant with stable phenotype. KB/BF2 cells were treated with 0.5 µg/ml of BFA, and the medium containing the drug was changed every 3 or 4 days. Colonies appearing in the presence of the drug after incubation for about 1 month were purified, and five independent clones were isolated. Each clone was grown and further incubated with 1.0 µg/ml of BFA, and colonies appearing after incubation for 2

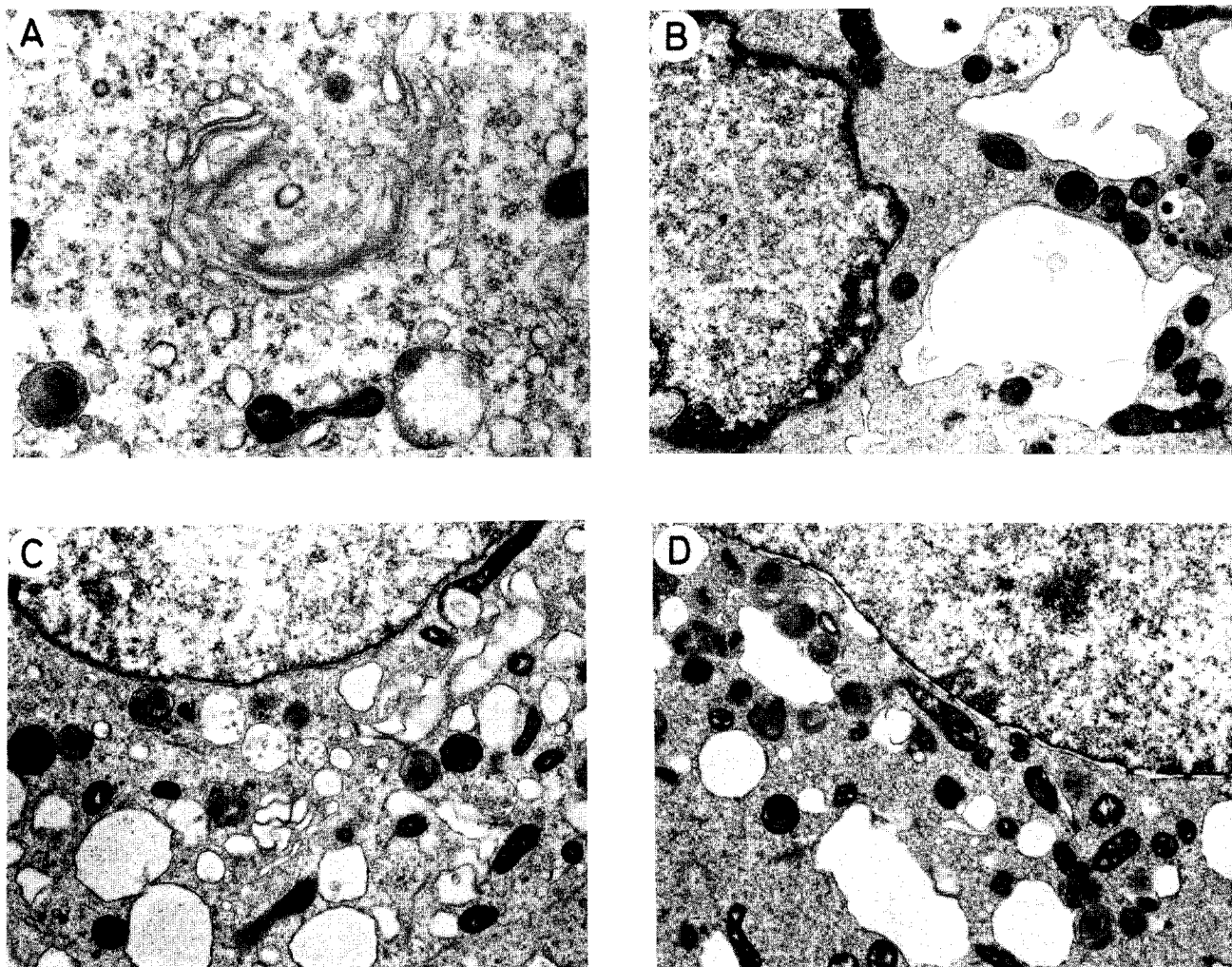


Fig. 1. Electronmicrographs of KB and KB/BF-2-2 cells. KB cells (A and B) and KB-BF-2-2 (C and D) cells were cultured in the absence (A and C) or presence (B and D) of 1.0 µg/ml BFA for 60 min. Magnification: $\times 25000$.

months were purified. We finally selected one BFA-resistant clone, KB/BF2-2, and its resistant phenotype was maintained stably for more than 2 months in the absence of the drug.

Materials. [^{35}S] Methionine (681 Ci/mmol) and TRANS-[^{35}S]-label® methionine and cysteine mixture (1123 Ci/mmol) were purchased from Du Pont-New England Nuclear; protein A-Sepharose was from Pharmacia Fine Chemical (Uppsala, Sweden); ricin (ricin D) was prepared as described previously (18); diphtheria toxin was from Swiss Serum and Vaccine Institute (Bern, Switzerland); Monensin was from Calbiochem Behring; BFA was from Epicentre Technologies. Anti- β -cop antibodies were raised in rabbits, for which the synthetic peptide EAGELKPEEEITVGPVQK (positions 496–513 of rat β -cop) was conjugated with keyhole limpet hemocyanin and injected into two rabbits (37). Anti- γ -adaptin and α -adaptin antibodies were kindly obtained from E. Ungewickell (1) and M. Robinson (31), respectively.

Immunofluorescence microscopy. Cells grown on glass

coverslips were briefly washed with phosphate-buffered saline (PBS) and fixed with methanol at -20°C for 5 min. After being rinsed with PBS, cells were incubated with rabbit anti- β -cop peptide antibodies or other antibodies for 30 min at room temperature. Immunolabeling was visualized by incubating the cells with the respective second antibodies conjugated with rhodamine (for mouse antibodies) or fluorescein (for rabbit antibodies) at the dilution of 1 : 50 for 30 min at room temperature. All antibodies were diluted with PBS containing 0.1% Triton X-100 and 5% normal goat serum. The cells then were washed with PBS, mounted on glycerin, and observed with a Nikon fluorescence microscope for α -adaptin and BIO-RAD laser scanning confocal imaging system for β -cop and γ -adaptin.

Electron microscopic study. Cells ($2\sim 3 \times 10^6/100\text{-mm}$ dish) were incubated with BFA ($1\text{ }\mu\text{g/ml}$) in MEM at 37°C for 1 hr. After being trypsinized and washed twice with PBS cells were fixed with 2% paraformaldehyde, and 2.5% glutaralde-

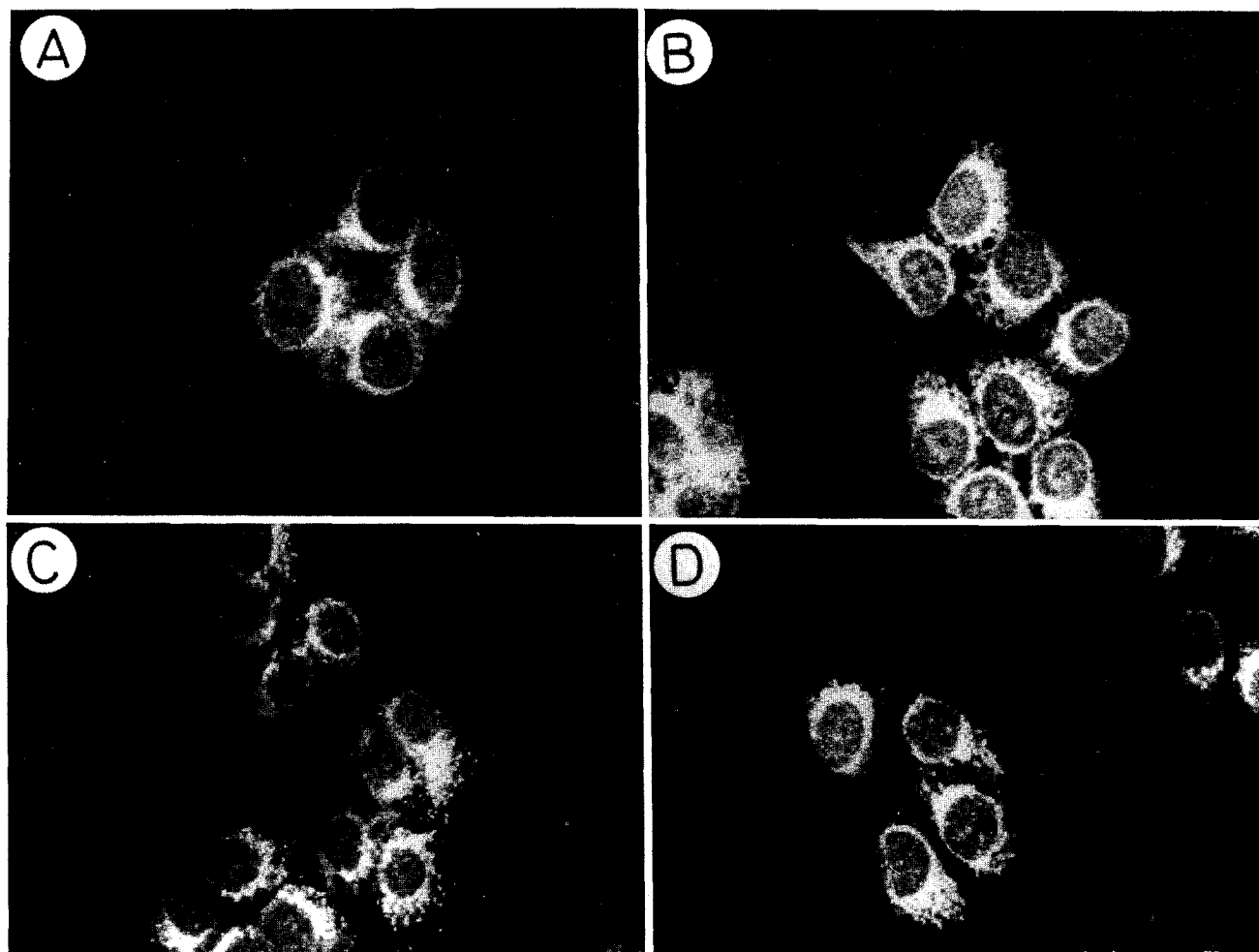


Fig. 2. Distribution of the β -cop in KB and KB/BF2-2 cell lines in the absence or presence of BFA. KB cells (A and B) and KB/BF2-2 cells (C and D) were incubated at 37°C for 60 min in the absence (A and C) or presence (B and D) of $1.0\text{ }\mu\text{g/ml}$ of BFA. The cells were fixed, stained with immunofluorescence, and subjected to immunofluorescence microscopy.

hyde in cacodylate buffer, pH 7.2 at 4°C for 2 hr, rinsed in the cacodylate buffer, and postfixed with 2% osmium tetroxide, and 0.5% potassium ferrocyanide in 0.1 M cacodylate-buffer, pH 7.4, at 4°C for 2 h as described previously (37, 40). The specimens were dehydrated through an ethanol series and embedded in Epok 812 epoxy resin. The sections prepared were prepared stained with uranyl acetate and lead citrate and examined by a JEM-100 CX electron microscope.

Iodination of ricin. ^{125}I -Ricin was made by the chloramine-T method as described previously (26, 27). ^{125}I -Ricin was purified by chromatography through a Sepharose G-75 column. The specific activity of our ^{125}I -ricin was in the range of 0.9×10^5 to 1.1×10^5 cpm per mg of ricin.

Binding and internalization assay. Approximately 4×10^5 cells in a 35-mm dish were washed twice with PBS and incubated with ^{125}I -ricin in 1 ml of MEM medium without serum at 0°C or 37°C in a CO_2 incubator as described previously (26, 27). To assay binding activity, cells incubated at 0°C were

washed three times with a cold PBS solution and treated with trypsin; the amount of cell-bound radioactivity was then counted with a gamma counter. To measure the amount of ^{125}I -ricin internalized, cells were incubated with ^{125}I -ricin at 37°C for 1 hr or 3 hr. The cells were then incubated for an additional 10 min in 1 ml of PBS containing 0.1 M lactose and washed three times with the same buffer. The amount of radioactivity inaccessible to the action of lactose was counted. In these binding and internalization assays, specific binding was calculated by subtracting the radioactivity of ^{125}I -ricin bound to the cells when the cells were incubated with MEM containing ^{125}I -ricin and 0.1 M lactose.

Measurement of cellular protein synthesis. Cells were incubated at a cell density of 1.5×10^5 cell/well for KB cells or 2.2×10^5 cells/well for KB/BF2-2 in 1.5 ml of medium using 24-well plate. Two days later, the monolayer culture was incubated with or without BFA for 30 min at 37°C. After being treated with ricin for 3 h, cell were incubated with [^{35}S] methio-

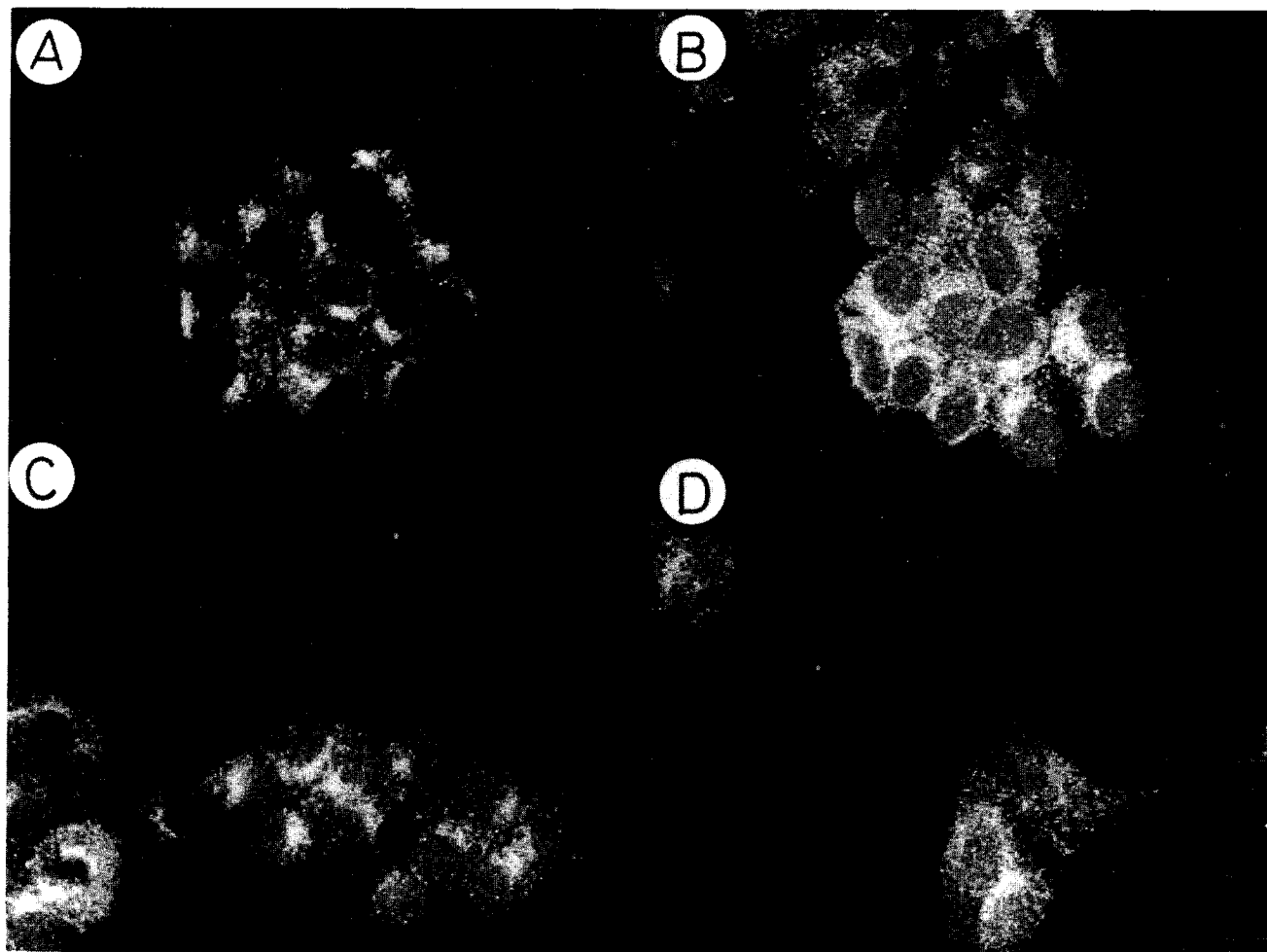


Fig. 3. Distribution of the γ -adaptin in KB and KB/BF-2-2 cells in the absence or presence of BFA. KB cells (A and B) and KB/BF-2-2 cells (C and D) were incubated at 37°C for 60 min in the absence (A and C) or presence (B and D) of 1.0 $\mu\text{g}/\text{ml}$ of BFA. The cells were fixed, stained with immunofluorescence, and subjected to immunofluorescence microscopy.

nine for 10 min at 37°C in a methionine-free medium. After removal of the medium, cells were suspended in 10% trichloroacetic acid, boiled for 10 min, filtrated through glass-fiber filters and washed twice with 5% (wt/vol.) trichloroacetic acid, and solubilized in 0.1 M KOH, followed by determination of the radioactivity.

RESULTS

We previously isolated KB/BF2 from human cancer KB cells, which showed about 10-fold higher resistance to BFA than that of the parent cells (37). The BFA-resistant phenotype of KB/BF2 cells was found to be unstable during serial culture in the absence of BFA (see, Materials and Methods), and we have further isolated a BFA-resistant cell line, KB/BF2-2, from KB/BF2 after long exposure to BFA. KB/BF2-2 was about 60-fold higher resistant to the cytotoxic effect of the drug, when assayed by colony formation, and its BFA-resistant phenotype was maintained for at least 2 months in the ab-

sence of drug. Figure 1 shows electronmicroscopic profiles of KB and KB/BF2-2 cells incubated at 37°C in the presence or absence of BFA. In the absence of the drug, the typical stack structure of the Golgi found in the parental KB cells (Fig. 1A) was not identified in KB/BF2-2 cells, which had various-sized vacuoles or dilated cisterna-like structures and small vesicles clustered in a perinuclear region (Fig. 1C). When being treated with BFA, the aberrant Golgi structures of KB/BF2-2 cells were not so significantly changed (Fig. 1D) as those in the untreated cells (Fig. 1C), in contrast to a dramatic change of the Golgi structures observed in BFA-treated KB cells (Fig. 1B).

We then examined dynamic structural features of the two cell lines by immunofluorescence microscopic techniques with antibodies raised against Golgi and plasma membrane markers. Antibodies against β -cop, a non-clathrin-coated protein subunit (9, 38), and those against γ -adaptin (1, 30) were used as probes for Golgi markers. Antibodies developed against α -adaptin was

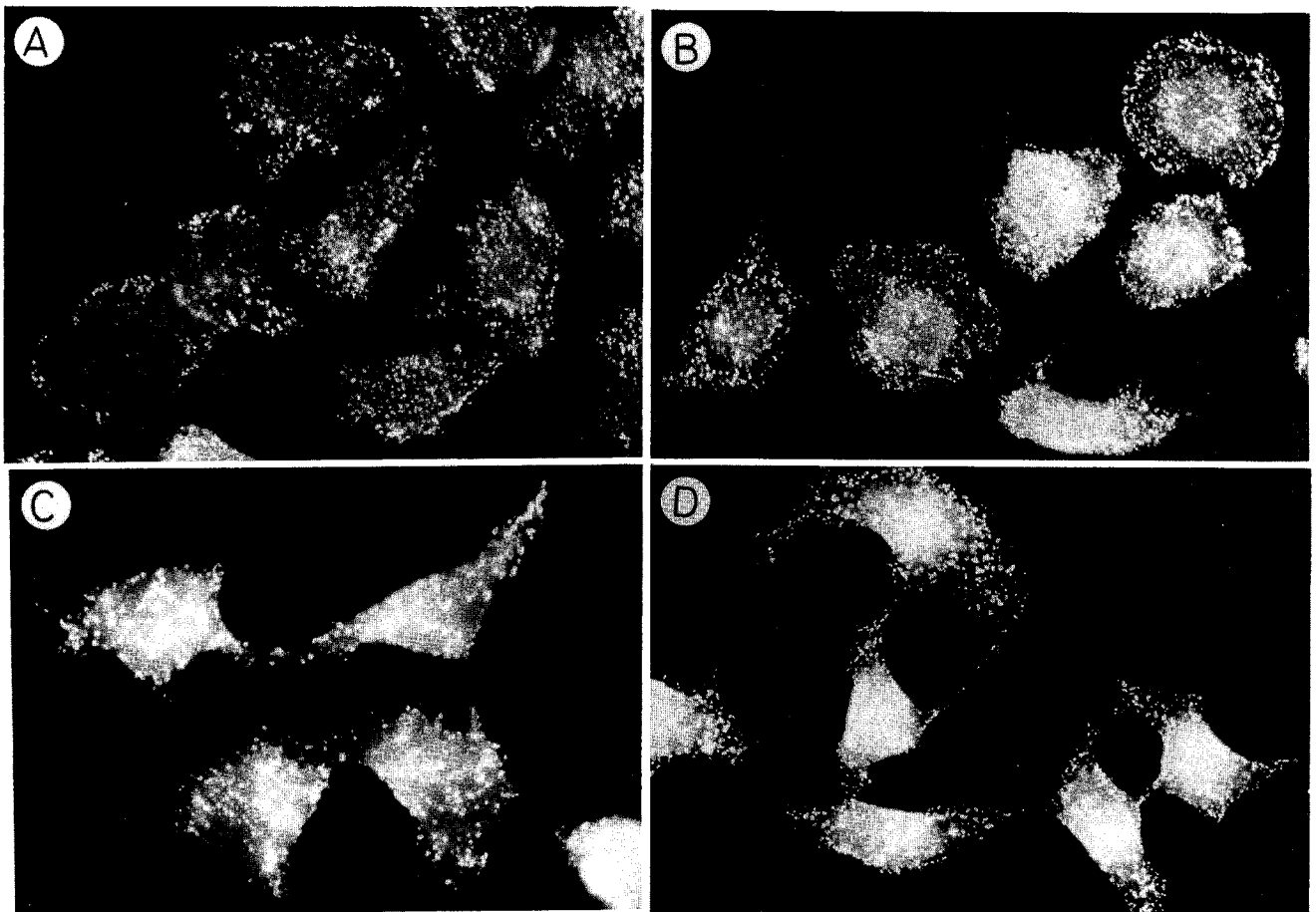


Fig. 4. Distribution of the α -adaptin in KB and KB/BF2-2 cells in the absence or presence of BFA. KB cells (A and B) and KB/BF2-2 cells (C and D) were incubated at 37°C for 60 min in the absence (A and C) or presence (B and D) of 1.0 μ g/ml of BFA. The cells were fixed, stained with immunofluorescence, and subjected to immunofluorescence microscopy.

also used as a plasma membrane-derived marker (1, 30). Two adaptin subsets of clathrin-coated vesicles are known: α -adaptin is a component of the plasma membrane adapter complex, and γ -adaptin is a component of the Golgi adapter complex (31). In control KB cells without BFA, the Golgi apparatus was labeled with anti- β -cop (Fig. 2) and anti- γ -adaptin (Fig. 3), although some punctuated staining profiles corresponding to Golgi-derived vesicles could also be seen. The antibody against α -adaptin stained numerous dots corresponding to clathrin-coated pits and endocytic vesicles (Fig. 4). When KB cells were treated with BFA, β -cop and γ -adaptin were redistributed from the Golgi into the cytoplasm. By contrast, distribution of α -adaptin was not changed between untreated and treated KB cells. BFA specifically affected the distribution of coat proteins associated with the Golgi complex, without changing the distribution of those associated with the plasma membrane. On the other hand, in untreated KB/BF2-2 cells,

β -cop and γ -adaptin were localized in many small vesicles which were diffusely distributed in the cytoplasm and also Golgi-like structures in the perinuclear regions (Figs. 2 and 3). Not so extensive changes in the distribution of both β -cop and γ -adaptin in KB/BF2-2 cells occurred even when BFA was present. Fig. 4 also demonstrated that α -adaptin was distributed to the plasma membranes in untreated and BFA-treated KB/BF2-2 cells.

Recent studies have demonstrated that BFA protects various cultured mammalian cell lines from the cytotoxicity of ricin possibly through the modulation of endocytic process of the toxin (36, 47). We examined whether BFA could protect KB and KB/BF2-2 cells from the cytotoxic action of ricin. The cytotoxicity of ricin was determined by its effect on protein synthesis through [35 S] methionine incorporation. As shown in Fig. 5, in the absence of BFA, protein synthesis in KB and KB/BF2-2 cell lines was blocked by ricin in a dose depend-

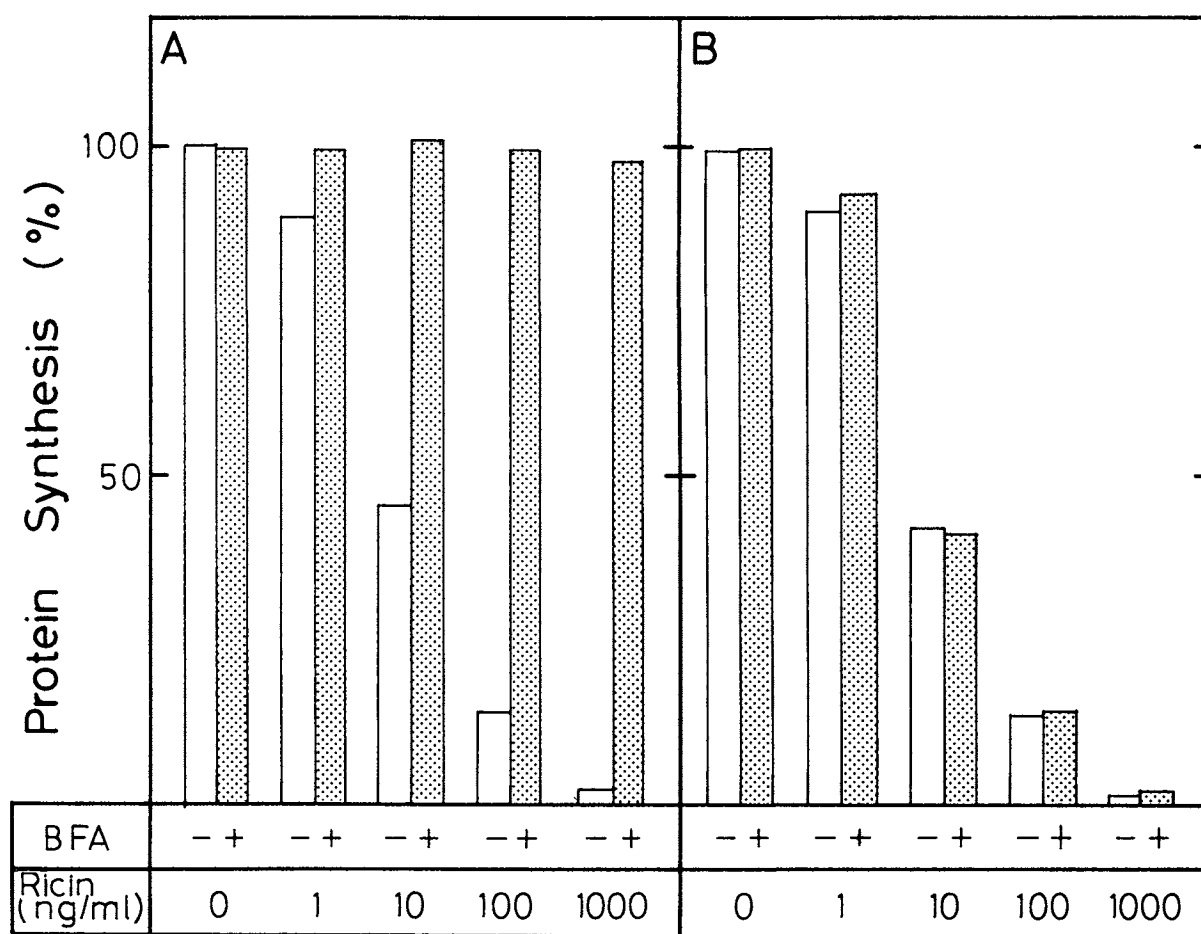


Fig. 5. The effect of BFA on the cytotoxicity of ricin in KB (A) and KB/BF2-2 (B). The monolayer culture was incubated in MEM with or without BFA (100 ng/ml) for 30 min at 37°C, followed by the addition of different concentrations of ricin. Cells were incubated for 3 hr at 37°C with or without BFA and then labeled with [35 S]-protein labeling mix for 10 min at 37°C in Met-free medium for the measurement of protein synthesis *in vivo*.

ent manner and completely blocked at 1 $\mu\text{g/ml}$ ricin in both cell lines. Thus, KB/BF2-2 was found to have essentially the same sensitivity to the cytotoxic effect of ricin as that of the parental KB cells. When being added to culture medium 30 min before the exposure to ricin, BFA at 100 ng/ml completely abrogated the cytotoxicity of ricin in KB cells, whereas no such protecting effect of BFA was observed in KB/BF2-2 cells (Fig. 5). We examined time kinetics for the removal effect by BFA of ricin toxicity. BFA at 100 ng/ml was added at various periods before or after exposure to 1 $\mu\text{g/ml}$ ricin. BFA almost completely protected KB cells from ricin intoxication when BFA was added at 30 min before ricin or at the same time with ricin, but not when BFA was added 30 min or more after the addition of ricin (Fig. 6). No significant protection was observed in KB/BF2-2 cells when BFA was added at any time point. Dose responses to BFA in its protective activity of ricin toxicity were examined in KB and KB/BF2-2 cells. As shown in Fig. 7, in KB cells BFA exerted the protective effect slightly at 10 ng/ml and BFA almost completely at

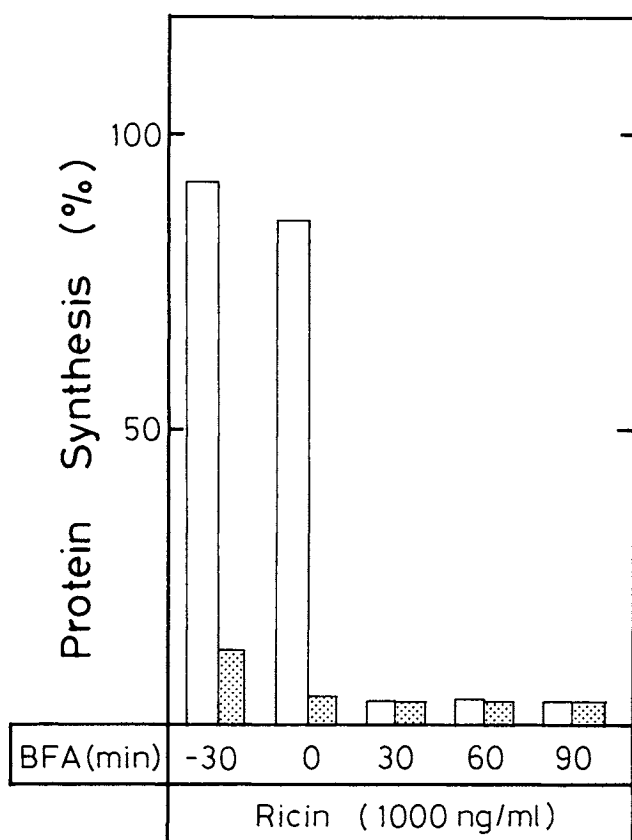


Fig. 6. The kinetics of the effect of BFA on ricin cytotoxicity in KB (\square) and BF 2-2 cells (\blacksquare). The monolayer cultures were treated with BFA (100 ng/ml) at different times relative to the addition of ricin. The cytotoxicity was expressed as percentages of [^{35}S] protein labeling mix as compared to that in cells treated with BFA only.

100 ng/ml and higher doses. By contrast, BFA at more than 400 ng/ml could partially inhibit the ricin toxicity in KB/BF 2-2 cells. Monensin, a Na^+/K^+ ionophoric antibiotic which inhibits Golgi functions, did not protect either KB or KB/BF2-2 cells from the ricin intoxication when 10 or 100 ng/ml monensin was used (Fig. 7).

Yoshida *et al.* (48) previously reported that BFA can block the ricin cytotoxicity, but not the diphtheria toxin cytotoxicity. We also tested whether BFA could protect the two cell lines from the diphtheria toxin-induced cytotoxicity. As shown in Fig. 8, BFA had no protection against the cytotoxicity of diphtheria toxin in either KB or KB/BF2-2 cell lines. No effect of BFA on the intoxication of diphtheria toxin in KB cells suggests that the intracellular transport system of diphtheria toxin is different from that of ricin. We examined whether the BFA-induced protection of ricin cytotoxicity might be due to altered binding or internalization of ricin. The specific binding of ^{125}I -ricin to the cell surface and its internalization were compared between KB and KB/BF2-2 cells in the presence or absence of BFA. As shown in Fig. 9, no significant difference in either binding or internalization of ^{125}I -ricin was observed between untreated KB and KB/BF2-2 cells. In addition, exposure to BFA did not appreciably change the binding and internalization of ^{125}I -ricin in either cell line (Fig. 9). These results suggest that the BFA-induced protection of ricin intoxication in KB cells might be responsible for an intracellular process after internalization of ricin, and that a possible site of this process is altered in KB/BF2-2 cells, resulting in no BFA-induced protection from the cytotoxicity of ricin.

DISCUSSION

Ricin is composed of two separate peptide chains, A and B, and induces cytotoxicity through the inhibition of protein synthesis in the cytosol fraction by removing a specific base from 28S rRNA (10, 25). The site of ricin A chain for ribosome inactivation has been determined (15). During its delivery to the cytosol, ricin is transported via endosomes to the Golgi apparatus and the trans-Golgi network (TGN) (43, 49). In hamster kidney BHK-21 cells, about 70–80% of endocytosed ricin molecules is present in the TGN after incubation for 60 min (44). The translocation of ricin to the cytosol appears to occur in the TGN. However, the translocation of the active diphtheria toxin chain appears to occur from the endosomes (33). BFA blocks the intoxication pathway of ricin in various cell lines (19, 36, 48). The inhibition of protein synthesis by ricin in KB cells was almost completely abrogated in the presence of BFA, consistent with previous studies (19, 36, 47). This protective effect of BFA was not observed in KB/BF2-2 cells, although only a slight protection was detected when a much

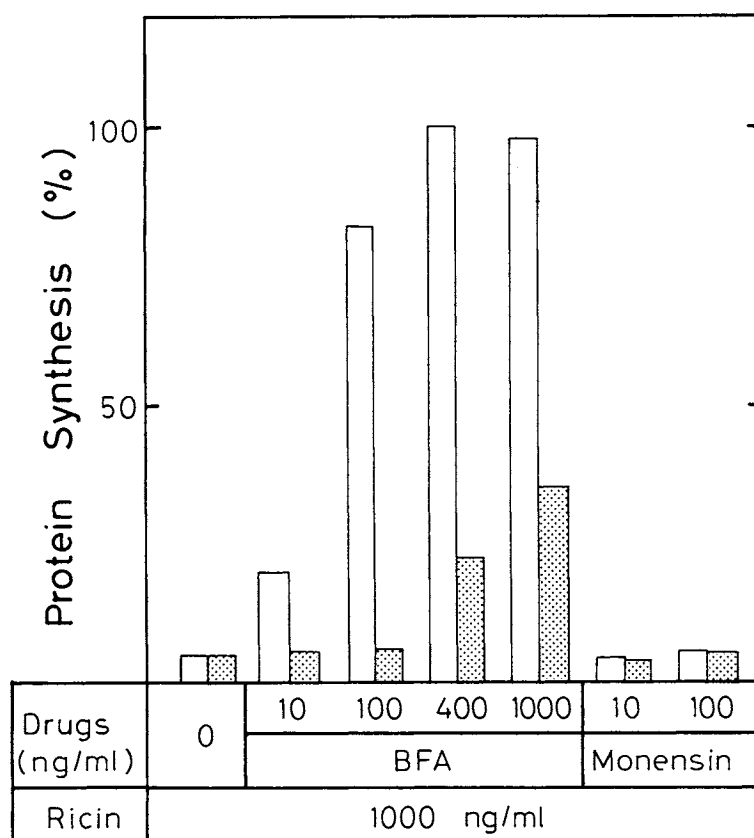


Fig. 7. Comparison of drug sensitivity of cytotoxicity of ricin in KB (□) and KB/BF 2-2 (■). The monolayer cultures were incubated in MEM with or without various doses of BFA and Monensin for 30 min at 37°C. The cells were exposed to ricin (1,000 ng/ml) for 3 hr at 37°C and then labeled with [³⁵S] protein labeling mix for 10 min in methionine-free medium for the measurement of protein synthesis.

higher dose (1 μ g/ml) of BFA was used (Figs. 5 and 7). BFA, however, did not protect either KB or KB/BF2-2 cells from the cytotoxicity of diphtheria toxin (Fig. 8). BFA does not affect binding or endocytosis of ricin in either KB or KB/BF2-2 cell lines (Fig. 9). Our present study also confirms the previous study by Yoshida and his colleagues (48) that Golgi function plays an essential role in the cytotoxicity of ricin, but not that of diphtheria toxin. Ricin is transported from the endosome to the Golgi region and released into the cytosol, whereas diphtheria toxin is directly released from the endosomes into the cytosol without involving the Golgi region (44, 48).

TGN/trans-Golgi cisternae are most likely the compartments where ricin (44) as well as horseradish peroxidase conjugates (16, 35, 43) accumulate in neurons and various cultured cells. In BFA-treated cells, many Golgi markers including the trans-Golgi enzyme galactosyl transferase are localized in the Golgi-ER fused vesicles (20) whereas the TGN markers are not distributed into the fused vesicles (4, 23, 45). Furthermore, in contrast to a dramatic change of morphology and distribution of ricin in the Golgi-ER by BFA, TGN remains intact and internalized ricin is still localized in the TGN when

BFA is added before or after the toxin (36). One could well ask why ricin, which internalized and transported to the TGN, cannot translocate into the cytosol in the presence of BFA. It is known that sialyltransferase is predominantly localized in TGN (14, 17, 32), but our previous study demonstrated that O-linked sugar chains of LDL receptors, but not their N-linked sugar chains, are sialylated in the presence of BFA (39). If ricin is activated through passing TGN/trans-Golgi to the cytosol, this pathway might be sensitive to BFA, as seen in sialylation of the N-linked sugar chains of LDL receptor. Alternatively, ricin is translocated and activated in an unidentified compartment of the Golgi where BFA can access it. Two kidney epithelial cell lines, MDCK and Ptk 2, are not protected against ricin by BFA (36). In our KB/BF2-2 cells, BFA could not protect the cytotoxicity of ricin. Furthermore, KB/BF2-2 cells had poor development of Golgi structures (Fig. 1), and Golgi-specific markers such as β -cop and γ -adaptin were localized in the cytoplasmic small vesicles and the Golgi structures (Figs. 2 and 3). The decreased sensitivity to the reversal effect of BFA on the intoxication of ricin in BFA-resistant cells appears to be closely correlated with the

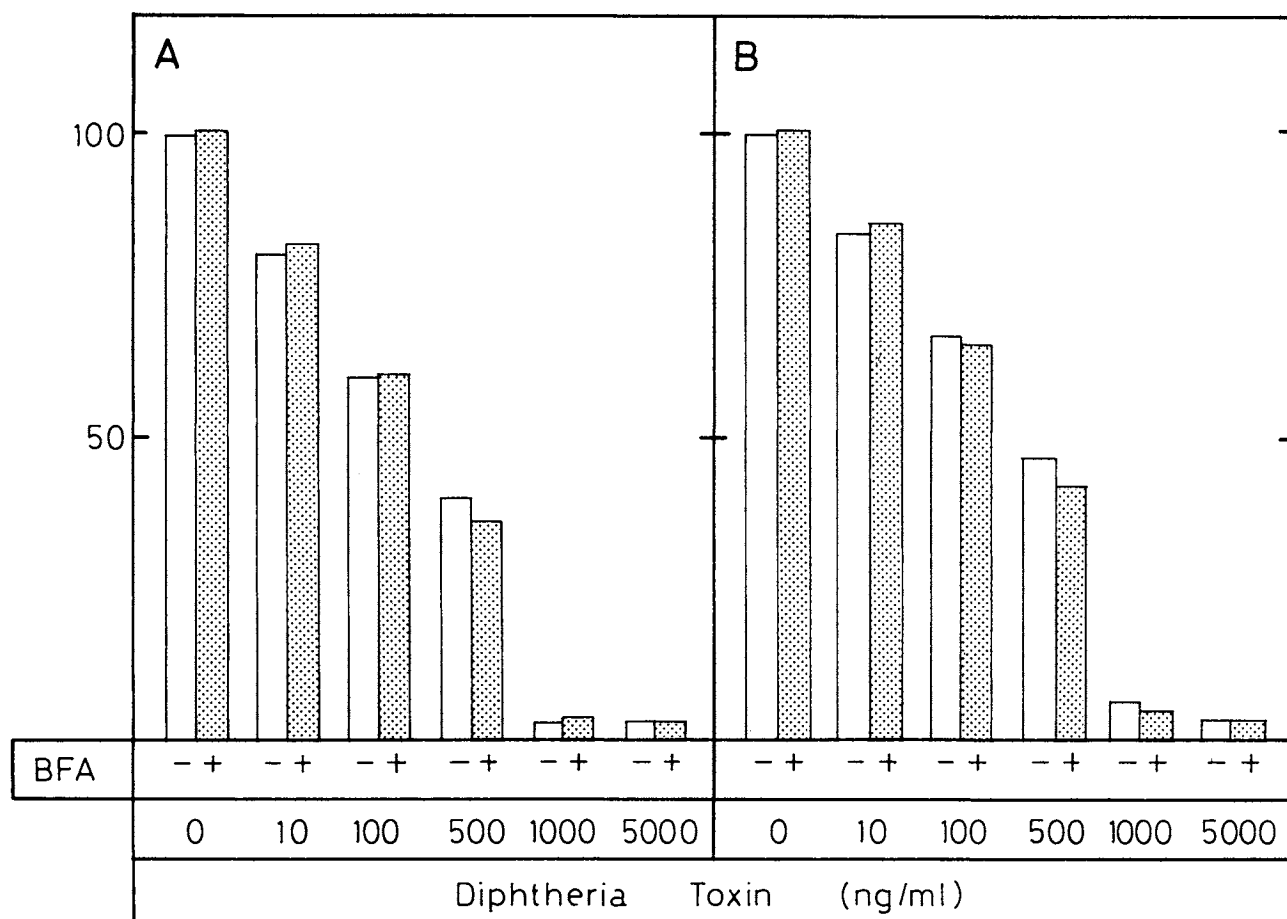


Fig. 8. The effect of BFA in the cytotoxicity of diphtheria toxin. The monolayer culture was preincubated in MEM with or without BFA at different concentrations for 30 min, followed by the addition of diphtheria toxin for 5 hr at 37°C, and then labeled with [³⁵S] protein labeling mix for 10 min in methionine-free medium for the measurement of protein synthesis.

morphological change in the Golgi apparatus or its related vesicles. If ricin is activated through passing TGN/trans-Golgi to the cytosol, the BFA-resistant mutation might confer a selective change in the intracellular transport pathway associated with TGN/trans-Golgi. Further studies are required to elucidate the question as to how BFA changes the membrane traffic of ricin and also the secretory pathway in KB and its BFA-resistant counterpart.

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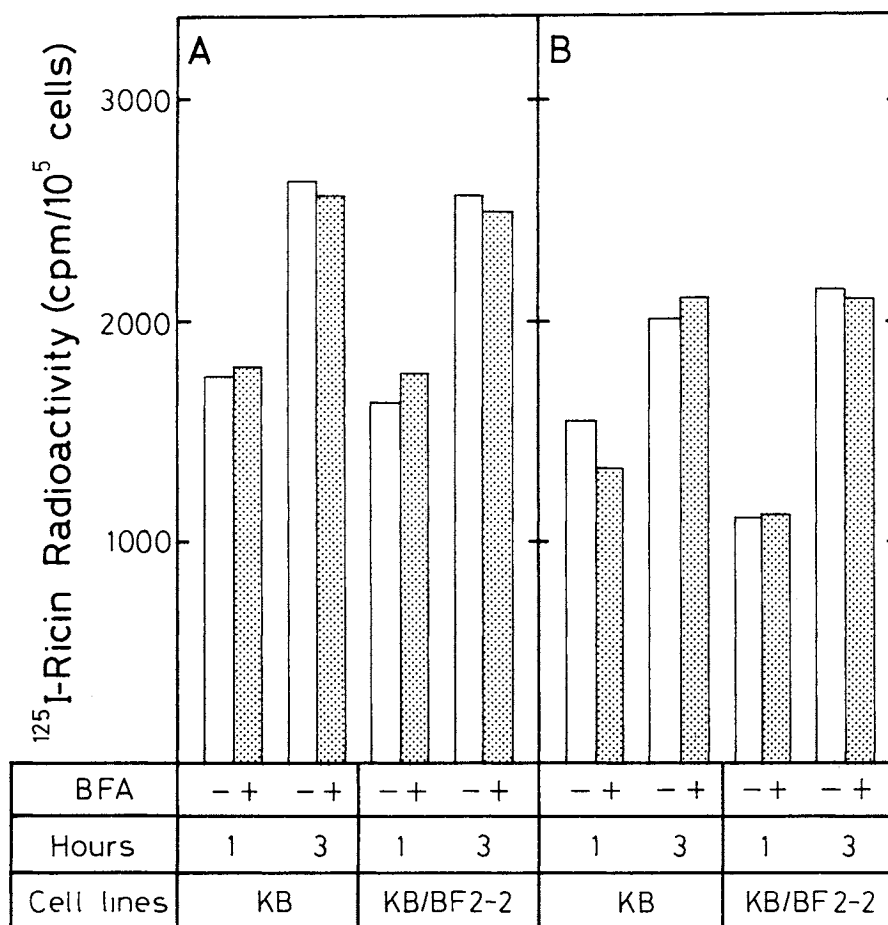


Fig. 9. The effect of BFA on the binding and internalization of ^{125}I -ricin in KB and KB/BF2-2. Cells were plated in a 24-well plate at a cell density 1×10^5 cells (KB) and 1.4×10^5 cells (KB/BF2-2) per well and incubated overnight. Cells were incubated at 4°C or 37°C with ^{125}I -ricin (1.0×10^6 cpm/well for 1 hr or 3 hr.)

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