Retinoic Acid Disrupts the Golgi Apparatus and Increases the Cytosolic Routing of Specific Protein Toxins

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Abstract. All-trans retinoic acid can specifically increase receptor mediated intoxication of ricin A chain immunotoxins more than 10,000 times, whereas fluid phase endocytosis of ricin A chain alone or ricin A chain immunotoxins was not influenced by retinoic acid. The immunotoxin activation by retinoic acid does not require RNA or protein synthesis and is not a consequence of increased receptor binding of the immunotoxin. Vitamin D_3 and thyroid hormone T_3, that activate retinoic acid receptor (RAR) cognates, forming heterodimers with retinoid X receptor (RXR), do not affect the potency of immunotoxins. Among other retinoids tested, 13-cis retinoic acid, which binds neither RAR nor RXR, also increases the potency of the ricin A chain immunotoxin. Therefore, retinoic acid receptor activation does not appear to be necessary for immunotoxin activity. Retinoic acid potentiation of immunotoxins is prevented by brefeldin A (BFA) indicating that in the presence of retinoic acid, the immunotoxin is efficiently routed through the Golgi apparatus en route to the cytoplasm. Directly examining cells with a monoclonal antibody (Mab) against mannosidase II, a Golgi apparatus marker enzyme, demonstrates that the Golgi apparatus changes upon treatment with retinoic acid from a perinuclear network to a diffuse aggregate. Within 60 min after removal of retinoic acid the cell reassembles the perinuclear Golgi network indistinguishable with that of normal control cells. C_6-NBD-ceramide, a vital stain for the Golgi apparatus, shows that retinoic acid prevents the fluorescent staining of the Golgi apparatus and eliminates fluorescence of C_6-NBD-ceramide prestained Golgi apparatus. Electron microscopy of retinoic acid–treated cells demonstrates the specific absence of any normal looking Golgi apparatus and a perinuclear vacuolar structure very similar to that seen in monensin-treated cells. This vacuolization disappears after removal of the retinoic acid and a perinuclear Golgi stacking reappears. These results indicate that retinoic acid alters intracellular routing, probably through the Golgi apparatus, potentiating immunotoxin activity independently of new gene expression. Retinoic acid appears to be a new reagent to manipulate the Golgi apparatus and intracellular traffic. As retinoic acid and immunotoxins are both in clinical trials for cancer therapy, their combined activity in vivo would be interesting to examine.

RETINOIC acid is a morphogen that defines certain cell fates during development and has the potential to treat cancer by inducing tumor cell differentiation (34, 39). Retinoic acid binds the retinoic acid receptor (RAR)[1] causing it to form heterodimers with the retinoid X receptor (RXR) and induce gene transcription (3, 19, 45). In addition to the well accepted role of retinoids in transcription activation, some retinoids may have direct effects on cell second messengers (9).

Monoclonal antibodies coupled to protein toxins, called immunotoxins, are being examined in numerous clinical trials for treatment of cancer and autoimmune diseases (36). Subsequent to cell surface binding by the monoclonal antibody, the toxic protein subunit crosses the membrane surrounding the cytosol to reach the intracellular substrate. Ricin, for example, enzymatically inactivates ribosomes inhibiting protein synthesis and causing cell death (8, 30, 31). How the hydrophilic enzyme crosses into the cytosol is unknown although endocytosis and intracellular routing to the proper compartment are required (17). The Golgi apparatus appears to be one compartment through which ricin must

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1. *Abbreviations used in this paper*: BFA, brefeldin A; C_6-NBD-ceramide, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminohexanoyl-D-*erythro*-sphingosine; DT, diphtheria toxin; PE, *Pseudomonas* exotoxin; RAR, retinoic acid receptor; rRA, recombinant ricin A chain; RXR, retinoid X receptor; Tfn, transferrin.

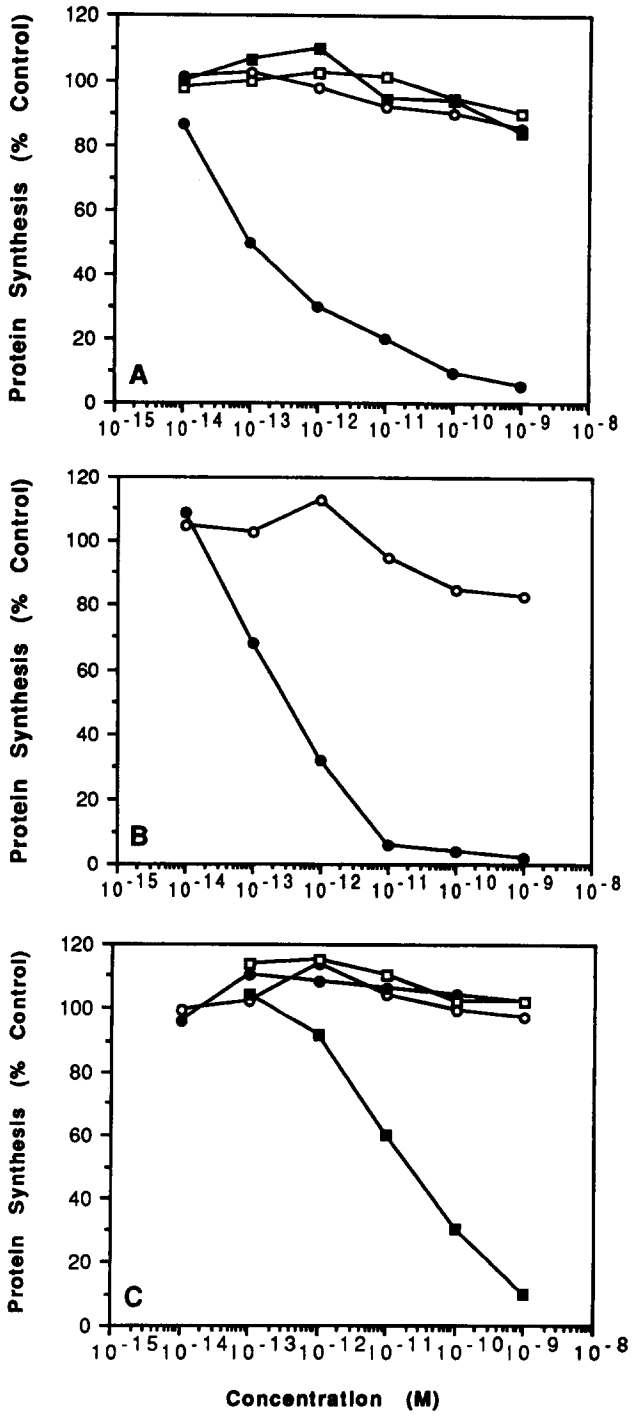

Figure 1. Potentiation of 454A12-rRA, Tfn-rRA, and rRA cytotoxicity by all-trans retinoic acid in U251, K562, and 9L cells. (*A*) U251 cells growing in 96-well plates were incubated with increasing concentrations of 454A12-rRA (*circles*) or rRA (*squares*) in the presence (●, ■) and in the absence (○, □) of 10 μM retinoic acid in leucine-free RPMI1640 medium; (*B*) K562 cells growing in 96-well plates were incubated with increasing concentrations of 454A12-rRA in the presence (●) or absence (○) of 10 μM retinoic acid; (*C*) 9L cells were incubated with increasing concentrations of 454A12-rRA (*circles*) or Tfn-rRA (*squares*) in the presence (●, ■) or absence (○, □) of 10 μM retinoic acid. After 3-h incubation, ^{14}C-leucine was added for another h. Cells were harvested and counted as described in Materials and Methods.

pass en route to the cytosol. Native ricin efficiently routes through the Golgi apparatus (12, 16, 38, 44) to the cytosol due to galactose-binding sites on the ricin B chain (17). When the ricin B chain is removed and enzymatically active A chain is linked to monoclonal antibodies reactive with cell surface molecules such as the transferrin receptor, much less effi- cient entry into the cytosol ensues (43). Although the immunotoxin is rapidly endocytosed via the transferrin receptor, it does not traffic such that the enzymatically active A chain rapidly reaches the cytosol. In addition to ricin B chain, some drugs that cause alterations in the Golgi apparatus such as the ionophore, monensin, and lysosomotropic amines cause a large increase in cell sensitivity to the immunotoxins (2). Chloroquine (21), a lysosomotropic agent, and the ricin B chain (14), have been tested in man for their ability to improve the anti-cancer activity of immunotoxins.

We find that retinoic acid alters the Golgi apparatus morphology and causes a tremendous potentiation of immunotoxin toxicity. This activity of retinoic acid is not the result of transcription activation but appears to be a direct effect of retinoids on the Golgi apparatus and intracellular traffic.

Materials and Methods

Materials

All-trans retinoic acid was purchased from Sigma Chem. Co. (St. Louis, MO) and Calbiochem Corp. (La Jolla, CA); brefeldin A (BFA), 13-cis retinoic acid, all-trans retinol, 13-cis retinol, all-trans retinal, 9-cis retinal, and 13-cis retinal all from Sigma Chem. Co.; *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminohexanoyl-D-*erythro*-sphingosine (C_6-NBD-ceramide) and fluorescein labeled goat anti-mouse IgG conjugate from Molecular Probe, Inc. (Eugene, OR); 53FC3 Mab against mannosidase II was a generous gift from Dr. Lippincott-Schwartz (National Institutes of Health); 1,25-dihydroxy vitamin D_3 and L-3,3',5-triiodothyronine(T_3) were from Calbiochem Corp.; 454A12-rRA was prepared as described (11); diphtheria toxin and Pseudomonas exotoxin were obtained from List Biological Co.; transferrin-CRM107 was prepared as described by Johnson et al. (18) and transferrin-PE was a generous gift from Dr. Aslak Godal (Hafslund Nycomed); 260F9-rRA (11) and M6-rRA (13) were prepared as described; and transferrin-rRA was a generous gift from Dr. Jerry Fulton (Inland Laboratories, Inc.).

Cell Lines

U251 (human glioma) cells and MCF-7 (human breast cancer) cells, and 9L (rat glioma) were grown in DMEM containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 10 μg/ml gentamycin. K562 (human erythroleukemia) cells were grown in RPMI1640 containing 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 10 μg/ml gentamycin; L_2C cells, a spontaneous transplantable B cell leukemia, were maintained by serial passage in inbred strain 2 guinea pigs as reported previously (13). L_2C cells were harvested from the peripheral blood and purified in Lymphocyte Separation Medium (Organon Teknika, Durham, NC), washed three times with HBSS, and resuspended in leucine-free RPMI1640 for cytotoxicity assay.

Protein Synthesis Assay

Protein synthesis inhibition by DT, PE, ricin, and immunotoxins was determined as described previously (41). Briefly, cells were plated at concentrations of 2×10^5 cells/ml in 96-well microtiter plates overnight in DMEM complete medium. Retinoic acid (15 mM in DMSO) and BFA (10 mg/ml in ethanol) stock solutions were diluted into leucine-free RPMI1640 medium without FCS to the appropriate concentrations. The same amount of DMSO and/or ethanol were added in the control solutions. After removing the complete DMEM medium, cells were incubated in the above leucine-free RPMI 1640 medium containing increasing concentrations of protein toxins with or without retinoic acid or other retinoids and/or BFA

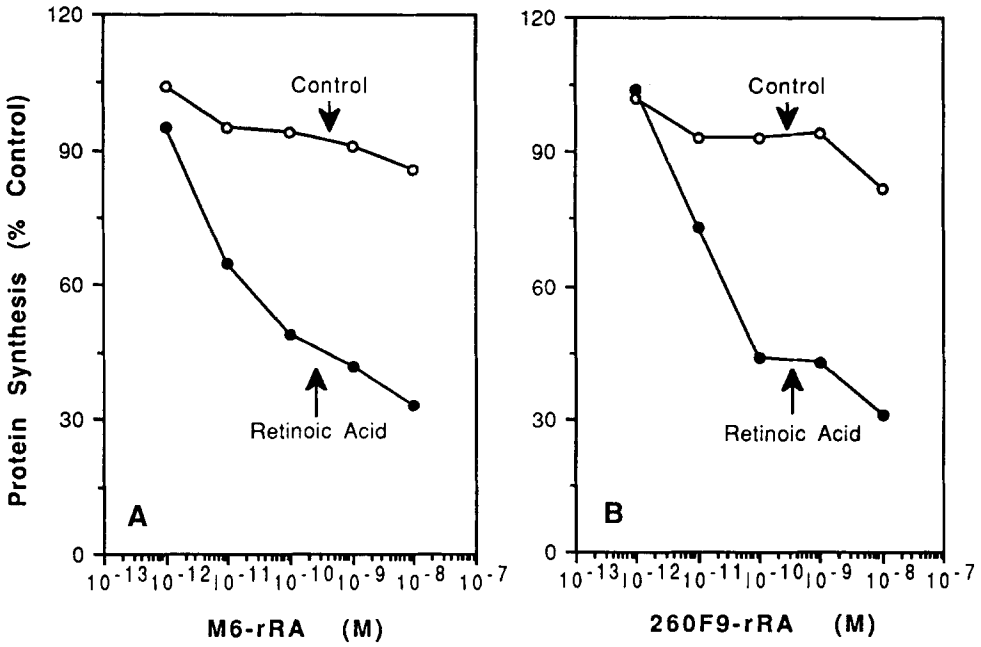

Figure 2. Potentiation of 260F9-rRA and M6-rRA cytotoxicity by all-trans retinoic acid in MCF-7 and L_2C cells. MCF-7 cells (*A*) or L_2C cells (*B*) growing in 96-well plates were incubated with increasing concentrations of 260F9-rRA with or without 10 μM retinoic acid as indicated. After 3 h, the medium was removed and the cells were pulsed with ^{14}C-leucine for another h. Cells were harvested and counted as described in Materials and Methods.

for 3 h followed by a 1-h pulse with 0.1 μCi ^{14}C-leucine. Cells were harvested onto glass fiber filters using a PHD cell harvester, washed with water, dried with ethanol, and counted. The results were expressed as the percentage of ^{14}C-leucine incorporation in mock-treated control cells.

Vital Staining of the Golgi Apparatus

C_6-NBD-ceramide was used to stain the Golgi apparatus in living cells (28). Cells were treated with retinoic acid either before C_6-NBD-ceramide staining or after staining. In the case of C_6-NBD-ceramide staining after retinoic acid exposure, cells plated on coverslips were incubated in leucine-free RPMI1640 without FCS containing 10 μM retinoic acid or media containing an equivalent amount of DMSO in control cells. After 2 h, the above medium was removed and fresh medium containing 5 μM C_6-NBD-ceramide was added and incubated at 2°C for 1 h followed by an additional incubation at 37°C for 30 min. After staining, coverslips with labeled cells were mounted for fluorescence microscopy. In the case of C_6-NBD-ceramide staining before retinoic acid exposure, cells were first incubated with 5 μM C_6-NBD-ceramide at 2°C for 60 min, the medium was removed and washed twice followed by an additional incubation at 37°C for 2 h in the presence or absence of 10 μM retinoic acid. Cells thus treated were mounted for fluorescence microscopy.

Immunostaining of the Golgi Apparatus for Light Microscopy

9L cells were cultured on coverslips in RPMI1640 medium with or without 10 μM retinoic acid for 3 h, and then fixed for 10 min in 2% formaldehyde in PBS at 25°C, washed in PBS containing 10% FCS. Cells were incubated with monoclonal antibody to mannosidase II in PBS containing 10% FCS and 0.2% saponin for 1 h, washed with PBS + 10% serum. Cells were then incubated with fluorescein-labeled goat anti-mouse IgG in PBS containing

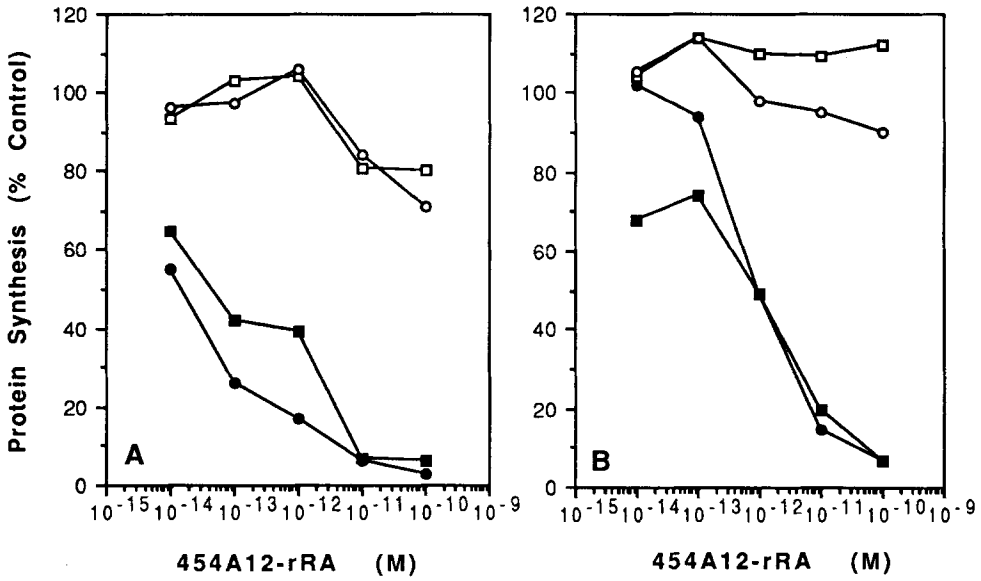

Figure 3. The effect of cycloheximide and actinomycin D on all-trans retinoic acid potentiated cytotoxicity of 454A12-rRA to U251 cells. Cells were preincubated for 30 min with (*squares*) or without (*circles*) 1.2 μg/ml cycloheximide (*A*), or 3 h with (*squares*) or without (*circles*) 5.0 μg/ml actinomycin D (*B*), and then further incubated with increasing concentrations of 454A12-rRA in the presence (●, ■) or absence (○, □) of 10 μM retinoic acid. After 3 h, the medium was removed and the cells were washed with fresh medium three times before pulsing with ^{14}C-leucine. Protein synthesis was measured as described in Fig. 1.

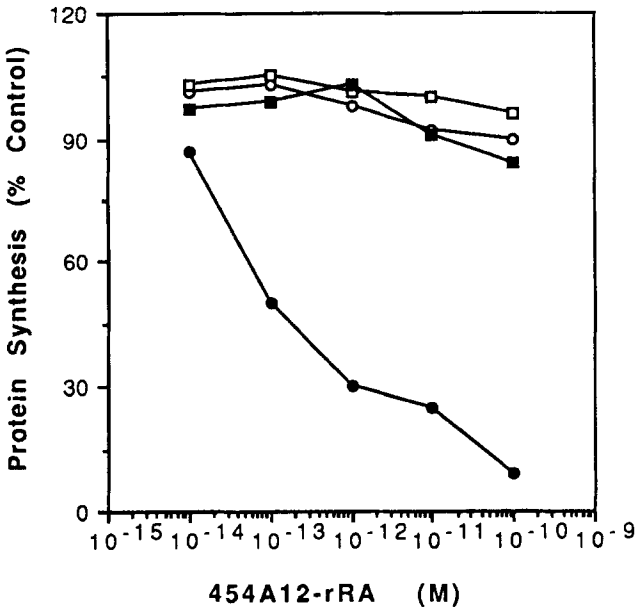

Figure 4. Brefeldin A blocks all-trans retinoic acid potentiated cytotoxicity of 454A12-rRA immunotoxin. U251 cells were incubated with increasing concentrations of 454A12-rRA at 37°C with (●, ■) or without (○, □) 10 μM retinoic acid in the presence (*squares*) or absence (*circles*) of 10 μg/ml brefeldin A. After 3 h, protein synthesis was assayed as described for Fig. 1.

10% serum and 0.2% saponin for 1 h, washed three times with PBS/serum, and then with PBS alone. The coverslips were mounted in 75% glycerol.

Electron Microscopy

Cells were grown in 4-well chamber slides overnight in DMEM complete medium, and then cells were incubated in leucine-free RPMI1640 medium containing 10 μM retinoic acid or an equivalent amount of DMSO in control culture. After 3 h, cells were washed twice and fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2 for 60 min at room temperature, cells then were further processed for electron microscopy.

Results

All-trans Retinoic Acid Potentiates Receptor Mediated Cytotoxicity of Immunotoxins

454A12-rRA, an immunotoxin made by a disulfide linkage between a monoclonal antibody against the human transferrin receptor (454A12) and recombinant ricin A chain (rRA), was incubated with the human glioma cell line, U251. After 3 h, there was no inhibition of protein synthesis up to 10^{-9} M 454A12-rRA. In the presence of 10 μM retinoic acid, cell protein synthesis was inhibited 50% at 10^{-13} M, a concentration more than 10,000 times lower than that which inhibited protein synthesis in the absence of retinoic acid (Fig. 1 *A*). AT 10^{-11} M immunotoxin, protein synthesis was only 20% of control after only 3 h. Human erythroleukemia cells, K562, were also more than 10,000 times more sensitive to 454A12-rRA in the presence of retinoic acid than in the absence of retinoic acid (Fig. 1 *B*). However, recombinant ricin A chain by itself was not detectably potentiated by retinoic acid (Fig. 1 *A*). 454A12-rRA was not detectably toxic to a non-target cell line (9L glioma) even in the presence of 10 μM retinoic acid (Fig. 1 *C*). However, transferrin-rRA, which can bind rat 9L cells, was potentiated at least 1,000-fold by 10 μM retinoic acid (Fig. 1 *C*).

Two other immunotoxins, 260F9-rRA, against a human breast cancer antigen (11) and M6-rRA against a B cell surface idiotype antigen (13), were examined for potentiation of toxicity by retinoic acid. Assayed against their respective target cell lines, MCF-7 and L_2C, both immunotoxins were potentiated at least several orders of magnitude by 10 μM retinoic acid (Fig. 2).

Thus, of three cell surface receptors examined, all deliver rRA to the cytosol much more efficiently in the presence of retinoic acid than in the absence of retinoic acid. In contrast to the dramatic effect on receptor-mediated toxicity of ricin immunotoxins by retinoic acid, no effect on fluid phase cytotoxicity of rRA or immunotoxin was seen in the presence of retinoic acid.

Comparison of the Effect of All-trans Retinoic Acid, Other Retinoids, Vitamin D_3 and Triiodothyronine (T_3) on Immunotoxin Potency

All-trans retinoic acid binds the RAR causing it to heterodimerize with the RXR and activate gene transcription (3, 19, 29, 45). 9-cis retinoic acid interacts with the RXR and also stimulates dimer formation and transcription activation (1, 15, 23). All other cis retinoic acids do not bind either RAR or RXR. We examined whether or not 13-cis retinoic acid, which binds to neither RAR nor RXR (1), would affect immunotoxin activity. Our results indicate that 10 μM 13-cis retinoic acid potentiates immunotoxins similarly to all-trans retinoic acid (data not shown). Among other retinoids tested, 10 μM all-trans retinol shows potentiation similar to that of all-trans retinoic acid, whereas 13-cis retinol, all trans-retinal, 13-cis retinal, and 9-cis retinal do not seem to increase 454A12-rRA immunotoxin potency at 10 μM concentrations (data not shown). All-trans retinol has been recently demonstrated to be a ligand of RAR, whereas all-trans retinal does not bind to RAR (35). Whether the cis-forms of retinol or retinal bind RAR or RXR receptor is not known. Thus there is some specificity among different retinoids in potentiating the cytotoxicity of immunotoxins, however retinoid receptor binding and the potentiation of the immunotoxin do not correlate. The thyroid hormone (T_3) receptor and the vitamin D_3 receptor are homologous with RAR and also form heterodimers with RXR to induce transcription activation (19, 45). Up to 1 μM thyroid hormone (T_3) or 1 μM 1,25-dihydroxy, vitamin D_3 had no affect on the sensitivity of U251 to 454A12-rRA immunotoxin (data not shown).

All-trans Retinoic Acid Potentiation of Immunotoxins Is Independent of Gene Expression

To test whether or not new gene products induced by retinoic acid result in immunotoxin sensitization, cells were incubated with cycloheximide (Fig. 3 *A*) or actinomycin D (Fig. 3 *B*) before exposure to retinoic acid and 454A12-rRA. Fig. 3 shows that neither actinomycin D nor cycloheximide prevented the potentiation of 454A12-rRA cytotoxicity by retinoic acid. Thus the well established transcription activation activity of retinoic acid does not appear to be the mechanism by which retinoic acid increases cell sensitivity to im-

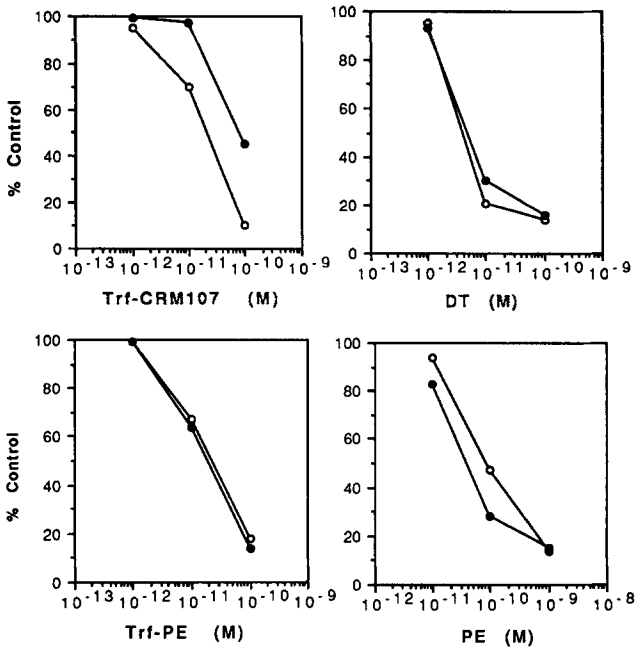

Figure 5. Effects of all-trans retinoic acid on the cytotoxicity of DT, PE, tfn-CRM107, and tfn-PE in U251 cells. U251 cells were incubated with increasing concentrations of DT, PE, tfn-CRM107, and tfn-PE as indicated in the figure with (●) or without (○) 10 μM all-trans retinoic acid for 3 h. Protein synthesis was assayed as described for Fig. 1.

munotoxins. This conclusion is also consistent with the rapid time course of the activation by retinoic acid. After only 3 h, the immunotoxin is 10,000 times more toxic to cells whereas many of the effects of retinoic acid on cellular differentiation occur days after exposure to retinoic acid. Apparently retinoic acid has a direct effect on cells that causes the increased sensitivity to immunotoxins.

All-trans Retinoic Acid Potentiates Immunotoxins at Steps Subsequent to Cell Surface Receptor Binding

Immunotoxins may be potentiated by increasing the amount of immunotoxin bound to cell surface receptors or by increasing the delivery of surface bound immunotoxin to the cytosol compartment. To examine if retinoic acid increases the binding of immunotoxins to target cells, cells were incubated with 454A12-rRA for 2 h at 4°C, and then washed to remove unbound immunotoxin. The cells were divided in half and one half was incubated in the presence and one half was incubated in the absence of retinoic acid for 3 h at 37°C, and then the cells were pulsed with ^{14}C-leucine and harvested. The results show that the potentiation of 454A12-rRA occurs even in cells washed before adding the retinoic acid (data not shown) indicating that the effect of retinoic acid is not a consequence of increased receptor binding. This leaves intracellular routing and passage into the cytosol as the likely effect retinoic acid has upon immunotoxin potency.

Brefeldin A Blocks the All-trans Retinoic Acid Potentiation of Immunotoxin Toxicity

The Golgi apparatus has been implicated in the efficient routing of native ricin to the cytosol through functions of the ricin B chain (17). To examine whether or not retinoic acid may affect routing of immunotoxins through the Golgi apparatus, the effect of BFA on the retinoic acid potentiation of 454A12-rRA was examined. BFA, by inhibiting vesicular transport from the ER to the Golgi, results in collapse of the *cis*-Golgi apparatus blocking the retrograde vesicular transport of vesicles from the Golgi to the ER (6, 24). BFA was incubated with U251 cells in the presence of 454A12-rRA and retinoic acid. Fig. 4 shows that BFA completely blocks the potentiation of toxicity by retinoic acid. This indicates that 454A12-rRA routes through a BFA sensitive compartment, possibly the Golgi apparatus or the ER, in the presence of retinoic acid.

The Effect of All-trans Retinoic Acid on the Potency of Other Protein Toxins and Immunotoxins

In contrast to rRA chain immunotoxins, which are potentiated by ionophores that disrupt the Golgi apparatus, diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PE) and their respective immunotoxins, are blocked by monensin, a carboxylic ionophore. We examined the effect of retinoic acid on the toxicity of PE, DT, and transferrin coupled to PE and transferrin coupled to a diphtheria toxin mutant, CRM107. Fig. 5 shows that, in contrast to 454A12-rRA, DT, PE, and transferrin-CRM107 (tfn-CRM107) and transferrin-PE (tfn-PE) are not potentiated by retinoic acid. DT and tfn-CRM107 are actually inhibited to a small extent by retinoic acid. These results are consistent with the model that retinoic acid alters the routing of immunotoxins through the Golgi apparatus with some degree of selectivity. The effect of retinoic acid differs markedly from that of monensin, however. Retinoic acid has little effect on DT and PE whereas monensin blocks DT over 1,000 times.

All-trans Retinoic Acid Alters the Golgi Apparatus Morphology Visualized by Immunostaining with an anti-Mannosidase II Monoclonal Antibody and by Vital Staining with C_6-NBD-Ceramide

Immunostaining of the Golgi apparatus with a monoclonal antibody against the Golgi marker, mannosidase II, shows that retinoic acid causes a marked perturbation in the Golgi apparatus (Fig. 6 *b*). In control 9L cells the Golgi apparatus has a typical perinuclear network appearance (Fig. 6 *a*). After treatment of 9L cells with 10 μM retinoic acid the Golgi apparatus becomes clumped and diffuse with no perinuclear distribution. Upon removal of the retinoic acid the typical perinuclear distribution of the Golgi apparatus reassembles by 60 min (Fig. 6 *c*). Thus retinoic acid causes a reversible dissolution of the perinuclear Golgi network when observed with an anti-mannosidase II antibody.

C_6-NBD-ceramide, a fluorescent dye, is another powerful tool to study the structure and function of the Golgi apparatus in living cells (28). We examined C_6-NBD-ceramide staining of the Golgi apparatus in U251 cells in the presence and absence of retinoic acid (Fig. 7). The Golgi apparatus in control cells (*a*) shows a perinuclear appearance as previously reported (28). In cells treated with 10 μM retinoic acid for 2 h there is a dramatic inhibition of Golgi fluorescence (*b*). If the Golgi apparatus is stained first with C_6-NBD-ceramide, and then incubated with retinoic acid (Fig. 7 *d*) or without (Fig. 7 *c*) for 2 h, cells show a dramatic decrease in fluorescence labeling. Thus retinoic acid disrupts the nor-

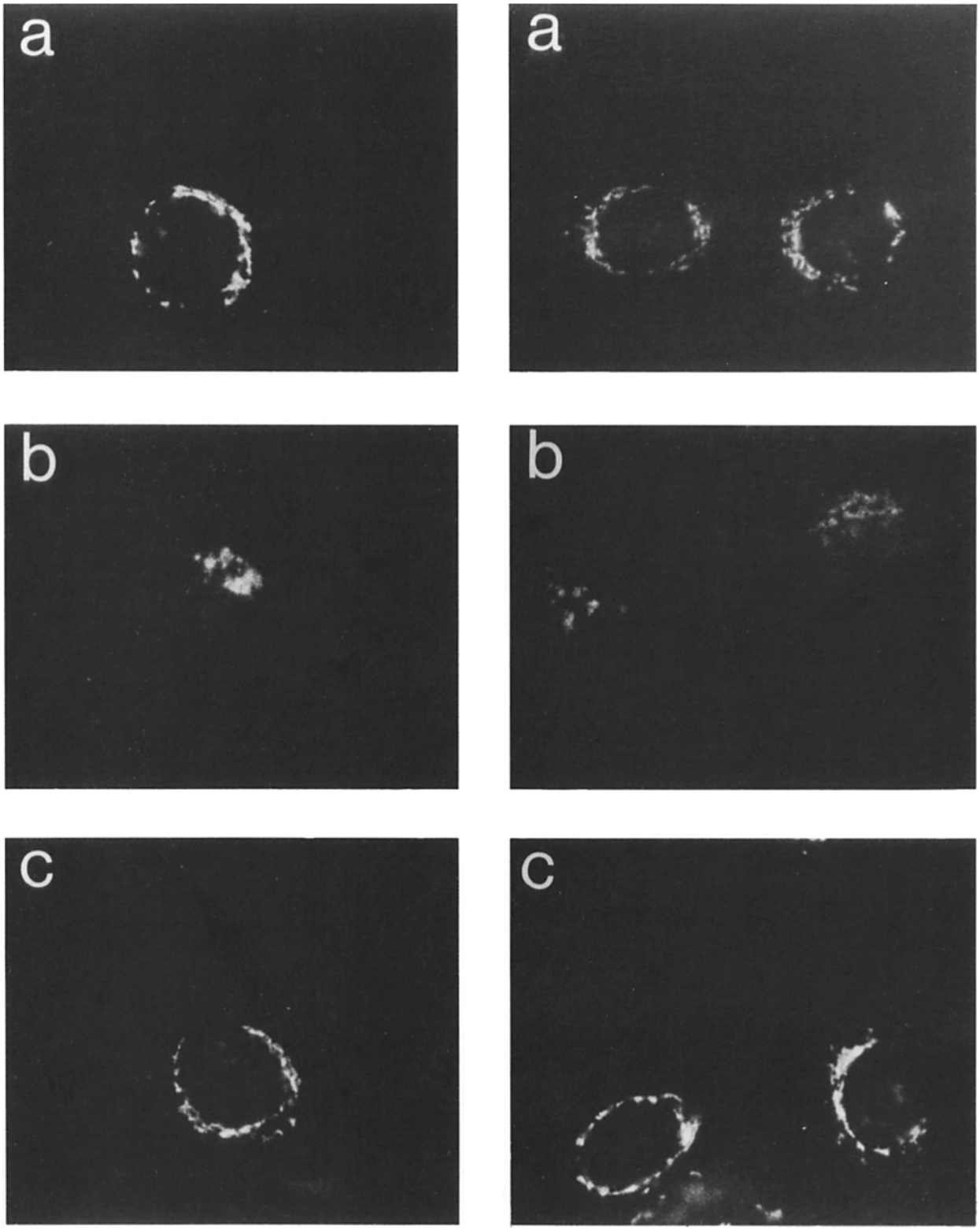

Figure 6. All-trans retinoic acid treatment causes redistribution of Golgi apparatus stained with mAb against mannosidase II. 9L cells grown on coverslips in leucine-free RPMI1640 medium were treated with (*b* and *c*) or without (*a*) 10 μM retinoic acid. After 2 h, the medium was removed and the cells were washed twice. Cells were then either fixed in 2% formaldehyde (*a* and *b*) or incubated further in DMEM complete medium for 60 min (*c*), and then fixed in 2% formaldehyde. Cells were incubated with mAb against mannosidase II in PBS containing 10% FCS and 0.2% saponin for 60 min, and washed. Cells were then incubated with fluorescein-labeled goat anti-mouse IgG in PBS containing 10% FCS and 0.2% saponin for another 60 min. Cells were washed and mounted in 75% glycerol.

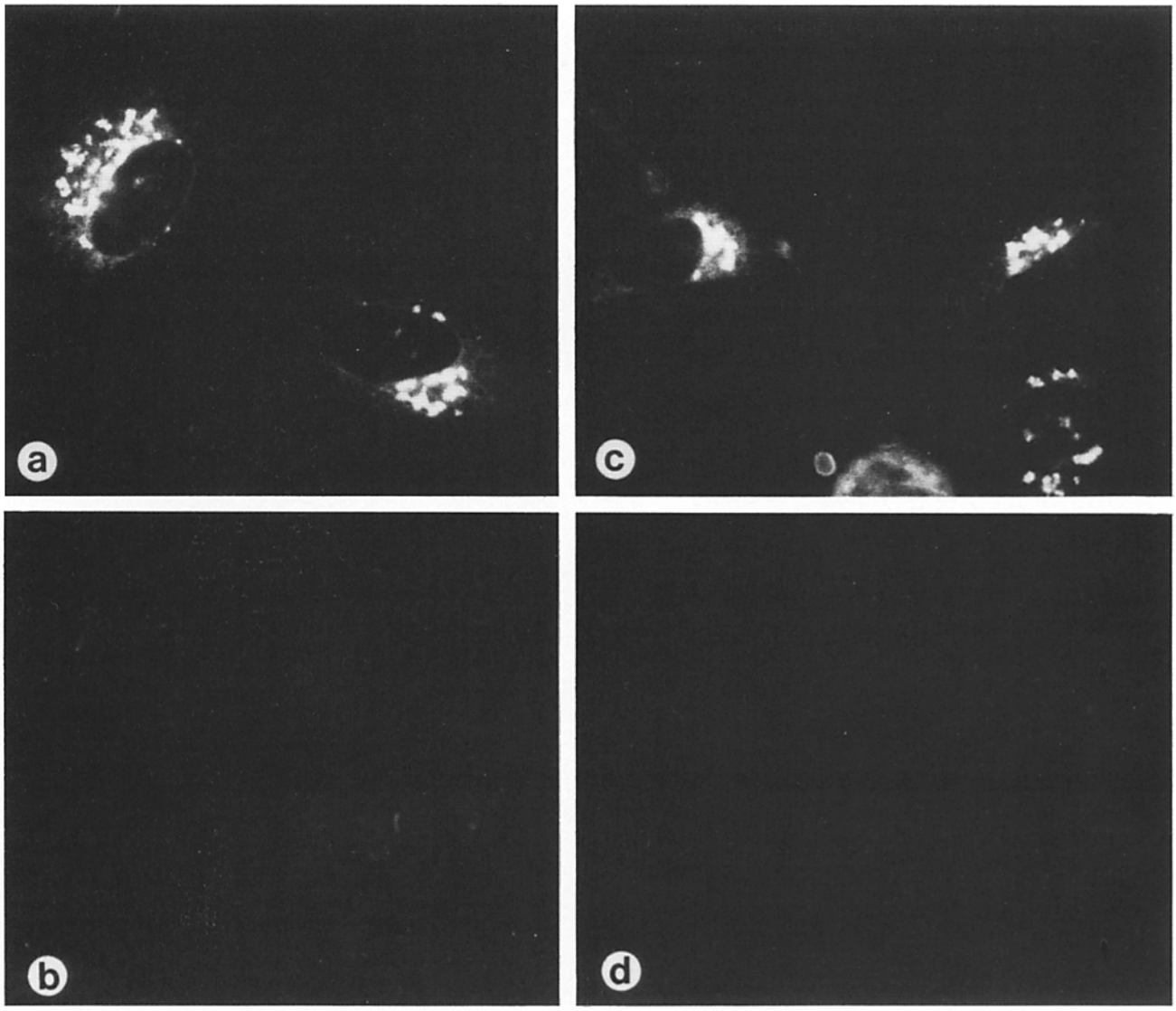

Figure 7. All-trans retinoic acid treatment either prevents or disrupts the specific vital staining of the Golgi apparatus with C_6-NBD-ceramide. U251 cells grown on glass coverslips in leucine-free RPMI1640 medium were treated with (*b*) or without (*a*) 10 μM retinoic acid. After 2 h, the medium was removed and the cells were washed twice. Cells were then incubated in the same medium with 5 μM fluorescently labeled C_6-NBD-ceramide in the absence of all-trans retinoic acid at 2°C for 1 h, washed twice, and incubated for 30 min at 37°C. Cells were carefully mounted on glass slides and photographed under a fluorescent microscope. In *c* and *d*, cells were first stained with 5 μM C_6-NBD-ceramide, washed twice, and further incubated at 37°C in the presence (*d*) or absence (*c*) of 10 μM all-trans retinoic acid for 2 h without C_6-NBD-ceramide. Cells thus treated were mounted and photographed as described above.

mal Golgi apparatus when examined with the vital dye, C_6-NBD-ceramide.

All-trans Retinoic Acid Treatment Causes a Reversible Disappearance of the Golgi Apparatus Observed by Electron Microscopy

To further examine the status of the Golgi apparatus in retinoic acid–treated cells we used electron microscopy. Retinoic acid treatment of U251 cells correlated with a complete disappearance of normal Golgi cisterna and the appearance of large perinuclear vacuoles (Fig. 8). Retinoic acid caused a similar disappearance of the Golgi apparatus and vacuolization in 9L cells (Fig. 9 *B*). Upon removal of the retinoic acid, normal Golgi stacking reappeared and the swollen vacuoles disappeared within 60 min. (Fig. 9 *C*). These results indicate that the vacuolized structures may at least partially be composed of dilated Golgi apparatus. Monensin causes massive dilation of the Golgi apparatus (22) similar to the appearance of retinoic acid–treated cells and also causes potentiation of rRA immunotoxins. The effect of retinoic acid on the Golgi may relate to the mechanism of immunotoxin potentiation.

Discussion

All-trans retinoic acid selectively increases the potency of certain immunotoxins. rRA containing immunotoxins, via three different receptors, on several different cell lines, are potentiated by retinoic acid whereas immunotoxins with diphtheria toxin and *Pseudomonas* toxin are not. Thus the

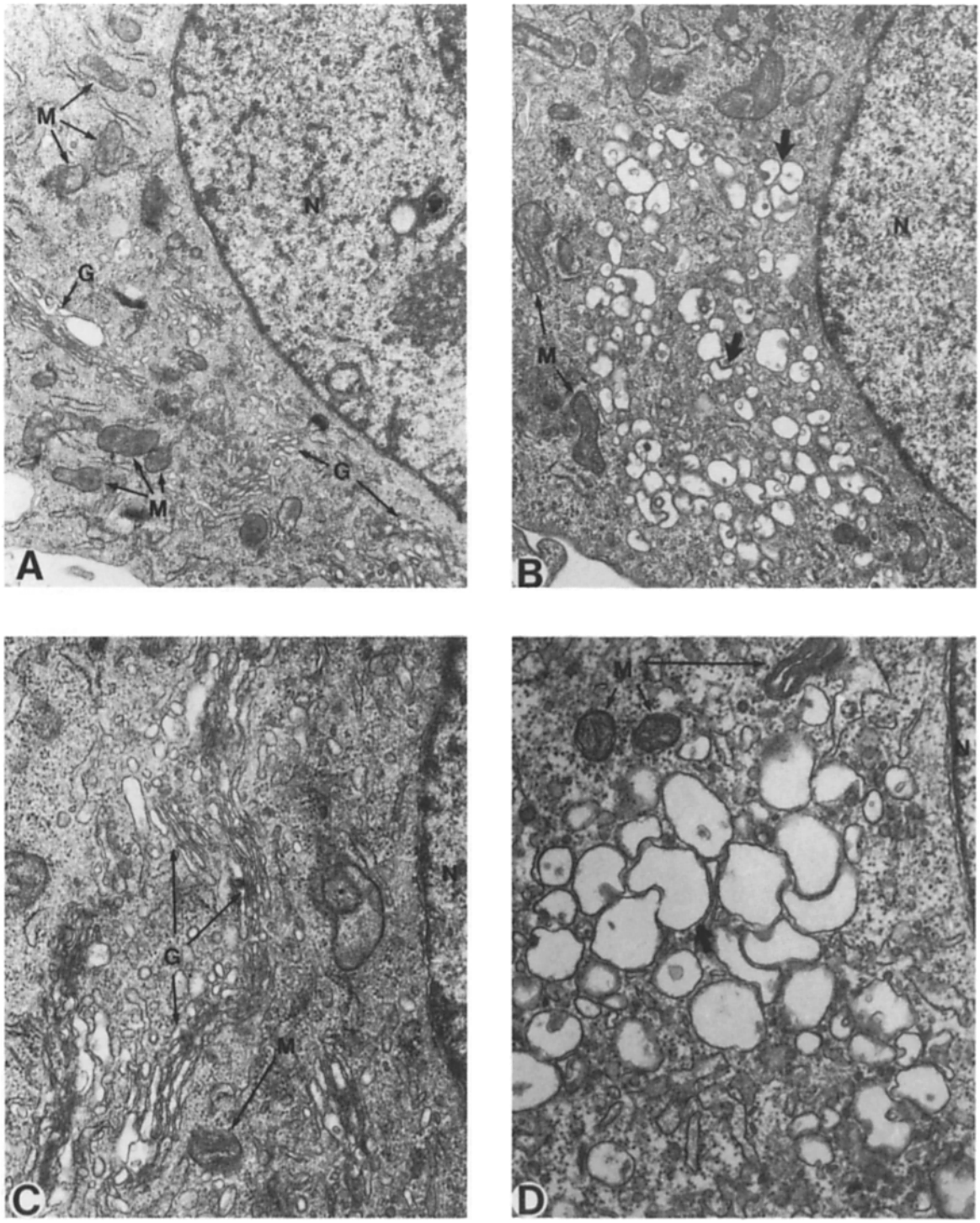

Figure 8. All-trans retinoic acid treatment causes disappearance of the Golgi apparatus and appearance of perinuclear vacuolization. Human U251 cells grown in 4-well chamber slides were incubated in leucine-free RPMI1640 medium with or without 10 μM retinoic acid, after 3 h, cells were washed twice and fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2 for 60 min at room temperature, cells then were further processed for electron microscopy (*M*, mitochondrium; *G*, Golgi apparatus; *N*, nucleus; *thick arrow*, vacuolized structures). *A*, control cell (× 10,000); *B*, retinoic acid-treated cells (× 10,000); *C*, control cells (× 20,000); *D*, retinoic acid-treated cells (× 20,000).

effect seems to be independent of the cell surface receptor yet specific to the toxin. Only receptor mediated pathways of intoxication appear to be affected by retinoic acid. rRA alone, and non-binding immunotoxins are not detectably potentiated by retinoic acid.

The sensitization of cells to immunotoxins by retinoic acid is completely blocked by BFA. BFA blocks the vesicular transport from the ER to the *cis*-Golgi apparatus causing a collapse of the *cis*-Golgi and a termination of the retrograde vesicular transport from the *cis*-Golgi back to the ER (24,

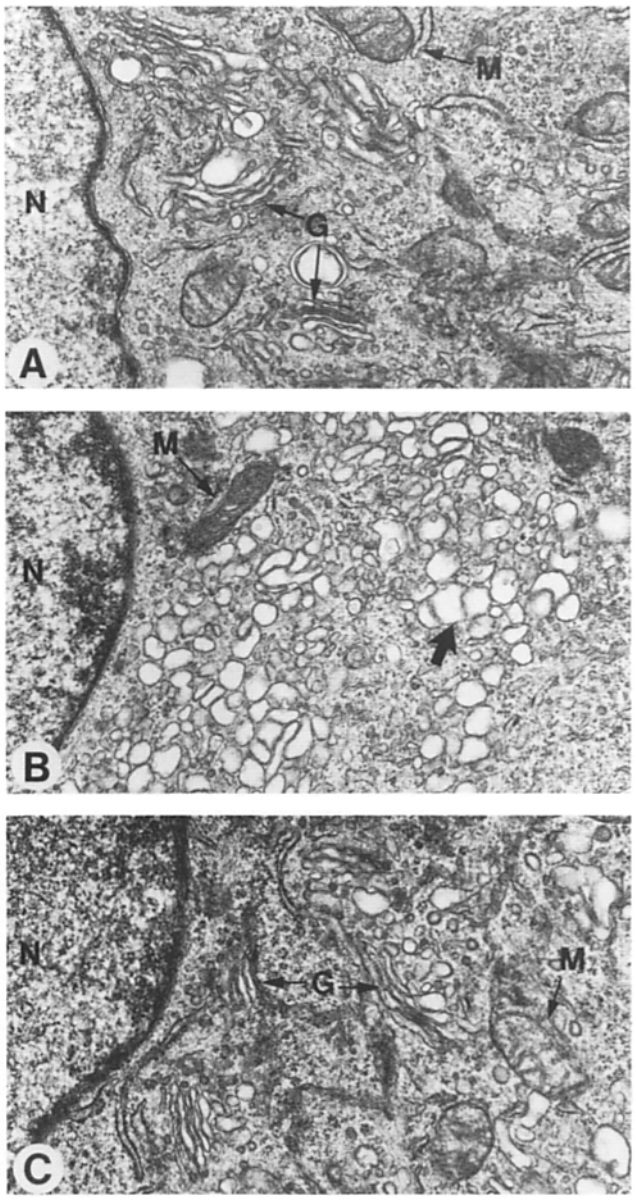

Figure 9. All-trans retinoic acid caused vacuolization disappears after removing the drug. Rat 9L cells cultured in 4-well chamber slides were incubated with (*B* and *C*) or without (*A*) 10 μM retinoic acid. After 3 h, cells were washed twice and either fixed (*A* and *B*) with glutaraldehyde as described in Fig. 8 or further incubated in complete DMEM medium for 60 min (*C*) before fixing. Cells were then processed for electron microscopy (*G*, Golgi apparatus; *M*, mitochondrium; *N*, nucleus; *thick arrow*, vacuolized structures; × 12,000).

25). The block of the retinoic acid potentiation by BFA suggests that retinoic acid stimulates transport of the immunotoxins through the *cis*-Golgi, possibly to the ER en route to the cytosol. BFA has also been shown to affect endosomes, lysosomes, and the *trans*-Golgi network (26, 33), and we cannot ascertain at this time which of the cellular effects of BFA cause the blockage of immunotoxin potentiation by retinoic acid.

Retinoic acid seems to be having an effect on cell sensitivity to toxins somewhat like that of lysosomotropic agents such as monensin. As monensin disrupts intracellular traffic and causes morphologic alterations in the Golgi apparatus (22), we examined retinoic acid–treated cells by immunostaining with an anti-mannosidase II Mab, with the vital Golgi stain, C_6-NBD-ceramide, and by EM. Examination of the Golgi apparatus with a lipid that specifically stains the Golgi in living cells (28), fluorescent C_6-NBD-ceramide, shows that retinoic acid treatment caused a dramatic disappearance of the Golgi apparatus staining. This is not because of decreased C_6-NBD-ceramide uptake since treatment with retinoic acid following C_6-NBD-ceramide staining also causes a dramatic decrease in fluorescence intensity of the Golgi apparatus. Immunostaining of mannosidase II demonstrates the disruption of the perinuclearly located Golgi apparatus by retinoic acid treatment, and this disruption is readily reversible upon removal of the retinoic acid. Electron microscopy also shows a complete disappearance of the Golgi stacking, and the appearance of a perinuclear vacuolization upon exposure of cells to retinoic acid. This vacuolization is reversed and normal Golgi apparatus reappears after removal of retinoic acid indicating that the vacuolized structures are derived at least partially from the swollen Golgi apparatus. The vacuolization seen by EM caused by retinoic acid in two different cell lines (U251, 9L) resembles that caused by monensin (22). However, the results of C_6-NBD-ceramide staining of retinoic acid–treated cells are opposite to those seen in monensin-treated cells. With monensin, C_6-NBD-ceramide staining of the Golgi is more intense and punctate (27) whereas retinoic acid eliminates C_6-NBD-ceramide staining. BFA causes diminution of the Golgi apparatus by EM and with NBD-ceramide in appropriate cell types (5, 20). Thus, the effects of retinoic acid on the Golgi presents a new pattern relative to that seen with previously described drugs.

The effects of retinoic acid on ricin, diphtheria toxin, and *Pseudomonas* exotoxin are consistent with this model that retinoic acid alters the endocytotic routing in cells. Native ricin, modeccin, and abrin toxicity are blocked a small amount by 10 μM retinoic acid in HeLa cells and Vero cells (37). Retinoic acid also protects U251 cells from ricin toxicity (data not shown). These results and the potentiation of rRA immunotoxins by retinoic acid are similar to the effects seen with lysosomotropic amines and ionophores. Native ricin contains a B chain that is thought to use a galactose-binding function, intracellularly, to route the toxin through the Golgi apparatus to reach the cytosol (17). The galactose-binding activity may allow ricin, in the *trans*-Golgi, to bind to KDEL receptor-like glycoproteins that cycle to and from the *cis*-Golgi and the ER (32, 40). Immunotoxins that lack a B chain are much less potent, apparently due to a deficiency in intracellular routing. rRA immunotoxins may recycle through the *trans*-Golgi back to the cell surface repeatedly in the absence of a B chain and disruption of the Golgi with monensin or retinoic acid may allow *trans*-Golgi to *cis*-Golgi movement, sensitizing the cell to the toxin. Retinoic acid, by disrupting the Golgi, may slightly disrupt the efficient B chain mechanism of Golgi transport resulting in a small inhibition of native ricin toxicity while potentiating the inefficient ricin A chain immunotoxin routing.

Diphtheria toxin requires a low intravesicular pH for the toxin B chain to mediate membrane transport to the cytosol (7). Endosomes or lysosomes may be the optimal intracellu-

lar site for DT entry into the cytosol and, as BFA does not inhibit DT, there is no evidence that routing to or through the Golgi is needed for DT toxicity. Monensin and lysosomotropic amines block DT presumably simply by neutralizing low pH in endosomes and lysosomes. Retinoic acid does not inhibit the intracellular routing of DT to these compartments nor does it appear to alter the intracellular pH of these compartments from which DT enters the cytosol.

PE, like DT, is blocked by lysosomotropic agents but appears to route through different intracellular compartments on its way to the cytosol (10). PE contains a KDEL-like COOH-terminal sequence that can bind the KDEL receptor in the Golgi and is proposed to route PE out of the Golgi to the ER for efficient transport to the cytosol (4). Consistent with this model BFA blocks PE toxicity (42). The inhibition of PE by monensin may result from its affect on the Golgi apparatus. Retinoic acid had little affect on PE arguing that retinoic acid is very specific in its cellular perturbation or that PE is already adept at Golgi to ER transfer and retinoic acid does not further facilitate this step. Even when different toxins enter cells via the same receptor they are affected differently by retinoic acid. Tfn-PE and tfn-CRM107, two immunotoxins that traffic into cells via the transferrin receptor, are not affected by retinoic acid whereas tfn-rRA and 454A12-rRA, rRA immunotoxins that use the transferrin receptor, are strongly potentiated.

The potentiation of immunotoxins by retinoic acid and monensin, however, shows some significant differences. Retinoic acid has little affect on DT or PE whereas monensin strongly blocks both DT and PE, and BFA can block retinoic acid mediated immunotoxin potentiation but not monensin-mediated immunotoxin potentiation (data not shown). Thus, similar to the results from morphology studies by EM, immunostaining and C_6-NBD-ceramide staining, retinoic acid shows some characteristics of immunotoxin potentiation similar to monensin and some very different from monensin.

The effect of retinoic acid on immunotoxin activity is not blocked by cycloheximide or actinomycin D indicating that the retinoic acid directly affects cells and does not function via induction of new gene expression. Furthermore, examining a series of agents that bind or heterodimerize members of the retinoic acid receptor, e.g., all-trans retinoic acid, all-trans retinol, thyroxine (T_3) and vitamin D_3, and retinoids that do not bind the receptors, e.g., all-trans retinal, 13-cis retinoic acid, and other cis-forms of retinol and retinal, shows a lack of correlation between immunotoxin activation and RAR or RXR receptor interaction. Thus the well established transcription activation activity of retinoic acid does not appear to be the mechanism by which retinoic acid increases cell sensitivity to immunotoxins. The morphological effects of retinoic acid on the Golgi apparatus as shown by vital staining and by EM study likely relate to the mechanism of immunotoxin potentiation. The molecular mechanisms of this new activity of certain retinoids remains to be discovered.

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