Arrest of Hormone-dependent Mammary Cancer Growth in vivo and in Vitro by Cholera Toxin

Yoon Sang Cho-Chung, Timothy Clair, Cedric Shepheard, and Bela Berghoffer

ABSTRACT

Growth of 7,12-dimethylbenz(a)anthracene-induced mammary carcinoma in rat was arrested by daily s.c. injections of cholera toxin. At a dose of 2 μg/200-g rat, tumors regressed to 50% of their initial size within 2 weeks, and 85% of tumors regressed completely within 4 to 5 weeks. The same response to cholera toxin was observed with another hormone-dependent mammary tumor, MTW9, but not with the hormone-independent tumors, DMBA No. 1 and MT 13762. The latter result was consistent with the lack of response of these hormone-independent tumors to hormone removal (ovariectomy) or N4',O6'-dibutyryl cyclic adenosine 3':5'-monophosphate treatment. The growth-inhibitory effect of cholera toxin was dose dependent, and upon cessation of treatment tumors resumed growth; after complete regression, however, tumors did not reappear until 6 months after termination of the treatment. An amount of cholera toxin as high as 10 μg/day/200-g rat s.c. injected over a 6-week period showed no systemic toxicity in the animals. The growth of human breast cancer cells (MCF-7) in culture was also inhibited by a daily supplement of cholera toxin. At a concentration of 100 ng/ml, the cell replication ceased completely within 2 days. The growth inhibitions, both in vivo and in vitro, were accompanied by marked increases in the cellular cyclic adenosine 3':5'-monophosphate content and type II cyclic adenosine 3':5'-monophosphate-dependent protein kinase activity as well as a decrease of estrogen binding activity. Thus, extinction of mammary cancer can be achieved by cholera toxin, an agent that stimulates the intracellular cyclic adenosine 3':5'-monophosphate system.

INTRODUCTION

Studies (2) from our laboratory have shown that DBcAMP inhibits growth of mammary carcinomas in the rat. Our subsequent studies (7) have shown that the growth-inhibitory effect of DBcAMP is enhanced by l-arginine which stimulates NAD-dependent ADP ribosylation and adenylate cyclase activation in the hormone-dependent mammary tumors in vivo.

The exoenterotoxin of Vibrio cholerae, cholera toxin, activates adenylate cyclase in many types of vertebrate cells (21). Cholera toxin binds with high affinity to ganglioside GM1 on the cell surface and releases its active fragment A1 subunit (13). The A1 subunit catalyzes a NAD-dependent ADP ribosylation of the regulatory component of adenylate cyclase resulting in strong, long-lasting activation of adenylate cyclase and increase in intracellular cAMP (13).

In this study, we examined whether cholera toxin can inhibit the growth of rat mammary tumors in vivo and human breast cancer cells (MCF-7) in culture and whether the effect of toxin on growth is correlated with the stimulation of the cAMP system.

MATERIALS AND METHODS

Chemicals and Reagents. cAMP was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Cholera toxin, 17β-estradiol, ATP, DEAE-cellulose, theophylline, and calf thymus histone (type II) were purchased from Sigma Chemical Co., St. Louis, Mo. [γ-32P]ATP (30.0 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. [3H]cAMP (26 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill., and [17β-3H]estradiol (17β-[2,4,6,7-3H]estradiol; 91.3 Ci/mmol) was from New England Nuclear, Boston, Mass.

Tumor Models. Primary DMBA-induced mammary carcinoma (15) in random-bred female Sprague-Dawley rats (220 to 250 g) and MTW9 mammary carcinoma (17) transplanted in inbred female Wistar-Furth rats (160 to 180 g) were used as models of hormone-dependent tumors (tumors regress upon hormone withdrawal). DMBA No. 1 (tumor received from Dr. W. F. Dunning in 1967, carried in the Laboratory of Pathophysiology by s.c. transplantation for 172 generations, and then stored at −80°) and MT13762 (23) mammary carcinomas transplanted s.c. into the lumbar region of 3-month-old female F344 rats (∼150 g) were used as models of hormone-independent tumors (tumors continued to grow and do not regress upon ovariectomy).

Human Breast Cancer Cells (MCF-7). The MCF-7 cells (Mason Research Institute, Rockville, Md.) were grown in McCoy's Medium 5A supplemented with bovine insulin (10 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal calf serum (10%); the medium was changed every 48 hr.

cAMP Assay. cAMP was measured by the competitive protein-binding method of Gilman (10) using a cAMP assay kit (Amersham Kit No. TRK 432). Samples (0.2 to 0.5 g of tissues or cells) were dipped into liquid nitrogen, weighed, and homogenized in 5 volumes of 0.25 M sucrose-1 mM MgCl2-1 mM CaCl2-10 mM KCI-20 mM Tris-HCl, pH 7.5. The homogenates were centrifuged at 105,000 × g for 60 min, and the resulting supernatants were used as cytosols.

Preparation of Tumor Cytosol. All procedures were performed at 0–4° by the method described previously (5). Tumors were excised, cleared of necrotic areas, weighed, minced, and then homogenized in a Teflon-glass homogenizer with 5 volumes of Buffer A (0.25 M sucrose-2 mM MgCl2-1 mM CaCl2-10 mM KCl-20 mM Tris-HCl, pH 7.5). The homogenates were centrifuged at 105,000 × g for 60 min, and the resulting supernatants were used as cytosols.

Protein Kinase Assay. Assay of protein kinase activity, with a histone mixture as the exogenous substrate, was carried out as described previously (9). The reaction mixture (0.2 ml final volume) of the assay contained: 100 mM potassium phosphate buffer, pH 7.5; 10 mM magnesium chloride; 1 mM theophylline; 0.6 mg calf thymus histone; 0.5 mM ATP, together with [γ-32P]ATP (19.2 Ci/mmol); and 20 μl enzyme. The mixtures ±1 μM cAMP were incubated at 30° for 5 min in a shaking water bath, and the reactions were stopped by the addition of 0.5 ml ice-cold 20% TCA. These samples were placed in an ice bath for 30 min

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2 The abbreviations used are DBcAMP, N4',O6'-dibutyryl cyclic adenosine 3':5'-monophosphate; cAMP, cyclic adenosine 3':5'-monophosphate; DMBA, 7,12-dimethylbenz(a)anthracene; TCA, trichloroacetic acid.
and then passed through Millipore filters (HAWP 02500, 0.45 μm) that had been premoistened with cold 5% TCA. The filters were washed 6 times with 5 ml cold 5% TCA and dissolved in 5 ml Filtron-X (National Diagnostics, Somerville, N. J.), and the radioactivity was counted in a Beckman LS-355 liquid scintillation spectrometer. The enzyme activity was linear for at least 7 min and proportional to the protein concentration over a range of 20 to 70 μg/reaction mixture.

**Estrogen-binding Assay.** Estrogen binding was measured by the modification (20) of the charcoal adsorption assay described originally by Korenman (18). Cytosol (200 μl; ~1 mg protein) was added to 50 μl Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 1.25 pmol (final concentration, 5 pmol) 17β-[3H]estradiol (91.3 Ci/mmol) ± 250 pmol unlabeled 17β-estradiol; the mixtures were incubated at 0–3°C for 16 to 18 hr. Reactions were stopped by the addition of 0.5 ml of the dextran-coated charcoal mixture (0.25% Norit A and 0.0025% dextran Grade C) (Sigma) in 0.01 M Tris-HCl, pH 7.8). The mixtures were incubated at 0°C for 20 min and then centrifuged at 2000 x g for 10 min at 4°C. The supernatant (0.5 ml) was transferred to scintillation vials, 10 ml of Ultralfluor (National Diagnostics) were added, and the radioactivity was counted in a Beckman LS-355 liquid scintillation spectrometer. The binding was expressed as the specific binding, calculated by the subtraction of a blank value (the amount of 17β-[3H]estradiol bound in the presence of excess nonradioactive 17β-estradiol alone) (20). The specific binding was proportional to the protein concentration over a range of 0.3 to 1.2 mg/0.25 ml reaction mixture.

Protein concentrations were measured by the method of Lowry et al. (19) with bovine serum albumin as standard.

**RESULTS**

Effect of Cholera Toxin on Tumor Growth in Vivo.** Daily injections of cholera toxin into rats bearing DMBA-induced tumors consistently produced growth inhibition of tumors. At a dose of 2 μg per 200-g rat s.c. per day, the growth inhibition was observed within 1 week, and the majority of tumors regressed to <50% of their initial sizes at 2 weeks posttreatment (Table 1). At 4 to 5 weeks after the treatment, 85% of tumors disappeared completely [the percentage changes in mean volume ± S.E. of the toxin-treated and untreated tumors (50 tumors each) were −76 ± 20 and +182 ± 45, respectively, at day 28].

Table 1

Effect of cholera toxin on the in vivo growth of DMBA-induced mammary carcinoma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total tumor no.</th>
<th>Mean tumor measurement (cm)</th>
<th>Mean % of change in tumor volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>50</td>
<td>Day 0: 1.5 ± 1.7</td>
<td>1.8 ± 2.0</td>
</tr>
<tr>
<td>Cholera toxin (2 μg/200-g rat s.c./day)</td>
<td>50</td>
<td>Day 0: 1.3 ± 1.9</td>
<td>1.1 ± 1.5</td>
</tr>
</tbody>
</table>

| a | Spontaneously static or regressing tumors were identified by measuring tumor growth for 7 to 10 days before start of experiment and were not included in the experiment. Tumors over 2 x 3 cm were also not included.
| b | Controls (50 tumors) were tested for hormone dependence; by Day 3, ovariectomy produced regression (>30% decrease in volume) in 42 tumors (85%), whereas it produced growth (>30% increase in volume) in 6 tumors (12%), and 2 tumors (3%) were static. Tumors regressed upon ovariectomy were found to be all adenocarcinomas (11).
| c | Mean ± S.E.
| d | The tumors in the rats receiving injections of heat-inactivated cholera toxin (10 μg per 200-g rat s.c. per day for 14 days) grew like those of untreated controls.

Tumors that did not regress (6 of 50 tumors treated) after cholera toxin treatment were the autonomously growing tumors (~12% of DMBA-induced tumors (4)) as evidenced by their unresponsiveness to ovariectomy (data not shown). The growth inhibition was dose dependent, and 50% inhibition of the growth rate was observed at a dose as low as 50 ng per 200-g rat s.c. per day. The tumor-bearing animals showed no systemic toxic effect, as evaluated by hematocrit levels, body weight, and food consumption, even when given injections of 10 μg per 200-g rat s.c. per day for 30 days. Upon cessation of cholera toxin treatment, tumors resumed growth, indicating that the growth-inhibitory effect is reversible; tumors that completely regressed (44 of 50 tumors treated), however, did not reappear up to 6 months after the treatment was terminated.

Cholera toxin, even injected at the dose of 10 μg/200-g rat s.c. for 11 days did not produce measurable amount of anti-cholera toxin antibody in the sera of animals. The antitoxin units ≤1.8/ml serum were measured in both cholera toxin-treated (20 sera) and untreated (15 sera) groups (negative titer, <1 unit/ml; positive peak titer, >2000 units/ml). Moreover, an infiltration of lymphocytes and macrophages (11), comparable, for example, with that of a skin transplant rejection, was never found in histological sections of regressing tumors (20 tumors examined) after cholera toxin treatment (10 μg/day/200-g rat s.c. for 11 days). These data suggest that the growth inhibition may not be attributed to the host-mediated effect.

Daily injections of cholera toxin (2 μg/200-g rat s.c. for 10 days) also produced regression of the hormone-dependent transplantable mammary tumor, MTW9; the percentage changes of the toxin-treated and untreated tumors (25 tumors each) were −60 ± 12 and +350 ± 80, respectively. No inhibitory effect by the toxin was, however, observed with ovariectomy-unresponsive DMBA No. 1 and MT13762 mammary tumors; the percentage changes of the toxin-treated (10 μg/day/200-g rat s.c. for 14 days) and untreated DMBA No. 1 tumors (25 tumors each) were +250 ± 55 and +255 ± 60, respectively, and those of MT13762 were +270 ± 65 and +265 ± 60, respectively. The latter result was consistent with the lack of response of these hormone-independent tumors to hormone removal (ovariectomy) or DBCAMP treatment (3).

Effect of Cholera Toxin on in Vitro Growth of Human Breast Cancer Cells (MCF-7). Growth of MCF-7 human breast cancer cells in culture was also inhibited by a daily supplement of cholera toxin. At a concentration of 100 ng/ml, the increase in cell number ceased completely within 2 days (Chart 1); at a concentration of 1 ng/ml, the cell number was 60% of that of untreated cells by Day 3 (data not shown). The growth arrest was maintained as long as the treatment was continued but was reversed upon cessation of the treatment (Chart 1). Viability of cells that were treated with cholera toxin was indicated by their exclusion of trypan blue; and the number of cells that were released into the medium during the 72 hr of culture was negligible (<3% of the seeding cell number) in both treated and untreated cells. Thus, growth inhibition of MCF-7 cells by cholera toxin appears to be due to a decrease in the rate of cell replication without affecting cell viability.

The growth arrest of MCF-7 cells by cholera toxin was accompanied by a striking change in cell morphology; the cytoplasm of the treated cells was greatly enlarged without appreciable change in the size of the nuclei (data not shown). This change in

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the decrease in estrogen binding activity was inversely related to the increase of protein kinase, especially the type II enzyme activity in the growth-arrested MCF-7 cells.

**DISCUSSION**

The evidence presented show that daily supplemented cholera toxin at nm concentrations produces a potent inhibition of rat mammary tumor growth *in vivo* and human breast cancer cell (MCF-7) growth *in vitro*. The *in vivo* growth inhibition accompanied neither the stimulation of anticholera toxin antibody production in the sera of animals nor the accumulation of the macrophages in the tumor cells. Thus, the growth-inhibitory effect could not be related to the host-mediated effect of the toxin but rather could be attributed to a direct action of the toxin on the tumor cells.

Indeed, the growth inhibition by cholera toxin correlated with manifold increases in the intracellular CAMP content and CAMP-dependent protein kinase holoenzyme activity as well as a decrease of estrogen binding activity. The similar inverse relationship between CAMP-dependent protein kinase activity and estrogen binding has been observed in hormone-dependent mammary tumor regression after hormone withdrawal (ovariectomy) or treatment with DBcAMP (1).

Cholera toxin failed to produce growth inhibition of hormone-independent mammary tumors that are also unresponsive to ovariectomy or DBcAMP treatment. Thus, the growth-inhibitory effect of cholera toxin correlates with the effect of DBcAMP or ovariectomy.

Cholera toxin was shown to stimulate the growth of human mammary epithelial cells in culture (24). If this action of cholera toxin was due to a stimulation of the intracellular CAMP system, it suggests that the action of CAMP involved in the growth regulation of normal cells is different from that in neoplastic cells; CAMP may promote the growth and differentiation of normal cells while inhibiting growth of neoplastic cells. It was shown (16) that certain combinations of hormones, such as progesterone and 17β-estradiol, produced very vigorous growth of normal mammary glands while completely destroying mammary cancer cells. It would appear that hormones or agents involved in normal cell growth and differentiation may often produce death of neoplastic cells.

It is striking that cholera toxin produced permanent arrest of mammary cancer growth by stimulating mammary cell differentiation and also by inhibiting cell proliferation. It is possible that cholera toxin might be used as an antitumor agent and that it might also be useful in the treatment of hormone-responsive tumors. It is hoped that further studies will elucidate the mechanism of action of cholera toxin in these systems.
mammary cancer growth in rat without producing any appreciable toxicity in the animals. Moreover, MCF-7 cells demonstrated full viability in dye exclusion tests after cholera toxin treatment. These results indicated that the growth-inhibitory effect of cholera toxin could not be related to a direct cytotoxic action.

It was previously reported (14) that one single injection of cholera toxin (1 µg/mouse i.p.) caused an almost complete inhibition of proliferation for up to 4 days of i.p. YAC lymphoma cells in mice, without noticeable toxic effects on the animals. In the present study of a rat system, daily injections of cholera toxin were required to produce growth inhibition even with a higher dose of the toxin. It seems that the metabolism and tolerance of cholera toxin may differ in different species of animals. Cholera toxin may have therapeutic potential for breast cancer in humans. Future studies on the immunological or toxicological tolerance of cholera toxin in higher organisms will facilitate such clinical trials.

ACKNOWLEDGMENTS

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REFERENCES

18. Y. S. Cho-Chung et al.

Table 2

<table>
<thead>
<tr>
<th>Protein kinase activity (pmol/5 min/mg protein)</th>
<th>Estrogen binding activity (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>cAMP (pmol/10^6 cells)</td>
</tr>
<tr>
<td>None</td>
<td>3.5</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>140.0</td>
</tr>
</tbody>
</table>

* - absence; +, presence of cAMP in the assay.

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