Correction. In the article "Specific disruption of vimentin filament organization in monkey kidney CV-1 cells by diphtheria toxin, exotoxin A, and cycloheximide" by Arlene H. Sharpe, Lan Bo Chen, John R. Murphy, and Bernard N. Fields, which appeared in the December 1980 issue of Proc. Natl. Acad. Sci. USA (77, 7267-7271), Figs. 1 and 3 were reproduced poorly. They are printed again here.

![Corrections](image_url)
Fig. 3. Effects of $10^{-11}$ M diphtheria toxin neutralized by antitoxin (A), CRM197 (B), $10^{-11}$ M *P. aeruginosa* exotoxin A (C), and 10 g of cycloheximide per ml (D) on the vimentin filament system. The effect of cycloheximide was reversed when growth medium containing cycloheximide was removed and replaced with fresh medium. The appearance of vimentin filaments at 0.5 hr (E), 1 hr (F), 2 hr (G), and 4 hr (H) after the removal of cycloheximide is shown. (A, F, G, and H, bar = 40 pm; B, C, D, and E, bar = 25 pm.)

Fig. 1 (on preceding page). (A–C) Organization of microtubules (A), microfilaments (B), and intermediate filaments (C) in control CV-1 cells. (D–F) Organization of microtubules (D), microfilaments (E), and intermediate filaments (F) in CV-1 cells treated with $10^{-11}$ M diphtheria toxin for 22 hr. Cells were subjected to indirect immunofluorescence microscopy with antibody against tubulin, actin, and vimentin. Note that only the intermediate filament system is affected by diphtheria toxin. (A, B, D, and E, bar = 10 μm; C, bar = 40 μm; F, bar = 30 μM.)
Specific disruption of vimentin filament organization in monkey kidney CV-1 cells by diphtheria toxin, exotoxin A, and cycloheximide

(INTERMEDIATE FILAMENTS)

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ABSTRACT We have examined the effect of diphtheria toxin, Pseudomonas aeruginosa exotoxin A, and cycloheximide on the CV-1 cell cytoskeleton. Within a few hours after producing an inhibition of cellular protein synthesis, all these agents specifically disrupted the organization of the vimentin filament system with no discernable effect on microtubules or microfilaments during the period of observation. Furthermore, just as the inhibition of protein synthesis by cycloheximide is reversible, so was the disruption of vimentin filaments by cycloheximide.

The mechanisms by which infectious agents and their toxic products damage cells are poorly understood. For example, despite extensive knowledge of the molecular biology of viral replication, little is known about how viruses produce cellular injury (1). Similarly, although it is well established that the primary lesion of diphtheria intoxication is the inhibition of cellular protein synthesis (2, 3), almost nothing is known about the complex series of events arising from the initial lesion at the molecular level which eventually leads to metabolic derangements, morphologic damage, and cell death (4). In an attempt to clarify the structural basis of the injury produced by infectious agents and chemicals, we have begun an investigation of the effects of these agents on the three major filamentous systems in the cell cytoplasm: microtubules, microfilaments, and intermediate filaments.

For our initial studies, we have chosen to examine the effects of diphtheria toxin, Pseudomonas aeruginosa exotoxin A (exotoxin A), and cycloheximide on the cytoskeleton because the molecular mechanisms of their actions are well characterized. Both toxins inhibit cellular protein synthesis by catalyzing the NAD-dependent, ADP-ribosylation of elongation factor 2 (2, 3, 5). Despite this detailed biochemical knowledge, it is not known whether these toxins affect the cytoskeleton during the process of cell injury. We report here that diphtheria toxin and P. aeruginosa exotoxin A specifically influence the organization of the vimentin class of intermediate filaments but have no discernable effect on microtubules or microfilaments. Furthermore, the cycloheximide-induced disruption of vimentin filaments is readily reversible.

MATERIALS AND METHODS

Cells and Media. CV-1 (African green monkey kidney) cells were grown as monolayers in roller bottles in Richter’s improved minimal essential medium containing zinc, insulin, and 10 mM Hepes (Irvine Scientific, Santa Ana, CA) supplemented with 10% (vol/vol) fetal calf serum (Sterile Systems, Logan, UT) at 37°C.

Toxins and Antitoxins. Corynebacterium diphtheriae strains C7 (βtox + ) and C7 (βtox− 197) and P. aeruginosa PA103 have been described (6, 7). C. diphtheriae strains were grown in C-Y medium under optimal conditions for tox gene expression (8). Diphtheria tox gene products were purified from culture supernatant fluids as described (9). P. aeruginosa PA103 was obtained from P.-C. Tai (Bacteriological Physic Unit, Harvard Medical School), and exotoxin A was partially purified from culture supernatant fluids as described (10).

Anti-diphtheria toxin and anti-exotoxin A were obtained from the Massachusetts Antitoxin and Vaccine Laboratory (Jamaica Plain, MA) and Barbara Iglewski (Department of Microbiology and Immunology, University of Oregon School of Medicine, Portland, OR), respectively.

Antisera to Cytoskeletal Filament Proteins. Rabbit antiserum prepared against tubulin (11), provided by Frank Solomon (Massachusetts Institute of Technology, Cambridge, MA), was used for the detection of microtubules. Antiserum to gerbil vimentin was prepared according to the procedure of Hynes and Destree (12). Rabbit antiseraum against actin (13), provided by Keith Burridge (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), was used to detect microfilaments. Fluorescein-conjugated goat anti-rabbit IgG was obtained from Meloy Laboratories (Springfield, VA).

Indirect Immunofluorescent Microscopy. CV-1 cells, grown at low density on 12-mm round glass coverslips (Rochester Scientific) in 60 × 15 mm culture dishes (2 × 10⁵ cells per dish), were incubated with various concentrations of diphtheria toxin, CRM197, exotoxin A, or cycloheximide for the indicated times. Prior to staining of cells with antibody to tubulin or vimentin, the cells attached to coverslips were fixed by the procedure of Osborn and Weber (14) and transferred to a humidified chamber. Ten microliters of an appropriate dilution of antisera was applied to each coverslip. The coverslips were incubated at 37°C for 1 hr, rinsed in phosphate-buffered saline, and returned to the humidified chamber. Ten microliters of an appropriate dilution of fluorescein-conjugated goat anti-rabbit IgG was applied to each coverslip. The coverslips were incubated at 37°C for 40 min, rinsed in phosphate-buffered saline and water, and mounted on glass slides in Gelvatol (Monsanto, St. Louis, MO). Prior to being stained with antibody to actin, the cells were fixed for 10 min in 3.5% (vol/vol) formaldehyde in phosphate-buffered saline, rinsed in phosphate-buffered saline and water, and incubated for 1 min in acetone that had been chilled to −20°C. After being rinsed in phosphate-buffered saline, the fixed cells were incubated with antisera to actin for 1 hr at 37°C. The cells were rinsed with phosphate-buffered saline and then incubated with fluorescein-conjugated goat anti-rabbit IgG as described above. All cells were examined with a Zeiss photomicroscope III equipped with epifluorescence and a Planapochromat objective lens (40× and 63×). Photomicrographs were made with Kodak Tri-X (ASA 400) at Exposure Index 1600.
**Determination of Rate of Cellular Protein Synthesis.** CV-1 cells were transferred from roller bottles to 35-mm plastic Falcon dishes (2 × 10^6 cells per dish). To measure the effects of diphtheria toxin, CRM197, exotoxin A, or cycloheximide on protein synthesis, we added various concentrations of these agents to CV-1 cells. At the times indicated, the growth medium was removed from the CV-1 cultures and replaced with Eagle’s minimal essential medium without methionine (GIBCO) containing 1 μCi of [35S]methionine per ml (Amersham/Searle; >400 Ci/mmol, 1 Ci = 3.7 × 10^{10} bequerels). The cultures were incubated for an additional hour at 37°C. Incorporation of radioactive methionine was stopped by the removal of the
RESULTS

Cytoskeleton of CV-1 Cells. After examining several types of cells, we found CV-1 cells to be ideal for these studies because of their high sensitivity to diphtheria toxin and ability to grow as well-spread and flattened cells in monolayers. Anti-tubulin staining revealed an extensive network of microtubules emanating from the nucleus (Fig. 1A). Antibodies to actin showed typical bundles of microfilaments in the cytoplasm (Fig. 1B). Staining of intermediate filaments with vimentin antibody revealed a cytoplasmic filamentous system emanating or terminating at one or more focal points surrounding the nucleus (Fig. 1C).

Effect of Diphtheria Toxin on CV-1 Cytoskeleton. CV-1 cells are exquisitely sensitive to diphtheria toxin (15, 16). To measure the rate of inactivation of cellular protein synthesis by diphtheria toxin, we incubated CV-1 cells with \([\text{S}]\)methionine for 1 hr at intervals after the addition of toxin. After the addition of toxin there was a characteristic lag period followed by an inhibition of protein synthesis (Fig. 2). As the concentration of toxin was increased from \(10^{-12}\) M to \(10^{-10}\) M, the lag period decreased and the rate of inhibition of protein synthesis increased. In these studies the effects of \(10^{-11}\) M diphtheria toxin on the cytoskeleton were examined after a 22-hr exposure to toxin. Diphtheria toxin had little or no effect on microtubules (Fig. 1D) or microfilaments (Fig. 1E). On the other hand, the organization of intermediate filaments was disrupted. Wavy filaments with no apparent organization were seen in the cytoplasm (Fig. 1F). This disruption of intermediate filaments was a specific effect of diphtheria toxin; antitoxin neutralized the effect (Fig. 3A).

Effect of CRM197 on CV-1 Cytoskeleton. In order to determine whether the enzymatic activity of diphtheria toxin was essential for the disruption of vimentin filaments, a mutant diphtheria toxin, CRM197, was examined for its effects on the cytoskeleton. CRM197 has a molecular weight identical to that of diphtheria toxin but is enzymatically inactive (6). Ittleson and Gill (17) have demonstrated that CRM197 is a competitive inhibitor of diphtheria toxin with a \(K_i\) of approximately \(10^{-8}\) M. It is presumed that, after binding to a eukaryotic cell receptor, CRM197 fragment A is transported normally into the cytosol. CRM197 added to cells at a concentration of \(10^{-11}\) M did not disrupt vimentin filaments (Fig. 3B). In addition, CRM197 had no effect on microtubules and microfilaments (data not shown). Thus, the enzymatic activity of diphtheria toxin appears to be necessary for disruption of vimentin filaments to occur.

Effect of \(P. aeruginosa\) Exotoxin A on CV-1 Cytoskeleton. \(P. aeruginosa\) exotoxin A inhibits protein synthesis in a manner identical to that of diphtheria toxin (5). To determine whether exotoxin A would also disrupt vimentin filaments, we added exotoxin A to CV-1 cells at a concentration of \(10^{-11}\) M. At this concentration, exotoxin A inhibited protein synthesis but had no effect on microtubules or microfilaments (data not shown). Vimentin filaments were disrupted in a manner identical to that of diphtheria toxin (Fig. 3C); the perinuclear organization sites disappeared and wavy fibers were visible in the cytoplasm. No effect on any filament system was observed when antibody to exotoxin A was preincubated with toxin prior to the addition of toxin to CV-1 cells.

Effects of Cycloheximide on CV-1 Cytoskeleton. Both diphtheria toxin and exotoxin A inhibit protein synthesis by the NAD-dependent ADP-riboseylation of elongation factor 2. To determine whether an antibiotic that inhibits eukaryotic protein synthesis would also disrupt vimentin filaments, we studied the effect of cycloheximide on the CV-1 cytoskeleton. Cycloheximide (10 \(\mu\)g/ml) inhibited protein synthesis by 80% within 5 min of its addition to cells. The intermediate filament system began to appear altered by 75 min. The filaments that had radiated from the perinuclear organization sites were no longer present at 3 hr, and only wavy fibers that differed in organization from vimentin filaments were visible (Fig. 3D). Microtubules or microfilaments were not affected (data not shown).

The inhibition of protein synthesis induced by cycloheximide is reversible (18). Protein synthesis resumed within minutes when growth medium containing cycloheximide was removed from CV-1 cells and replaced with fresh medium. Vimentin fibers began to reappear (Fig. 3E and F) by 1 hr after replacement of medium. Multiple focal sites were present by 2 hr (Fig. 3C). The intermediate filament system resumed its normal appearance (Fig. 3H) by 4 hr after the removal of cycloheximide.
DISCUSSION

The addition of diphtheria toxin, P. aeruginosa exotoxin A, or cycloheximide to CV-1 cells leads to a disruption of the organization of cytoplasmic vimentin filaments. This effect occurs in every treated CV-1 cell and is specific; the alteration does not occur when diphtheria toxin and exotoxin A are incubated with their respective antitoxins prior to addition to cells. The disruption is not simply related to the binding of toxin to the cell surface and the translocation of fragment A into the cytosol because CRM197, a missense mutant of diphtheria toxin that binds normally to diphtheria toxin receptors but is devoid of enzymatic activity, does not disrupt vimentin filaments.

Diphtheria toxin, exotoxin A, and cycloheximide disrupt the organization of vimentin filaments but not that of microtubule or microfilament bundles within a few hours after inhibiting cellular protein synthesis. Thus, intermediate filaments appear to be more sensitive than microtubules or microfilament bundles to the cellular injury induced by certain bacterial exotoxins.
The disruption of intermediate filaments after the inhibition of protein synthesis may thus play a significant role in the pathogenesis of exotoxin-mediated cellular injury.

The cell cytoplasm is a highly organized three-dimensional lattice of chemically distinct filaments in which cytoplasmic inclusions and organelles are positioned. Intermediate filaments are thought to function in the spatial organization of the contents of the cell cytoplasm (19). For example, intermediate filaments may play a role in the anchoring of the polyomes to the cytoskeleton (20, 21) as well as in influencing the distribution and orientation of mitochondria (unpublished results). The results reported here indicate that vimentin filaments respond very quickly to alterations in cellular metabolism.

Why might inhibition of protein synthesis lead to the disruption of vimentin filaments? Rapid turnover of vimentin protein would seem to be an unlikely explanation because vimentin protein does not disappear, but rather its organization is altered. We have used Penman's extraction procedure (21) to isolate the cytoskeleton, to resolve the components in a two-dimensional gel with isoelectric focusing and NaDodSO4/polyacrylamide gel electrophoresis, and to quantitate the destruction of "assembly" protein are sensitive to this effect. Labile filaments within the cytoplasm may disappear, but not that of microtubules and microfilaments. Although there was no significant reduction in the other major cytoskeletal components (such as actin, tropomyosin, and α-actinin), we did observe the disappearance, or decrease, of several polypeptides normally associated with cytoskeleton as established by Penman's procedure in both the diphtheria toxin- and cycloheximide-treated cells. It is possible that rapidly turning over regulatory proteins may exist to assemble vimentin filaments within their proper orientation. Inhibition of protein synthesis could lead to the depletion of such "assembly" proteins, resulting in a disorganized vimentin filament system.

Other investigators have reported that cycloheximide does not alter the cytoskeleton (22). We have examined several types of cells for their response to the addition of this antibiotic. Disruption of vimentin filaments was observed in kangaroo rat epithelial cells (PtK2 cells) but not in gerbil fibroma cells (CCL 146) or in BALB/c 3T3 cells. Possibly, cells that have a more labile "assembly" protein are sensitive to this effect.

We have also found that reovirus infection of CV-1 cells leads to the destruction of vimentin filaments although other changes were observed as well. We do not know if the disruption of vimentin filaments is related to a virally mediated inhibition of protein synthesis in CV-1 cells. Thus, viruses as well as toxins may exert their pathologic effects, at least in part, not only through interference with intermediary metabolism and the biosynthesis of macromolecules but also through disruption of vimentin filaments.

This study demonstrates a means to specifically disrupt the organization of vimentin filaments but not that of microtubules or microfilaments. Other agents that alter the cytoskeleton affect more than one filament system. Colchicine, for example, causes the breakdown of microtubules and induces the formation of juxtanuclear aggregates of vimentin-containing filaments (23). Because diphtheria toxin, exotoxin A, and cycloheximide disrupt the organization of vimentin filaments only, these agents may help to elucidate the precise biologic functions of vimentin filaments.

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