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Preclinical pharmacology of cholera toxin

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Abstract Cholera toxin was selected for pharmacologic evaluation by the National Caner Institute on the basis of antiproliferative activity against small-cell and non-small-cell lung-cancer cell lines. A feature common to the sensitive cell lines was abundant expression of G_{M1} ganglioside, the cellular receptor for cholera toxin. A sandwich enzyme-linked immunosorbent assay (ELISA) was developed to quantitate cholera toxin in biological fluids. A sigmoidal relationship was observed between the cholera toxin plasma concentration and the absorbance at 490 nm (OD_{490}) of the product of horseradish peroxidase-catalyzed oxidation of *o*-phenylenediamine over the range of 6.25–1,600 ng/ml. Logit transformation of the OD_{490} data was linear over the entire concentration range and assay variability was less than 25%. Cholera toxin was stable in murine and human whole blood and plasma. Following i.v. administration of 1,500 μg/kg to male $CD2F_1$ mice, cholera toxin plasma elimination was described by a two-compartment open model. The half-lives ($t_{1/2}\alpha$, $t_{1/2}\beta$), plasma clearance, and steady-state volume of distribution were 0.7 min, 49 min, 24 ml min^{-1} kg^{-1} 912 ml/kg, respectively. Cholera toxin was not detected in plasma following an s.c. dose of 1,500 μg/kg. Urinary recovery following intravenous drug administration was less than 0.1%.

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Introduction

Cholera toxin (NSC 629801), a protein produced by the bacteria *Vibrio cholerae*, has been extensively used in cellular pharmacology as a reagent to probe for the involvement of specific heterotrimeric G proteins in the coupling of cell-surface receptors to their effectors [19]. It is composed of two distinct subunits. The A subunit (28,000 Da) is noncovalently bound to an aggregate of five B subunits (11,600 Da). The B-subunit aggregate is responsible for binding to G_{M1} ganglioside, the cell-surface receptor for cholera toxin. The A subunit penetrates the cell membrane and activates adenylate cyclase through adenosine diphosphate (ADP) ribosylation of the stimulatory G protein G_{α_s}. In intestinal epithelium, the resulting increase in cyclic adenosine monophosphate (cAMP) causes diarrhea and fluid loss by inhibiting sodium chloride uptake and by stimulating active chloride secretion [11].

Cholera toxin can also be involved in other cellular pathways. Recent reports have indicated that cholera toxin can inhibit cellular responses to multiple mitogens in human small-cell lung-cancer cell lines by a mechanism independent of cAMP generation and uncoupling of receptor/effector interaction [14, 27]. In addition, nanomolar concentrations of cholera toxin inhibit the in vitro growth of several human small-cell and non-small-cell lung-cancer cell lines expressing the G_{M1} ganglioside. Since $G_{\alpha s}$ also appears to be involved in the regulation of cell-membrane turnover [22], it is possible that perturbations of membrane dynamics are involved in a global disruption of mitogen-induced signals with consequences on growth of cells dependent on paracrine and autocrine stimulation.

On the basis of the unique mechanism of action and in vitro growth inhibition for chemotherapy-resistant small-cell and non-small-cell lung-cancer cell lines, the

National Cancer Institute conducted preclinical studies with cholera toxin as a potential chemotherapeutic agent. In this report, we describe an enzyme-linked immunosorbent assay (ELISA) developed for determination of cholera toxin in biological samples. We also report the stability of cholera toxin in aqueous and biological solutions and pharmacokinetic data obtained following administration of cholera toxin to mice.

Materials and methods

Cholera toxin (azide-free) was provided by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (NCI, Bethesda, Md). Goat anti-choleragenoid antisera were obtained from List Biologicals (Campbell, Calif.). Avidin-horseradish peroxidase was purchased from Vector Laboratories (Burlington, Calif.). Sulfo-NHS-biotin and avidin-HABA (4-hydroxyazobenzene-2-benozic acid) were purchased from Pierce Chemicals (Rockford, Ill). Dialysis tubing (12,000 to 14,000-Da molecular-weight cutoff) was purchased from Baxter Scientific Products (McGraw Park, Ill.). Multiwell plates were purchased from Corning Scientific (Corning, N.Y.) Dynatech Laboratories (Chantilly, Va.), and Costar (Cambridge, Mass.).

Biotinylation of goat anit-choleragenoid antiserum

Goat anit-choleragenoid antisera (11,600 AU/ml) containing 0.1% azide were desalted on a G-25 Sephadex column. Elution volumes of sodium azide and goat anti-choleragenoid antisera were determined with potassium ferrocyanide and blue dextran, respectively. A 1-mg/ml solution of lyophilized goat antisera was prepared in 50 m*M* sodium bicarbonate buffer (pH 8.5), loaded on the G-25 column, and eluted with 1-ml fractions of sodium bicarbonate buffer (pH 8.5).

Antisera biotinylation was performed according to a published method [10] by adding sulfo-NHS-biotin (0.32 mg) to a 20-mg/ml solution of antisera in 50 m*M* sodium bicarbonate buffer (pH 8.5). The mixture was incubated on ice for 2 h and unreacted biotin was removed by centrifugation using Centricon-30 microconcentrators. The extent of biotinylation was determined using the avidin-HABA reagent. Moles of biotin per mole of antisera were determined by the difference in absorbance (500 nm) of the avidin-HABA reagent achieved with and without the biotinylated antisera. Biotinylated antisera contained 0.6–1.0 mol biotin/mol IgG.

Solutions

Stock solutions of cholera toxin (1 mg/ml) were prepared by diluting the lyophilized product provided by the NCI with distilled water and were stored in refrigerator (4°C). Solutions of cholera toxin for standard curve samples were prepared by serial dilution of the cholera toxin stock solutions with phosphate-buffered saline (5 m*M* potassium phosphate, pH 7.4, 150 m*M* sodium chloride; PBS) containing 0.05% Tween 20. To 100 µl of the sample medium (cell-culture medium, PBS buffer, plasma, whole-blood lysate, or urine) we added 10 µl of a standard curve dilution and 890 µl of PBS-Tween 20 (0.05%) buffer containing 0.11% goat sera for final cholera toxin concentrations of 1.6 µg/ml to 6 ng/ml.

Solutions of σ-phenylenediamine dihydrochloride (10 mg/ml) were prepared in methanol. The peroxidase substrate solution was prepared by diluting 1 ml of the σ-phenylenediamine dihydrochloride solution and 0.01 ml of a 30% hydrogen peroxide solution to 100 ml with distilled water.

Cholera toxin ELISA

A published sandwich immunoassay procedure [9] was modified for quantitation of cholera toxin as described below. Polystyrene microtiter plates (Corning) were precoated with goat anti-choleragenoid IgG (200 µl/well, 1 µg/ml) diluted in PBS (pH 7.4). After incubation of the plates at 4°C overnight, the wells were washed three time with 0.15 *M* NaCl containing 0.05% Tween 20 (200 µl/well), and the plates were incubated at room temperature for 1 h with a solution of 1% fetal calf serum in 0.15 *M* NaCl containing 0.05% Tween 20. The wells were washed three times with 0.15 *M* NaCl containing 0.05% Tween 20 (200 µl/well). The appropriate standards, unknown plasma, red-blood-cell (RBC), or urine samples (100 µl) were diluted 1:10 with PBS containing 0.05% Tween 20 and 1.0% goat serum, after which 200-µl aliquots were added to wells in triplicate and plates were incubated for 1 h. After washing of the wells three times with 0.15 *M* NaCl containing 0.05% Tween 20 (200 µl/well), 200 µl of a solution of biotinylated goat anti-choleragenoid antiserum diluted 1:1000 in PBS (pH 7.4) was added and plates were incubated for 1 h. After washing of the wells three times with 15 *M* NaCl containing 0.05% Tween 20, 200 µl of a solution of avidin-horseradish peroxide diluted 1:200 in PBS (pH 7.4) was added. After a 1-h incubation, wells were washed three times with PBS containing 0.05% Tween 20 and freshly prepared peroxidase substrate solution (200 µl/well) was added to each well. Microtiter plates were incubated for 20 min at room temperature in a dark environment and then acidified with 25 µl of 8 *N* sulfuric acid. The absorbance value for each well was determined at 490 nm.

Stability in buffered solutions and biological fluids

Cholera toxin was incubated for 24 h at 37°C in 50 m*M* potassium phosphate (pH 7.4), thawed and fresh human plasma, fresh mouse plasma and whole blood, and mouse urine. Aliquots were removed at 0.5, 1, 2, 4, 8, and 24 h following addition of drug to the incubation medium and were stored frozen until analysis.

As part of whole-blood stability studies, cholera toxin concentrations were determined in plasma and RBCs. Following separation of whole blood, 2 vols. of distilled water were added to 1 vol. of the packed RBCs to lyse the cells. The supernatant was isolated by centrifugation (10,000 rpm × 3 min) and frozen immediately.

Pharmacokinetics of cholera toxin

Non-tumor-bearing male $CD2F_1$ mice (20–30 g), supplied by the NCI, were housed five per cage on commercially obtained pure wood-shaving bedding in an on-site facility, with light being provided from 6:00 a.m. to 8 p.m. Food (Purina Rodent Chow) and tap water were provided ad libitum.

Cholera toxin was prepared in normal saline to a final concentration of 0.3 mg/ml. Mice received a 1,500-µg/kg dose of cholera toxin i.v. through a lateral tail vein or s.c. through a skin fold behind the neck. Standard Broome-type restraints were used for i.v. injections, which were completed in approximately 15 s using a 1-cc tuberculin syringe fitted with a 27-gauge needle. Blood samples were obtained prior to and at 3, 6, 9, 12, 15, 20, 30, 60, and 120 min following drug administration from mice (anesthetized with ether vapors) by cardiac puncture using citrated syringes (170 µl CPDA) anticoagulant/ml whole blood) fitted with 19-gauge needles. The whole blood was transferred to microcentrifuge tubes. Plasma was isolated by centrifugation (10,000 rpm × 3 min), and 200-µl aliquots were immediately frozen.

For 24-h urinary recovery studies following i.v. injections of cholera toxin (1,500 μg/kg), mice were placed in a glass metabolism cage (four per cage) and urine was collected in Erlenmeyer flasks kept on dry ice. At the end of the collection period, the urine volume was recorded and the samples were stored frozen (− 20°C) until analysis.

Data analysis

Mean absorbance values (490 nm) were obtained from triplicate determinations of each standard or unknown sample containing cholera toxin. Data were fit by nonlinear least-squares regression to a four-parameter logistic function [7]:

$$OD = \frac{OD_{min} - OD_{max}}{1 + (C/C_{50})^b} + OD_{max} \quad (1)$$

where OD is the mean sample absorbance at 490 nm; C, the sample concentration; OD_{max}, the asymptotic maximal absorbance; OD_{min}, the asymptotic minimal absorbance; C_{50}, the concentration at $(OD_{max} + OD_{min})/2$; and b, a slope factor that determines the steepness of the curve. The asymptotic absorbance values were used for logit data transformation according to the following equation:

$$\text{logit } p = \ln \frac{p}{1-p}, \quad (2)$$

$$\text{where } p = \frac{OD - OD_{min}}{OD_{max} - OD_{min}}.$$

A graph of logit p versus log C was linear and fit to Eq. 3 by least-squares regression as follows:

$$\text{logit } p = a + b \log C, \quad (3)$$

where a is the y-intercept and b is the slope that corresponds to the slope factor of the four-parameter logistic equation. Unknown concentration values were calculated from the corresponding mean OD data following rearrangement of Eq. 3 to give Eq. 4;

$$C = \text{antilog}\left[\frac{(\text{logit } p) - a}{b}\right]. \quad (4)$$

Plasma standard curve samples containing cholera toxin were prepared fresh each day. The day-to-day reproducibility of the OD_{490} values obtained for each standard concentration was determined from the standard error of the mean OD_{490} values from six standard curves. The day-to-day assay reproducibility was assessed by comparing fitted logistic function parameters and the logit transformation slope values of six standard curves prepared with the same batch of goat anti-choleragenoid IgG and biotinylated material. Plasma concentration-time data were fitted by nonlinear least-square regression analysis using the program PCNONLIN.

Results

During our initial investigations of the applicability of a sandwich ELISA technique (data not shown), cholera toxin concentrations of less than 10 ng/ml were detected in buffer samples. However, when samples were prepared in mouse plasma the assay sensitivity was greatly reduced and the reproducibility of repeated analyses was poor. We evaluated several modifications to the treatment of plasma before addition of samples to microtiter-plate wells. The optimal sensitivity and reproducibility occurred when plasma samples were diluted 1:10 with PBS and normal goat serum was added to plasma samples at a final concentration of 0.1% in the plasma/PBS mixture. Sigmoidal relationships between cholera toxin concentration and OD_{490} values were observed over the range of 1.5–400 ng/ml when samples were prepared in buffer and over the range of 6.25–1,600 ng/ml when samples were prepared in diluted fresh mouse plasma (Fig. 1).

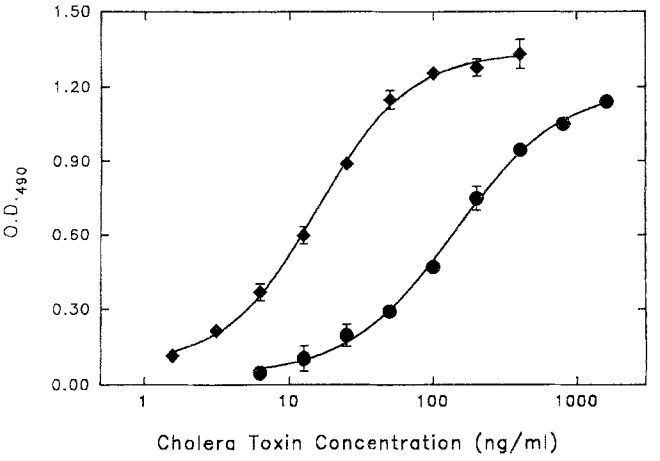

Fig. 1 ELISA dose-response curves generated for cholera toxin in phosphate buffer (*solid diamonds*) and diluted mouse plasma (*solid circles*). Data represent mean values ± SD for triplicate determinations of the OD_{490}. Data were fitted by logistic regression

Substantial variability [median coefficient of variation (CV), 38%; range, 12%–140%] was observed in mean OD_{490} values obtained for the lowest concentration standards (6.25 and 12.5 ng/ml). Excellent reproducibility (median CV, 4.8%; range, 0.7%–25%) of the OD_{490} values was observed for individual standards containing ≥ 25 ng cholera toxin/ml. Sigmoidal relationships were evident from each plasma standard curve, and graphs of the logit transformation of the mean OD_{490} values versus cholera toxin concentration were linear over the entire concentration range. In addition, mean OD_{490} values were within the 95% confidence intervals determined from linear regression of the data (Fig. 2).

The day-to-day reproducibility of the low concentration standards was poor (CV, > 39%), but excellent day-to-day reproducibility was evident from standards containing ≥ 25 ng cholera toxin/ml (median CV, 8.8%; range, 7.0–24%). The day-to-day assay reproducibility determined from fitted logistic parameters or logit slope values indicates that assay variability was < 25% (Table 1). Because of the 10-fold dilution required for plasma samples and the lower inherent sensitivity, the assay was ~ 40-fold less sensitive for murine plasma samples in comparison with buffer samples. Mouse urine was also diluted 1:10 prior to ELISA analysis. The ELISA dose-response curve overlapped

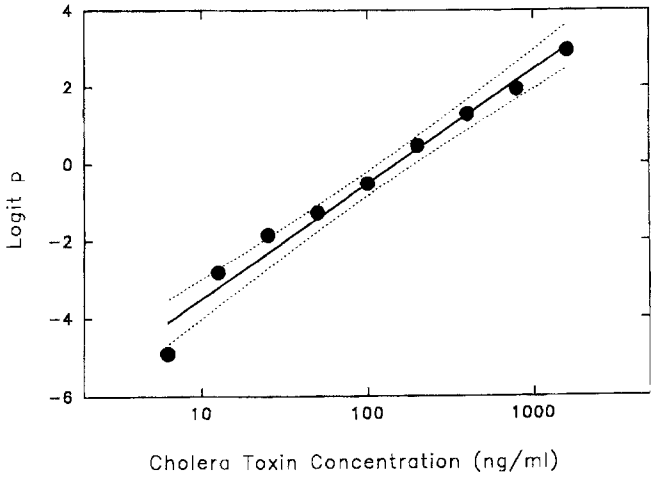

Fig. 2 Logit transformation of the mean OD_{490} values for the murine plasma samples illustrated in Fig. 1. The linear regression and 95% confidence intervals are represented by teh *solid line* and *dahsed lines*, respectively

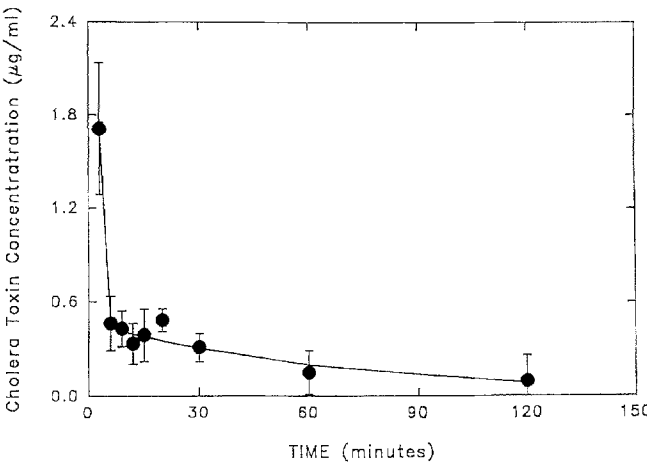

Fig. 3 Plasma profile of cholera toxin following i.v. administration of 1,500 μg/kg to male $CD2F_1$ mice. The *symbols* represent mean values ± SD for four determinations of the concentration at each time point

Table 1 Regression estimates for the four parameter logistic function and logit equation

	Mean (± CV)
Od_{max}	1.22 (0.11)
Od_{min}	0.04 (0.07)
C_{50}	164 (35)
b	− 1.01 (0.24)
b (logit)	2.47 (0.59)

with that of buffer samples. Therefore, the limits of quantitation for the urine and plasma assays were 62.5 and 250 ng/ml, respectively.

Cholera toxin stability was evaluated in pH-7.4 potassium phosphate buffer and biological fluids. Within 1 h of incubation in phosphate buffer the cholera toxin concentration decreased to 10% of the initial value and remained constant for the remainder of the incubation period (data not shown). The presence bovine serum albumin (BSA), (2%) or Tween 20 (0.05%) in the incubation mixture prevented the dramatic decrease in the cholera toxin concentration, and the concentration remained unchanged during the 24-h incubation period. During incubation of cholera toxin in murine plasma, the concentration decreased by 60% in the 1st hand remained constant for the remainder of the incubation period. Accumulation by RBCs was also evaluated. Approximately 25% and 35% of the cholera toxin added to mouse and human whole blood, respectively, was recovered in the packed RBC fraction following centrifugation of whole blood.

Since plasma samples were stored overnight prior to analysis, the effect of a single freeze-thaw cycle on the assayed concentration of cholera toxin was addressed in two experiments. Overnight storage of frozen plasma samples did not appear to a affect the concentration of cholera toxin detected in murine plasma samples.

Following an i.v. 500-μg/kg dose, little cholera toxin was detected in plasma. The dose was increased to 1,500 μg/kg for later experiments. Although this dose is 3-fold greater than the LD_{100} (dose lethal to all mice), acute toxicities were not observed during these studies. Following i.v. administration of 1,500 μg/kg cholera toxin, the plasma profile (Fig. 3) displayed biphasic disappearance, with drug being detected in two of three mice at 1 h after administration and in one of three mice at 2 h after administration. The values calculated for $t_{1/2}\alpha$, $t_{1/2}\beta$, plasma clearance (Cl_p), and steady-state volume of distribution (V_{ss}) by non-linear least-squares regression analysis of the data from four experiments were 0.7 min, 49 min, 77ml min^{-1} m^{-2} (24 ml $min^{-1}kg^{-1}$), and 2,920 ml/m^2 (912 ml/kg), respectively. Interestingly, plasma concentrations of cholera toxin increased at 15–20 min after i.v. drug administration in all experiments. Following s.c. administration of cholera toxin at the same dose (1,500 μg/kg) employed for the i.v. studies, no drug was detected in plasma during a 24-hour period after administration. Less than 0.1% of the delivered i.v. dose was recovered in urine collected over a 24-h period after drug administration.

Discussion

Cholera toxin has been given to several mammalian species for investigative purposes [3, 4, 12, 16, 21, 26], although the plasma concentrations and plasma disposition of this macromolecule were not characterized in those studies. Several immunoassay methods that

afford low picogram-per-milliliter determination of cholera toxin have been reported [1, 8, 20, 23, 24], but none has been utilized for determination of this macromolecule in biological fluids.

Our ELISA procedure afforded low nanogram-per-milliliter detection of cholera toxin in cell-culture medium and buffer. The assay's sensitivity and reproducibility were reduced 40-fold when analyses were performed with murine plasma samples. Although addition of normal goat serum (to bind mouse anti-goat antibodies) and dilution of samples with PBS-Tween (to prevent adsorption) improved the sensitivity and reproducibility of the cholera toxin ELISA, the sensitivity was nonetheless lower than desired, most likely due to components in plasma that react in a specific manner with cholera toxin and may interfere with antigen-antibody reactions important for immunoassays [2, 5]. For example, plasma proteins may bind nonspecifically to unprotected adsorption sites or to antibodies, and plasma may contain antibodies to goat sera or to IgG. It may not be possible to prevent such interactions during in vivo studies of cholera toxin.

Several procedures have been used to fit immunoassay dose-response data [25]. The four-parameter logistic equation [7] is most useful since it uses the full range of dose-response data. Furthermore, logit transformation of the OD_{490} data plotted versus log concentration yields a linear graph providing a simple, accurate means of determining cholera toxin concentrations in murine plasma samples [13].

While the sensitivity of the ELISA was lower than desired the assay provided sufficient sensitivity for determination of cholera toxin in plasma following i.v. administration to mice. The cholera toxin dose used for the pharmacokinetics investigations (1,500 μg/kg) was 3-fold greater than the i.v. dose lethal to non-tumor-bearing mice (500 μg/kg; J. Plowman, personal communication). However, toxicity was not observed following i.v. or s.c. administration of drug during the period required for the pharamacokinetics studies.

Characteristic features of the plasma disposition of cholera toxin in mice included rapid distribution, slow elimination, and an apparent increase in plasma concentration at approximately 20 min after drug administration. The rapid distribution may be due to binding of drug to membranes of liver, fat, and red blood cells and plasma glycoproteins [6]. As expected, cholera toxin was not detected in urine. Urinary excretion of macromolecules is dependent on the molecular size and diminishes substantially as the molecular size is increased from 20 to 70 KDa [15]. Cholera toxin, an 84-kDa protein, may exceed the permeability threshold of the glomerular membrane and, therefore, glomerular filtration would not play a role in its elimination. Alternative routes of elimination may include metabolism in the kidney [17] or liver [18]. The transient increase in plasma concentrations observed at 20 min after administration of cholera toxin may have been due to dissociation of the molecule from plasma proteins or target tissues or to other unknown mechanisms.

Of particular interest in preclinical studies is the relationship between pharmacokinetics and antitumor activity in murine tumor models. The antitumor activity of a single i.v. or i.p. dose of 1 μg(~ 50 μg/kg) cholera toxin has been evaluated in mice bearing i.p. YAC murine lymphoma cells [12]. The antitumor activity of multiple i.v. doses of 30–150 μg/kg cholera toxin has been evaluated in mice bearing s.c. NCI-N592 human small-cell lung cancer cells and s.c. NCI-H460 human non-small-cell lung cancer cells using short-term (every day for 5 days) or intermittent (one a week for 6 weeks) schedules (J. Plowman, personal communication). The tumor-growth delay was modest but not significant, and no tumor-free servivor was observed following treatment with cholera toxin according to these doses and schedules.

Extrapolation of the pharmacokinetic data obtained following single high doses of drug in our studies to low multiple-dose regimens employed for antitumor activity studies, although speculative, may provide insight into the antitumor activity of cholera toxin in those studies. Following i.v. administration of 1,500 μg/kg cholera toxin in our pharmacokinetics studies, the peak plasma concentrations were ~ 14 μg/ml, and plasma concentrations fell below 200 ng/ml at ~ 2 h after cholera toxin administration. Assuming linear pharmacokinetics at doses below 1,500 μg/kg, the peak plasma concentrations would be less than 500 ng/ml and plasma concentrations would fall below 25 ng/ml at 1 h after administration of i.v. doses comparable with those examined during in vivo activity studies, e.g., ≤ 150 μg/kg. In vitro 50% growth-inhibitory (IC_{50}) concentrations of cholera toxin against sensitive cell lines were 27–700 ng/ml following a 7-day exposure [14, 27]. Therefore, extrapolation of pharmacokinetic data suggests that the doses and schedules examined during in vivo antitumor studies ([12]; J. Plowman, personal communication) yielded plasma concentrations far below the optimal concentrations necessary to maintain in vitro antiproliferative activity [14, 27]. On the basis of these observations, we hypothesize that continuous exposure or long-term repeated-dose schedules may be more effective than single-dose or short-term multiple-dose schedules in reproducing antiproliferative activity in in vitro studies. Data from rat studies using s.c. administration of cholera toxin are consistent with such a hypothesis. Daily s.c. administration of cholera toxin (10 μg/200-g rat) for 6 weeks to female rats bearing s.c. hormone-dependent mammary tumors produced tumor regression during the administration period [4].

In summary, the ELISA procedure and pharmacokinetic data presented herein are of importance to future in vivo investigations of the pharmacologic and therapeutic effects of cholera toxin or its derivatives. Anti-choleragenoid IgGs are specific for epitopes on

the B subunits of the cholera toxin molecule and, thus, this assay may be useful for quantitation of choleragenoid and cholera toxin conjugate molecules.

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