[CANCER RESEARCH 52, 5096–5099, September 15, 1992]

Advances in Brief

Cytotoxicity of *Clostridium difficile* Toxin A for Human Colonic and Pancreatic Carcinoma Cell Lines[1]

Vladimir M. Kushnaryov,[2] Philip N. Redlich, J. James Sedmak,[3] David M. Lyerly, Tracy D. Wilkins, and Sidney E. Grossberg

Departments of Microbiology [V. M. K., J. J. S., P. N. R., S. E. G.] and Surgery [P. N. R.], The Medical College of Wisconsin, Milwaukee, Wisconsin 53226; Department of Anaerobic Microbiology, Virginia Polytechnic Institute, Blacksburg, Virginia 24061 [D. M. L., T. D. W.]

Abstract

The use of bacterial exotoxins may constitute novel adjuncts to treatment of gastrointestinal tract malignancies. *Clostridium difficile* toxin A was evaluated for its cytotoxic effect *in vitro* on 24 human cell lines and strains including carcinomas of the colon, pancreas, prostate, lung, breast, and lymphoid malignancies, as well as nonmalignant tissues. All nine colon and five pancreas cell lines were extraordinarily sensitive to the cytotoxic effect of *Clostridium difficile* toxin A at very low concentrations. This effect, which occurred rapidly and was dose dependent, was observed in all cells of seven colon and two pancreas cell lines at concentrations as low as 1–5 ng/ml (10^{-12} to 10^{-11}M), whereas cells derived from other sites required 60 to greater than 500 ng/ml to achieve an equivalent effect. The data suggest that *Clostridium difficile* toxin A may have potential therapeutic value in the treatment of some gastrointestinal tract cancers.

Introduction

Carcinomas of the gastrointestinal tract are a leading cause of cancer deaths in the United States. Colorectal and pancreatic cancer are estimated to constitute over 75% of all gastrointestinal tract cancers to be diagnosed in 1992 (1). Surgical resection may be adequate therapy for early stage disease; however, effective therapeutic modalities are not available for patients with advanced disease. Novel therapeutic approaches must be considered in order to improve significantly the prognosis of patients with gastrointestinal tract carcinomas. One such approach may be treatment with plant and/or bacterial toxins; however, a major drawback of toxin therapy has been nonspecificity requiring a mechanism for targeting such toxins to malignant cells (2). Nevertheless, other toxins may exist which have inherent selectivity for certain cell types. One such toxin may be the enterotoxin of *Clostridium difficile*, an anaerobic bacterium naturally populating the colon of some individuals (3). The target site of infection by *C. difficile* in clinical disease is clearly the large intestine, with toxin A[4] considered to be the major contributor to the syndrome of pseudomembranous colitis in patients and experimental animals (3, 4). Therefore, we sought to determine whether cells of colonic origin were more sensitive to the cytotoxic effect of toxin A than cells derived from other gastrointestinal and non-gastrointestinal tract sites. We show a selective cytopathic effect *in vitro* of toxin A for human cell lines derived from colonic and pancreatic carcinomas compared to human cells from non-gastrointestinal tract origins.

Materials and Methods

Toxin A. Toxin A was purified by the method of Sullivan *et al.*(5). The toxin was homogeneous, as determined by polyacrylamide gel electrophoresis and crossed immunoelectrophoresis, and was stored as a filter-sterilized solution at 4°C. The level of endotoxin as measured by the *Limulus* lysate assay (Sigma, St. Louis, MO) was below the detection limit (0.5 endotoxin units/ml) for a 500-ng/ml solution of toxin A in minimal essential medium containing 2% fetal bovine serum.

Cells. The designation, origin, and source of human cell lines and strains used in this study are listed in Table 1. The source and cultivation of A549 human lung carcinoma cells, Daudi lymphoblastoid cells, and CHO cells have been described previously (6, 7). Cells were cultivated according to instructions from the provider and were maintained at 36°C in a CO_2 incubator.

Cytotoxicity. The primary method of cytotoxicity testing has been described (7). Briefly, cells were distributed in 96-well plates at 5×10^4 cells/well and incubated at 36°C overnight. The medium was exchanged for identical medium with 2% serum that contained serial 2-fold dilutions of toxin A starting at 500 ng/ml. Cells were incubated at 36°C and toxicity was assessed by phase-contrast microscopy; cultures were observed every 1–2 h for 8 h and at 24 h. The cytotoxic dose, CTD-24, was defined as the lowest concentration that would lead to a cytopathic effect, *i.e.*, cell rounding, of 100% of cells at 24 h. The kinetics of the cytotoxic effect was evaluated by counting a minimum of 200 cells at frequent intervals as noted, and the percentage of rounded cells calculated. Additional methods to assess cytotoxicity included cell counting and viability determinations, electron microscopy (7), and the MTT colorimetric assay performed on selected cell lines, in triplicate, following 1, 2, and 3 days of incubation with toxin A as described (8). CHO cells, previously characterized with respect to its sensitivity to toxin A (7), were used as a reference control cell line. The data were evaluated statistically in the two-tailed Student's *t* test.

Results

Evaluation of Toxin A Cytotoxicity. Incubation of cells with toxin A leads to a cytopathic effect manifested by cell rounding and eventual cell death. CHO cells, a cell line sensitive to the cytopathic effect of the toxin, manifest rounding of all cells at concentrations of 100–150 ng/ml by 24 h as previously described (7). The selectivity of the cytopathic effect was studied on 24 human cell strains and lines derived from breast epithelial cells, diploid fibroblasts, carcinomas of the prostate, lung, breast, colon, pancreas, and malignant lymphoid tissue. The typical effect of the toxin on cell morphology is shown for SKCO-1 colon carcinoma cells in Fig. 1. Incubation of cells in the presence of toxin A caused an increase in refractility and retraction of the cytoplasm toward the nucleus, resulting in

Received 3/5/92; accepted 7/24/92.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported, in part, by a grant from The Cancer Center of The Medical College of Wisconsin.

[2] To whom requests for reprints should be addressed.

[3] Present address: Universal Foods Corporation, Milwaukee, WI 53218.

[4] The abbreviations used are: toxin A, *Clostridium difficile* enterotoxin; CHO, Chinese hamster ovary; CTD-24, the dose having a cytotoxic effect on 100% of cells by 24 h; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Table 1 *Designation and origin of human cell lines and strains*[a]

Origin	Cell lines or strains
Colonic carcinoma	SW1116, HCT116, SKCO-1, HT-29, KM12C, KM12SM, KM12L4, SW480
Pancreatic carcinoma	BxPC-3, AsPC-1, Capan-2, MIA PaCa-2, Hs766T
Colon adenoma	VaCo 235
Lung carcinoma	A549
Prostate carcinoma	PC-3, DU-145
Breast carcinoma	009P, 013T
Lymphoma	Daudi, Raji
Breast epithelium	006FA
Diploid fibroblast	HCS (human corneal stroma), MRC-5

[a] The SW1116, HT-29, SW480, Raji lymphoblastoid cells, and the pancreatic cell lines were obtained from the American Type Culture Collection. The KM12C, KM12L4, and KM12SM cells were obtained from Dr. J. Fidler, University of Texas, Houston, TX. The HCT116 and SKCO-1 cells were obtained from Dr. J. Schiller, University of Wisconsin, Madison, WI. VaCo 235 cells were obtained from J. Willson, University Hospitals of Cleveland, Cleveland, OH. The PC-3 and DU-145 cells were obtained from Dr. G. Wilding, University of Wisconsin, Madison, WI. The 009P, 013T, and 006FA cells were provided by Dr. M. Hancock, Triton Biosciences, Alameda, CA. HCS fibroblasts were provided by Drs. W. O'Brien and J. Taylor, Medical College of Wisconsin, Milwaukee, WI. The MRC-5 diploid fibroblasts were provided by Dr. D. Carrigan, Medical College of Wisconsin, Milwaukee, WI.

complete rounding of the cells and eventual cell death. The concentrations of toxin A that led to a cytotoxic effect on 100% of cells measured at 24 h (CTD-24) on different cell lines and strains are presented in Fig. 2. All eight lines of colonic carcinoma and five lines of pancreatic carcinoma were extraordinarily sensitive to the toxin. Seven colon and two pancreas cell lines were affected at concentrations as low as 1–5 ng/ml (10^{-12} to 10^{-11} M), whereas cells from non-gastrointestinal tract sites required 60 to greater than 500 ng/ml to achieve an equivalent cytotoxic effect.

Colon carcinoma cell lines having different metastatic behavior in a nude mouse model were equally sensitive to the toxin *in vitro*. The highly metastatic KM12L4 and KM12SM cell lines were affected at the same concentration (1–2 ng/ml) as the poorly metastatic parental line KM12C originally derived from a Dukes' B_2 tumor (9). In addition, a colon cell line derived from a villous adenoma of the rectum, VaCo 235 (10), was affected at a mean concentration of 16 ng/ml. Thus, the high sensitivity to the cytopathic effect of toxin A was a uniform characteristic of the colon cell lines evaluated regardless of their degree of malignant differentiation or metastatic potential.

Daudi and Raji cell lines, both of lymphoblastoid origin, were unaffected by toxin A at the highest tested concentration of 500 ng/ml during 5 days of culture as determined by counting of viable cells (Fig. 2).

To confirm the results of the visual method of cytotoxicity testing, we used the MTT colorimetric assay measuring cell respiratory activity to assess cell killing on selected sensitive (SKCO-1, HCT116, AsPC-1, and Capan-2) and resistant (DU-145 and PC-3) cell lines at different toxin concentrations. At 12.5 ng/ml, the absorbance of the sensitive cell lines incubated with toxin for 3 days ranged from 15 to 30% of untreated controls compared to 93 to 100% for the resistant cell lines. Furthermore, the decrease in absorbance, *i.e.*, diminished cell respiratory activity, correlated with cell rounding. Similar results were observed at higher toxin concentrations.

To evaluate the morphological changes over time associated with cell rounding, we followed selected sensitive cell lines by

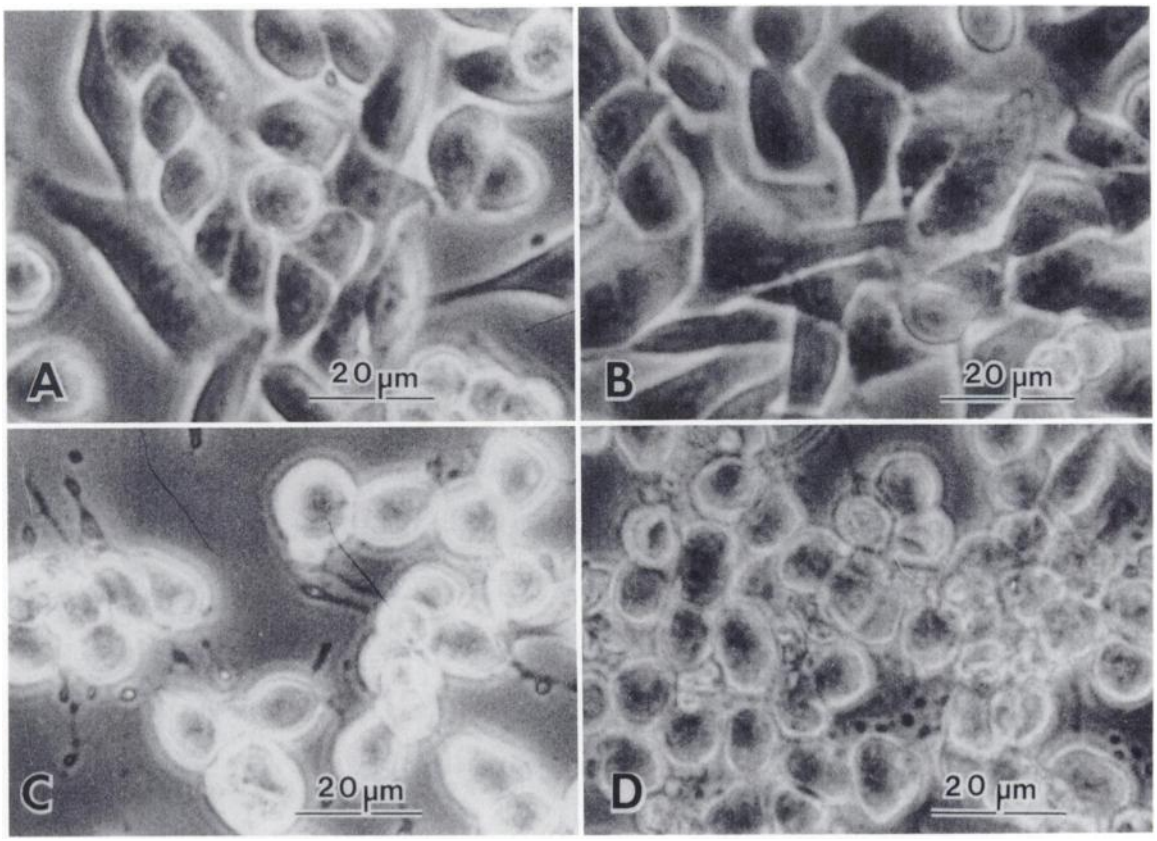

Fig. 1. Phase-contrast microscopy of SKCO-1 colon carcinoma cells. Photomicrographs were taken at the initiation of the culture (*A*), 24 h of incubation without toxin A (*B*), 2 h of incubation with 40 ng/ml of toxin A (*C*), and 24 h of incubation with 40 ng/ml of toxin A (*D*). Gradual contraction of the cytoplasm and rounding of the cells with increasing refractility, especially around the nuclei, are noted in the toxin-treated cells.

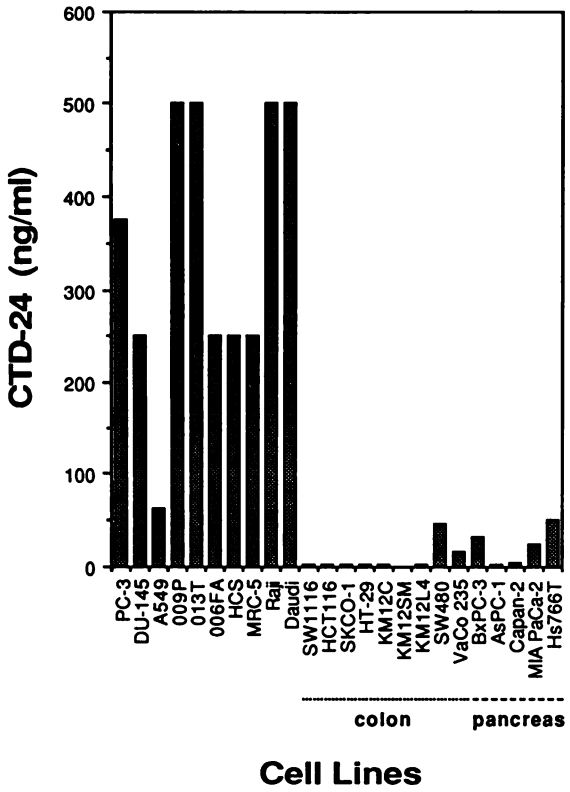

Fig. 2. Sensitivity of different human cells to the cytotoxic effect of *Clostridium difficile* toxin A. When cultures became nearly confluent, they were treated with serial 2-fold dilutions of toxin A starting at 500 ng/ml. The cytoxic effect was recorded at 24 h as determined by cell rounding, or counting of viable cells for Daudi and Raji cell lines, and the minimum dose of toxin required to affect 100% of the cells was calculated (CTD-24). Data are presented as the mean CTD-24 of triplicate cultures. The SE was ≤17% for all groups. Cell lines (derived from carcinomas) and cell strains (diploid cells) are represented.

electron microscopy. Rounded cells demonstrated vacuolization, autolysis, and complete disintegration by 48–72 h following toxin exposure, confirming that rounding resulted in cell death (not shown).

Kinetics of Cytotoxicity. The kinetics of the cytopathic effect of toxin A for SKCO-1 and CHO cells is shown in Fig. 3. An effect on SKCO-1 cells was noted within 30 min of exposure to 1 μg/ml of the toxin, equivalent to 500 × CTD-24. At a concentration of 10 ng/ml (5 × CTD-24), rounding of cells was less rapid; nevertheless, most cells were rounded by 3 h of exposure. The kinetics of cell rounding for CHO cells at 1 μg/ml (9 × CTD-24) was comparable to that observed for SKCO-1 cells exposed to the lower toxin concentration. In separate experiments, HCT116 cells were rapidly affected at toxin concentrations of 10 ng/ml and 1 μg/ml in a manner identical to that observed for SKCO-1 cells (results not shown).

Discussion

Current therapy of advanced gastrointestinal tract carcinomas has little impact on the disease process or overall patient survival. In an effort to improve patient outcome, clinical trials are evaluating new treatment regimens that include biological agents by themselves and in combinations with chemotherapeutic drugs. For colorectal carcinoma, preliminary data reporting a 63% response rate in patients with advanced disease to a regimen combining α-interferon and 5-fluorouracil are encouraging (11); however, others have reported only a 26% response to the same regimen (12). Novel treatment approaches must continue to be sought to improve the outcome of such patients.

An important property of an effective antitumor agent is selective toxicity toward the disease with little or no toxicity to normal tissues. In search of such an agent for colon carcinoma, we evaluated the cytotoxicity of *C. difficile* toxin A since the colon is the site of clinical disease attributable to this toxin (4). The use of plant or bacterial toxins in the therapy of malignant disease is not new. Diphtheria toxin has been tested in experimental animals and humans with encouraging results (13, 14). However, toxins studied to date suffer the drawback of nonspecific cytotoxicity, requiring a targeting vehicle such as antibody, hormones, growth factors, or cytokines (2).

Among the 24 human cell lines and strains tested that were derived from different sites and/or malignancies, cells derived from colonic and pancreatic carcinomas as well as a rectal adenoma were extraordinarily sensitive to the cytopathic effect of toxin A at very low concentrations. Indeed, colorectal and pancreatic cell lines were 50- to 500-fold more sensitive to the toxin than the non-gastrointestinal tract cell lines evaluated, suggesting that susceptibility to low doses of toxin A may be a characteristic of neoplasms of the gastrointestinal tract. Analysis of the kinetics of the cytopathic effect of toxin A demonstrated a rapid effect on colon carcinoma cells that was dose dependent and related to the CTD-24. To confirm the observed differential sensitivity based on cell rounding, additional assays for cell killing were used. Results of the MTT colorimetric assay correlated with the cell rounding at low toxin concentrations, although rounding was observed earlier than inhibition of cell respiration. Additionally, colon cells rounded by toxin A were followed by electron microscopy which demonstrated cell death and autolysis of all rounded cells by 48–72 h.

Differential sensitivity to the cytotoxic effect of *C. difficile* toxins on other cultured cells has been previously noted (15); morphological changes in mouse adrenal tumor cells due to toxin A were observed at 80 ng/ml compared to more than 1 μg/ml required for CHO and HeLa cells to achieve a similar effect. More recently, the effects of toxin A on CHO and T-84

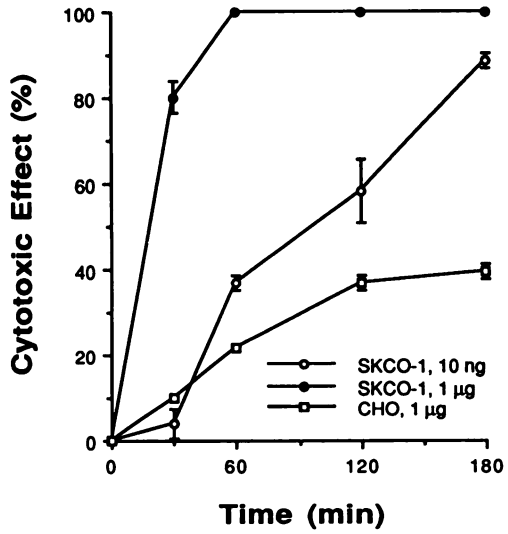

Fig. 3. Kinetics of cell rounding. SKCO-1 and CHO cells were incubated in the presence of either 10 ng/ml or 1 μg/ml of toxin A as noted. At the times indicated, cells were counted, and the cytotoxic effect was calculated. The cytotoxic effect is expressed as the number of rounded cells as a percentage of total cells. Data are represented as the mean ± SEM of 10 random fields with a minimum of 200 cells counted. CHO cells were observed for additional 3 h at which time 87 ± 4% of cells were rounded (not shown).

human colonic carcinoma cells were compared, but concentrations of toxin A lower than 1 μg/ml were not tested on T-84 cells (16).

With respect to the effect of toxin A on normal intestine mucosal epithelial cells, histological examination of the mucosa of intact small and large intestine exposed to the toxin at relatively high concentrations (1–10 μg/ml) has been studied in experimental animals (16). Toxin A, at 10 μg/ml, had no effect on protein synthesis in isolated rabbit small intestine mucosal cells compared to untreated controls in short-term experiments (17); however, neither detailed microscopic analysis nor quantitative evaluation of cytotoxicity on colonic mucosal cells was described. The sensitivity *in vitro* of normal human intestinal epithelial cells to the cytotoxic effect of toxin A has not been reported.

The mechanism of the rapid cytopathic effect of toxin A has previously been studied on CHO cells (7, 18, 19). Toxin A was found to be internalized by receptor-mediated endocytosis subsequently affecting the cytoskeleton and the nuclei (7, 18). Nuclear filaments comprised of actin, lamin, and vinculin transiently appeared between 2.5 and 4 h of exposure to the toxin (1 μg/ml) and their appearance coincided chronologically with irreversibility of the cytopathic effect (18). The working hypothesis that was suggested postulates that the toxin affects the distribution and/or synthesis of lamins in the cells, thereby disturbing the complex regulation of mitosis and maintenance of the cytoskeleton (20). No mitoses were observed in CHO cells treated with the toxin (7).

One explanation for the observed differential cytotoxicity of toxin A may be that colonic and pancreatic carcinoma cells possess a greater density of cell surface receptors specific for toxin A compared to less sensitive cell lines. Analysis of receptor densities by quantitative immunogold electron microscopy on colonic and pancreatic carcinoma cell lines and on cells derived from other gastrointestinal and non-gastrointestinal tract epithelial malignancies are in progress.

The remarkable cytotoxicity of low concentrations of *C. difficile* toxin A for cells derived from colonic and pancreatic carcinomas constitutes a basis for future investigation of the mechanism of its selective action. These future studies may be aided by the recent cloning of toxin A (21). Investigations of the cytotoxic effect of toxin A on cells derived from additional gastrointestinal and non-gastrointestinal tract malignancies as well as assessment of its therapeutic efficacy *in vivo* are under way.

Acknowledgments

The authors express their appreciation for the excellent technical assistance of Irene Hernandez, Mary Faculjak, and Amy Loebel.

References

1. Boring, C. C., Squires, T. S., and Tong, T. Cancer Statistics, 1992. CA Cancer J. Clin., *42:* 19–38, 1992.
2. Pastan, I., and FitzGerald, D. Recombinant toxins for cancer treatment. Science (Washington DC), *254:* 1173–1177, 1991.
3. Lyerly, D. M., Krivan, H. C., and Wilkins, T. D. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev., *1:* 1–18, 1988.
4. Bartlett, J. G. Pathogenesis and treatment of pseudomembranous enterocolitis. Front. Gastrointest. Res., *13:* 370–386, 1986.
5. Sullivan, N. M., Pellett, S., and Wilkins, T. D. Purification and characterization of toxins A and B of *Clostridium difficile*. Infect. Immun., *35:* 1032–1040, 1982.
6. Redlich, P. N., and Grossberg, S. E. Analysis of antigenic domains on natural and recombinant human IFN-β by the inhibition of biologic activities with monoclonal antibodies. J. Immunol., *143:* 1887–1893, 1989.
7. Kushnaryov, V. M., and Sedmak, J. J. Effect of *Clostridium difficile* enterotoxin A on ultrastructure of Chinese hamster ovary cells. Infect. Immun., *57:* 3914–3921, 1989.
8. Carmichael, J., DeGraff, W. G., Gasdar, A. F., Minna, J. D., and Mitchell, J. B. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res., *47:* 936–942, 1987.
9. Marikawa, K., Walker, S. M., Nakajima, M., Pathak, S., Jessup, J. M., and Fidler, I. J. Influence of organ environment on the growth, selection, and metastasis of human colon carcinoma cells in nude mice. Cancer Res., *48:* 6863–6871, 1988.
10. Willson, J. K. V., Bittner, G. N., Oberley, T. D., Meisner, L. F., and Weese, J. L. Cell culture of human colon adenomas and carcinomas. Cancer Res., *47:* 2704–2713, 1987.
11. Wadler, S., and Wiernik, P. Clinical update on the role of fluorouracil and recombinant interferon α-2a in the treatment of colorectal carcinoma. Semin. Oncol. (Suppl. 1), *17:* 16–21, 1990.
12. Kemeny, N., Younes, A., Seiter, K., Kelsen, D., Sammarco, P., Adams, L., Derby, S., Murray, P., and Houston, C. Interferon α-2a and 5-fluorouracil for advanced colorectal carcinoma. Cancer (Phila.), *66:* 2470–2475, 1990.
13. Raso, V., and McGrath, J. Cure of experimental human malignant mesothelioma in athymic mice by diphtheria toxin. J. Natl. Cancer Inst., *81:* 622–627, 1989.
14. Buzzi, S. Diphtheria toxin treatment of human advanced cancer. Cancer Res., *42:* 2054–2058, 1982.
15. Donta, S. T., Sullivan, N., and Wilkins, T. D. Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin. Microbiol., *15:* 1157–1158, 1982.
16. Lima, A. A. M., Lyerly, D. M., Wilkins, T. D., *et al.* Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine *in vivo* and on cultured cells *in vitro*. Infect. Immun., *58:* 582–588, 1988.
17. Mitchell, T. J., Ketley, J. M., Burdon, D. W., *et al.* The effects of *Clostridium difficile* toxins A and B on membrane integrity and protein synthesis in intestinal cells *in vivo* and *in vitro* and in McCoy cells *in vitro*. J. Med. Microbiol., *23:* 205–210, 1987.
18. Kushnaryov, V. M., Sedmak, J. J., Markwald, R. R., Faculjak, M. L., and Loo, P. M. Actin and lamin comprised filaments in the nuclei of Chinese hamster ovary cells affected with *Clostridium difficile* enterotoxin A. Cytobios, *64:* 181–196, 1990.
19. Florentini, C., Malorni, W., Paradisi, M., Giuliano, M., Mastrantonio, P., and Donelli, G. Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. Infect. Immun., *58:* 2329–2336, 1990.
20. Steinert, P. M., and Roop, D. R. Molecular and cellular biology of intermediate filaments. Annu. Rev. Biochem., *57:* 593–625, 1988.
21. Phelps, C. J., Lyerly, D. L., Johnson, J. L., and Wilkins, T. D. Construction and expression of the complete *Clostridium difficile* toxin A gene in *Escherichia coli*. Infect. Immun., *59:* 150–153, 1991.