Effects of Cytochalasin B in Culture and in Vivo on Murine Madison 109 Lung Carcinoma and on B16 Melanoma

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ABSTRACT

Cytochalasin B (CB), at 100 or at 10 mg/kg single dose s.c. in carboxymethyl-cellulose (2%)/Tween-20 (1%) 24 h after s.c. challenge of B6D2F1 mice with trocar implants of B16F10 tumor s.c., delayed the appearance of measurable tumor nodules by 157 and 93%, respectively, and extended host survival by 65 and 26%. Tumor growth was also delayed when CB treatment was given 1 day after the appearance of palpable tumor nodules. By in vivo bioassay, in vitro cloning, and dye exclusion measurements, solid tumor nodules treated in vivo with CB at either 100 or 10 mg/kg showed the same viability and tumorigenicity as did vehicle-treated nodules 4 and 6 days after drug treatment, at which time growth inhibition was still apparent. This indicates that growth inhibition by CB is not dependent on a gross cytotoxic effect. CB itself has ever been evaluated in mice for in vivo pharmacodynamic effects of any kind. Some direct attempts at cancer chemotherapy against L1210 leukemia, Ehrlich carcinoma, sarcoma 180, and some other neoplastic model systems in rodents have been made using cytochalasin D (27–30). However, these were tests designed to evaluate single-agent cytotoxic antitumor activity, a property unlikely to be important in cytochalasins, which are not especially cytotoxic. These early chemotherapeutic studies did not reveal direct cytotoxic antitumor activity in vivo.

In experimental designs more relevant to the unusual properties of the cytochalasins, B16F10 murine melanoma cells (31) and TA3 murine mammary ascites carcinoma cells (32) were treated with CB in vitro and transplanted in vivo, but, unfortunately, without the effect-sustaining direct administration of CB in vivo. Intravenous challenge with B16F10 cells treated with CB under these conditions showed increased extrapulmonary metastases (31). However, many of the important morphological and functional effects of cytochalasins in vitro are known to be readily reversible (references detailed in Ref. 2). Thus, in vitro alteration of cells with cytochalasins followed by implantation in vivo without the additional administration of cytochalasins in vivo is not likely to reveal the full potential of cytochalasin effects on cell functions in vivo.

A full evaluation of the in vivo utility of these agents will require that bioactive concentrations be achieved in target tissues and maintained for times sufficient to produce pharmacodynamic effects. Such in vivo evaluation necessarily requires that the agents be available in amounts that are generally impractical to obtain from commercial sources. It also requires knowledge concerning formulation, administration, and dose-limiting host toxicities as a function of formulation, route of administration, and dose scheduling. Moreover, evaluation of in vivo interactions of cytochalasins with other antitumor agents necessarily requires that the effects of the cytochalasins as single agents be known. We have developed improved procedures for the production of CB and have determined its distribution as a function of time in 14 tissues following single dose bolus i.p. injection (2). In the present work we have determined the maximum tolerated doses of CB administered by different routes as a bolus drug in a variety of formulations. The earlier CB production and tissue distribution work along with the toxicity studies presented here permit us to examine the anti-

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The abbreviations used are: CB, Cytochalasin B; CM-cellulose, carboxymethylcellulose; M109, Madison 109; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

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neoplastic cells (6–26). Effects on cell motility, adherence, secretion, and drug efflux, among other effects, suggest to us that the cytochalasins might produce significant responses in experimental cancer chemotherapy model systems in vivo either as individual agents or, more likely, as agents amplifying responses produced by known antitumor drugs.

For reasons detailed earlier (2), very few in vivo studies have been carried out with the cytochalasins. In vivo toxicities of various cytochalasins, cytochalasin derivatives, and chaetoglossins (27–29) have been investigated, but it does not appear that CB itself has ever been evaluated in mice for in vivo pharmacodynamic effects of any kind. Some direct attempts at cancer chemotherapy against L1210 leukemia, Ehrlich carcinoma, sarcoma 180, and some other neoplastic model systems in rodents have been made using cytochalasin D (27–30). However, these were tests designed to evaluate single-agent cytotoxic antitumor activity, a property unlikely to be important in cytochalasins, which are not especially cytotoxic. These early chemotherapeutic studies did not reveal direct cytotoxic antitumor activity in vivo.

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tumor effects of cytochalasin B as a single agent in vivo in anticipation of later studies with in vivo drug combinations.

The present work details our results in culture, in vivo with the B16F10 melanoma syngeneic in C57BL/6 mice, and in vivo with the Madison 109 murine lung carcinoma syngeneic in BALB/c hosts, a carcinoma which is invasive and metastatic from a s.c. implant. Using these systems we have determined effects on growth kinetics, localized invasion, spontaneous metastases, and host survival.

MATERIALS AND METHODS

Animals and Tumors. The M109 lung carcinoma arose spontaneously in 1964 in the lung of an 18-month-old BALB/c mouse. The tumor was characterized as an alveologenic carcinoma and has been highly consistent in its histology and growth characteristics (33, 34). Its value as a chemotherapeutic model system has been examined using virtually every known clinically active antineoplastic agent in a thorough analysis by Rose (35). The M109 lung carcinoma used in this work was obtained from Dr. William Rose (Bristol Laboratories). All of our work with M109 was conducted with BALB/c and CDF1 (BALB/c female × DBA/2 male) mice. The M109 carcinoma was maintained by serial s.c. passage of 20% tumor brei (approximately 1 × 10^6 total cells) in BALB/c mice every 12 days (35). Experimentation was done on CD2F1 mice of Cancer Treatment, Tumor Depository. This subline was maintained generally around 20%.

Suspension. Viability of the tumor suspension by dye exclusion was tested, the 20% cell suspension was diluted to allow injection of 1 × 10^5 trypan blue-excluding cells per mouse. Trypan blue exclusion was determined using 0.2% dye solution in a 1:1 (v/v) ratio with cell suspension. Viability of the tumor suspension by dye exclusion was generally around 20%.

B16F10 murine melanoma selected by Fidler (36) for high lung-colonizing potential from i.v. challenge was received from the Division of Cancer Treatment, Tumor Depository. This subline was maintained in culture in RPMI 1640 medium, containing 20 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 100 μg/ml streptomycin, 100 units/ml penicillin, 250 μg/ml fungizone, 10% newborn calf serum (Grand Island Biological Company), and 2 g/liter sodium bicarbonate (hereafter called complete medium), and 5% carbon dioxide at 37°C (pH 7.2). All media were filter-sterilized immediately prior to use. Cells were harvested in log phase by detaching with 0.25% trypsin/0.02% EDTA for 1 min or less and then adding 10 ml of complete medium. The line was maintained by subculturing late log phase cells by 1:10 dilution every 5–6 days. The original cell sample from the National Cancer Institute was expanded and frozen in complete medium brought up to 50% fetal bovine serum, 10% DMSO, v/v. After 10 passages, new stock was thawed, washed in complete medium, and maintained in vitro. B16BL/6, a subline selected for bladder connective tissue invasiveness (37), was also obtained from the Division of Cancer Treatment, Tumor Depository, and maintained as above for the F1O subline.

The F1O and BL6 sublines were used as challenge tumors either as suspensions prepared from cultured cells, or as trocar implants of solid tumor pieces prepared from s.c. tumors elicited by injection of suspended cultured tumor cells. Subcutaneous trocar implantation of a solid piece of tumor (0.5 mm³) on the ventral surface of C57/BL6 mice was performed under Nembutal anesthesia with a 13-gauge tumor implant needle (trocar). In experiments where in vivo bioassay of treated tumors was performed, single-cell suspensions were prepared by aseptically removing the treated tumors, mincing in sterile isotonic NaCl solution, and passing the suspension through a sterile nylon filter to remove cell clumps and connective tissue.

Cytochalasin B. The compound was prepared in lots of 400 to 600 mg from mold matts of Drechslera dematioides by the method of Lipski et al. (2), was purified by preparative TLC, and was analytically pure at the 99% level as detailed in that work. Radioactive CB was prepared as described previously (2).

In Vivo Growth Curves. B16F10 cells were routinely harvested from flasks at late log phase, seeded into 24-well culture plates (Corning) at a concentration of 2.5 × 10^5 trypan blue excluding cells/cm² into wells containing 2 ml complete medium, and allowed to attach overnight. After 24 h the medium was removed and replaced with 2 ml of fresh complete medium. The wells were treated with various concentrations of CB dissolved in DMSO. CB solutions were prepared from a stock solution of 1 mg/ml, which was stored frozen and protected from light. CB was added in 5- to 20-μl aliquots and all growth experiments involved control wells, which received the equal volume of DMSO (0.25% to 1% final DMSO concentration). Identical wells were harvested in triplicate or quadruplicate, counted in a Coulter counter, and averaged to determine absolute cell number for each CB concentration. Viability was determined by trypan blue exclusion.

Reversibility in vitro. Cells were grown and monitored as in growth curve experiments but were washed at various times. Washing involved removal of medium, addition of 2.0 ml fresh complete medium, gentle pipetting, removal of wash medium, and addition of fresh medium. This procedure was repeated two additional times. Determination of CB in Treated Cells and Media. Plates were seeded and treated as in in vivo growth curve studies. For each concentration three wells were harvested and pooled at the indicated times. Medium with residual CB was separated from cells and each was analyzed separately. For analysis of CB in the medium, 5 ml of HPLC grade methanol were added to the 6 ml of pooled medium and centrifuged at 1000 × g for 5 min and the supernatant was collected. HPLC grade water (15 ml) was added to give a 20% methanol final concentration. This was then passed through a C18 Sep-Pak (Waters) that had been washed with 4 ml methanol followed by 6 ml HPLC grade water.

Analysis of CB in cells, deriva cells were harvested as in growth experiments and 5 ml methanol were added. This mixture was homogenized with a Polytron and centrifuged and the supernatant was collected. This solution was made 20% methanol by adding water and passed through a Sep-Pak cartridge as above. Extracts from cells or media were treated identically after this point. After drying overnight in a vacuum desicator, the samples were eluted from the C18 cartridge with 2 ml HPLC grade ethyl acetate. The eluates were dried down under nitrogen and redissolved in 70 μl methanol and 50 μl were loaded onto an analytical Linear K silica gel TLC plate with a sample-concentrating pre-run zone. The plate was run in a solution of 15% methanol/85% toluene (v/v) or 3/1 benzene/acetone, dried, and stained with p-anisaldehyde as detailed previously (2). CB (1 and 5 μg) was added to parallel cell and media samples as controls and to TLC plates directly as a marker.

In Vivo Chemotherapy Testing. When testing was done on animals challenged with either B16F10 or M109 tumors passaged in vivo, the animals were challenged either with a single suspension (1 × 10^5 trypan blue-excluding cells for B16F10 or 1 × 10^6 trypan blue-excluding cells for M109) s.c. or i.p., or s.c. with an implant of tumor pieces (0.5-mm cubes) with a 13-gauge trocar on day 0. Tumor treatment was initiated either on day 1 or at the time of appearance of palpable tumor nodule. Animals were sedated for trocar implant with Nembutal at 40 mg/kg. One day prior to tumor challenge, hair in the region of trocar entry and at the site of tumor deposition was removed with electric clippers and chemical depilatory to permit sterile sealing of the entry wound and to facilitate caliper measurement of tumor dimensions.

CB Treatment. The CB was administered in solution in DMSO (s.c.) or suspended in 0.85% sterile NaCl solution (i.p. and s.c.) or in sterile pyrogen-free 2% CM-cellulose/1% Tween-20 (s.c., i.p., or i.v.) (a gift from Dr. Thomas Rice, Southern Research Institute. The CM-cellulose/Tween was found to be the most reliable vehicle for administration of CB in suspension, since this vehicle minimized adherence of CB to the syringing walls and permitted delivery of 80 to 90% of the nominal dose, as determined by TLC analysis of the residual CB in the syringes. For i.v. administration of CB in solution, the CB was dissolved in...
ethanol and diluted to 48% ethanol using warm pyrogen-free NaCl solution immediately prior to tail-vein injection (0.1 ml total vehicle). Dilution to 33% ethanol also retained CB in solution but was not used in this work.

Suspensions were achieved by weighing CB into a syringe, loading the vehicle into a second syringe, connecting the syringes with a double-hubbed emulsifying needle, forcing the suspension back and forth repeatedly through the coupling tube until flow was impeded, and then progressively decreasing the bore diameter from 21 gauge to 23 gauge and finally to 25 gauge. Syringes and couplers were washed with methanol and the methanol wash was analyzed by TLC to determine residual CB that was not actually delivered to the test animals.

CB Toxicity in Vivo. Maximum tolerated single bolus doses (LD_{50-10}) as a function of vehicle and route of administration were estimated by cumulative experience with CB treatment of tumor-bearing animals (either M109 in CD2F1 or B16 melanoma in B6D2F1, mice). Maximum tolerated doses estimated in tumor-bearing animals were confirmed in non-tumor-bearing CD2F1, B6D2F1, or C57B1/6 mice, 20 to 24 g, of either sex. The effects of subacute CB doses were monitored by determination of animal weights daily for 2 weeks. The vehicles employed [CM-cellulose/Tween or 1:1 (v/v) 95% ethanol/0.85% NaCl solution] were pyrogen free (Limulus amebocyte lysate assay; Whittaker M. A. Bioproducts, Walkersville, MD).

Effects on Primary Tumor and Metastases. Primary s.c. tumor growth was monitored by noting the day when tumor first became measurable. Growth rate was monitored by calculating tumor weight, using measurement of two perpendicular dimensions and the equation \((L \times w^2)/2\) (38), and by host survival where animals were terminated when tumors reached 25 mm in one dimension or when an animal appeared to be in distress. The validity of the tumor growth rate measurements was tested by comparing actual weights of tumors obtained at autopsy to values obtained prior to the terminal stages. Intrapertoneal and i.v. tumor growth was determined by host survival, which included animals terminated at the point where distress was apparent and end stages predictable within 2-3 days. Invasion from s.c. implants of M109 tumor was readily apparent as breaking through the abdominal wall and into the peritoneum. Metastases in the liver, spleen, lungs, and pancreas were determined either by necropsy of terminal stage animals or by necropsy of animals sacrificed at a constant time point, generally 23 to 30 days after tumor implant, as indicated. Organs were fixed in Bouin’s solution (75 ml saturated picric acid, 25 ml formalin, and 5 ml glacial acetic acid) and metastatic foci were counted under a dissecting microscope. Metastases are presented as the proportion of animals with one or more metastatic nodules and also as the mean number of nodules per organ.

Measuring Viability of Tumor Cells in Vivo. Tumor cell viability was determined by three methods: trypan blue exclusion, plating efficiency, and growth of tumor in secondary recipients. In all cases, s.c. tumors were removed from initial experimental mice, minced, and dissociated to form a single-cell suspension in sterile isotonic saline. A 0.1-ml aliquot of suspension was added to 0.1 ml of 2% trypan blue. Total cells and viable cell counts were made on a hemacytometer. Secondary isogenic recipients were inoculated with 1 x 10^6 viable cells s.c. and monitored for tumor appearance and host survival.

To determine plating efficiency, cells were plated in 6-well tissue culture plates (Costar) at a density of 1 x 10^3 cells/cm². Cell colonies (50 or more cells clustered) were counted on day 4 of culture. Plating efficiency was defined as the ratio of the number of colonies to the number of cells plated, expressed as percentage.

Statistics. Significance was determined by Student’s t test and by Wilcoxon-Mann-Whitney two-sample test.

RESULTS

Effect of CB on B16F10 Growth and Morphology in Vitro. B16F10 cells were treated in vitro with concentrations of CB ranging from 100 nm to 4 μM 24 h after seeding in vitro, at which time the cells had a density of 3 x 10^4/cm². Fig. 1 shows the dose-response effects of continuous exposure to CB for 3 and 4 days. A sharp cytostatic dose-response effect was observed, where cells treated by continuous exposure to 200 nm CB grew to 70% of the density reached by DMSO-treated controls after 4 days, while cells treated at 1 μM grew to only 10% of the control cell density. CB at 4 μM was completely cytostatic, but no evidence of cell destruction was seen even after 4 days of drug exposure in vitro. An IC_{50} value of 460 nm was determined after 3-day exposure and 370 nm after 4 days. A departure from linearity was observed at the IC_{50} point when exposure to CB was for 3 days. This does not affect comparisons of IC_{50} values but does suggest a concentration-dependent self-limiting effect, possibly cell cycle based, and has been observed by us with other neoplastic cell lines.

Final DMSO concentrations in vitro ranged from 0.25 to 1.0% and produced a maximum inhibition compared to untreated control cells of less than 30%. Cells exposed to CB at levels above 100 nm showed alterations in morphology. The cells became rounded, rather than exhibiting the fibroblastic spreading characteristic of DMSO-treated control cells or of untreated cells, and the CB-treated cells had additional pseudopodal appendages. These morphological changes appeared to be directly proportional to both the concentration and the time of exposure (data not shown).

Reversibility of the Cytostatic Effect in Vitro. According to a number of studies (cited in Ref. 2), most of the effects of CB on cells are rapidly reversible upon removal of the CB-containing growth medium followed by washing and refeeding of the cells with fresh medium. In order to test the reversibility of the cytostatic effects produced by CB, cells were exposed to 1.0 or 5.0 μM CB for 7, 24, or 48 h prior to removal of the CB-containing medium, washing, and refeeding with fresh medium (Fig. 2). After a 24-h period to allow attachment and equilibration of the washed cells, growth rates for the various cell treatment groups were determined. The reversibility of the effects of CB in this system was a function both of CB concentration and of duration of exposure to CB. Cells exposed to 1.0 μM CB (Fig. 2A) for either 7 or 24 h and then washed grew within 3 days after CB removal to a 4-fold greater density than cells continuously exposed to 1.0 μM CB (unwashed controls). Under the same conditions, the cells which were exposed to 1.0 μM CB for 48 h prior to removal of CB took 6 days for the washed cells to achieve a 4-fold increase in density compared to cells continuously exposed to CB at 1 μM. A drug exposure
of only 7 h at 5.0 μM CB (Fig. 2B) was sufficient to produce the same level of growth inhibition as was produced by 1.0 μM CB after 48 h of exposure. When cells were treated with 5.0 μM CB for 48 h prior to washing, there was virtually no regrowth even 6 days after reseeding in the absence of CB.

TLC Analysis of Washed and Unwashed Cultures. To determine the effectiveness of the washing procedure, cell cultures were treated with CB and washed. CB levels in the original drug-treated medium, in the combined washings, and in the washed cells were determined by TLC analysis (see “Materials and Methods”). CB was detectable only in the original drug-treated medium, and the combined washings. It was below the limit of detection, that is less than 1 μg of CB/10^6 cells, in the methanol extracts of the washed cells.

The completeness of CB removal by washing was also confirmed using tritium-labeled CB. More than 99% of the added radioactivity was recovered in the medium and washings when cells were treated with labeled CB and washed with complete medium (data not shown).

Maximum Tolerated Doses, Routes, and Vehicles. The maximum tolerated doses of CB by i.p., s.c., or i.v. bolus injection were established for various drug formulations as suspensions or solutions. The vehicles tested for drug suspensions were 2% CM-cellulose/Tween 20, 0.85% NaCl solution, and 10% Intralipid (Cutter Laboratories). All vehicles were pyrogen-free. CB solutions were prepared in DMSO for i.p. or s.c. testing and, for i.v. injection, in 1/1 (v/v) ethanol/sterile pyrogen-free 0.85% NaCl solution, after initial solution in 95% alcohol. Suspensions in NaCl solution or in Intralipid proved to be unsatisfactory, since variable proportions of the suspended drug up to 80% on occasion remained adsorbed to the delivery system. More than 80% of the drug was consistently delivered to test animals with the CM-cellulose/Tween vehicle, as determined by quantitative TLC analysis of drug that remained trapped in the delivery system.

Table 1 presents the detailed cumulative results obtained in our study of the in vivo toxicity of CB. Where deaths occurred at a given dose level in the first two mice tested, no additional testing was done at that dose. Maximum weight loss in groups of animals where no deaths occurred generally was observed 2 or 3 days after drug treatment and was less than 5%. Drug suspension in CM-cellulose/Tween administered at 50 mg/kg/ day of CB to normal or tumor-bearing mice was the maximum consistently tolerated i.p. dose (<LD0). The presence or absence of tumor did not appear to affect drug toxicity, but there did appear to be slightly greater sensitivity to the drug in CD2F1 mice. The drug was consistently administered by i.v. injection in DMSO solution in 1/1 (v/v) 95% ethanol/0.85% NaCl solution, pyrogen-free. CB was administered over the venal wall of peritoneum except as noted. Drug administered over the lateral wall of the peritoneum.

Table 1 Cytochalasin B: bolus dose-limiting acute toxicities in mice

<table>
<thead>
<tr>
<th>CB treatment (mg/kg/day)*</th>
<th>Vehicle</th>
<th>Drug deaths fraction</th>
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<tr>
<td>CB administered i.p.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>CMC/Tw</td>
<td>2/34^d</td>
</tr>
<tr>
<td>50^a</td>
<td>CMC/Tw</td>
<td>0/24^d</td>
</tr>
<tr>
<td>100</td>
<td>CMC/Tw</td>
<td>5/9^d</td>
</tr>
<tr>
<td>25</td>
<td>DMSO</td>
<td>1/5</td>
</tr>
<tr>
<td>50</td>
<td>DMSO</td>
<td>6/7</td>
</tr>
<tr>
<td>CB administered i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CMC/Tw</td>
<td>0/5</td>
</tr>
<tr>
<td>20</td>
<td>CMC/Tw</td>
<td>0/5</td>
</tr>
<tr>
<td>40</td>
<td>CMC/Tw</td>
<td>2/2</td>
</tr>
<tr>
<td>50</td>
<td>CMC/Tw</td>
<td>2/2</td>
</tr>
<tr>
<td>10</td>
<td>EtOH/NaCl</td>
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</tr>
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</tr>
<tr>
<td>40</td>
<td>EtOH/NaCl</td>
<td>2/2</td>
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<tr>
<td>CB administered s.c.</td>
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<td></td>
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<td>100</td>
<td>CMC/Tw</td>
<td>0/31^d</td>
</tr>
<tr>
<td>150</td>
<td>CMC/Tw</td>
<td>4/28</td>
</tr>
<tr>
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<td>DMSO</td>
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<tr>
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<td>0/16^f</td>
</tr>
<tr>
<td>150</td>
<td>DMSO</td>
<td>6/10^f</td>
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* Day 1 only unless noted otherwise.
^a CM-cellulose, 2%/Tween-20, 1%, tested pyrogen-free.
^b CD2F1, normal or M109 tumor-bearing.
^c B6D2F1, normal or B16F10 tumor-bearing.
^d Days 1 and 7 or days 1, 4, and 7.
^e C57Bl/6.
^f 1/1 (v/v) 95% ethanol/0.85% NaCl solution, pyrogen-free.
^1 Drug administered over ventral wall of peritoneum except as noted.
^2 Day 1 or days 1 and 4.
^3 Drug administered over the lateral wall of the peritoneum.
Animals under nembutal anesthesia were implanted with a solid piece of B16F10 tumor s.c. using a 13-gauge trocar. Treatment with CB in 2% CM-cellulose/Tween 20 at 10 or 100 mg/kg or with vehicle alone was given s.c. peritumorally 1 day after tumor implant. Animals were considered positive for tumor nodule appearance when a definite mass could be palpated. Either treatment was significantly different from vehicle controls (P < 0.005, n = 6).

Fig. 3. Effect of CB s.c. on the time of B16F10 tumor nodule appearance. Animals receiving 150 mg/kg CB s.c. and were sacrificed 12 days after tumor implant to permit direct measurement of tumor burden, there was also a substantial inhibition of tumor growth. The treated group had tumors with mean weights of 159 mg, compared to vehicle controls which had mean tumor weights of 1.25 g.


doses, weight loss was minimal in animals surviving the acute initial response to CB. Multiple daily doses of CB were administered i.v. with no apparent cumulative toxicity (data not shown). Since there is a very sharp dose-response effect for the toxicity of CB administered i.v., the drug was tested for in vivo effects on tumor growth at 5 mg/kg, which is one half the maximum tolerated dose in CD2F1 mice.

CB in CM-cellulose/Tween suspension at 150 mg/kg administered s.c. ventrally over the peritoneal wall was approximately the LD15 dose, and this dose could be used for in vivo testing, but 100 mg/kg was more reliably tolerated. CB dissolved in DMSO and administered s.c. was also tolerated at 100 mg/kg, but only if injected laterally on the peritoneal wall. Injection in DMSO s.c. at 100 mg/kg ventrally over the peritoneum was relatively more toxic, presumably due to rapid penetration into the peritoneal cavity by the dissolved drug.

Effects of CB on B16F10 Implanted s.c. CB administered as a single bolus dose at either 100 or 10 mg/kg s.c. under the tumor challenge site, 24 h after trocar implant of B16F10 solid tumor s.c., had marked effects on tumor latency, tumor growth rate, and host survival. Fig. 3 shows the delay in the appearance of measurable tumor nodules in the treated groups compared to vehicle (CM-cellulose/Tween 20)-treated controls. The median of tumor appearance in the controls was 7, whereas treatment with CB at 10 mg/kg delayed the median appearance day to 13.5, and at 100 mg/kg to day 18 (P < 0.005). Once the tumor nodules began to grow, their growth rates were comparable in either of the treated groups, compared to controls (data not shown), but there was a marked increase in the duration of host survival in both of the treated groups (Fig. 4). Animals receiving 100 mg/kg CB on day 1 exhibited a median survival of 28 days, compared to vehicle control survival of 17 days (165% survival treated/control). At 10 mg/kg CB, survival was 126% (treated/control) (P < 0.005).

In a separate experiment where animals received 150 mg/kg CB s.c. and were sacrificed 12 days after tumor implant to permit direct measurement of tumor burden, there was also a substantial inhibition of tumor growth. The treated group had tumors with mean weights of 159 mg, compared to vehicle controls which had mean tumor weights of 1.25 g.

Cytochalasin B Effects on M109 Lung Carcinoma. Since neither the B16F10 nor the B16BL6 lines exhibited spontaneous invasion and metastases from s.c. implants, we investigated the effects of CB in vivo on invasion and spontaneous metastasis using the M109 lung carcinoma model. The pattern of growth and metastases of the Madison 109 lung carcinoma is dependent upon the route of tumor injection. Table 2 presents the median survival times and the patterns of metastatic spread observed for the different routes of tumor challenge. Intraperitoneal tumor spread more extensively and killed more rapidly than either i.v. or s.c. tumor challenge. The i.p. tumor spread...
primarily to peritoneal sites, whereas the i.v. injection produced tumor colonies (artificial metastases) in the lungs of all challenged animals and in the livers of some of them. The s.c. challenge metastasized mainly to the lungs. These results are consistent with the findings of other investigators (33, 39, 40).

When the s.c. M109 tumor was treated s.c. with CB suspended in CM-cellulose/Tween, following procedures used for the B16F10 tumor models, tumor appearance was delayed by 4 days (appearing by day 6 in the vehicle controls and in untreated controls compared to day 10 in the CB-treated animals). As shown in Fig. 5, CB treatment produced a significant decrease in tumor growth rate when animals were treated with CB either on day 1 or on the day when a measurable tumor nodule was detected (generally around day 6). Increased median survival time was seen in CB-treated animals, either those treated on day 1 or those treated when a tumor nodule was detected (Table 3). Of the animals treated in this latter group, 33% (2 of 6) were tumor-free on day 50. In all experiments a lower tumor weight at death was consistently observed in all of the treated groups. The difference was significant when tumors excised from treated and control animals sacrificed on day 23 were compared. The difference was no longer statistically significant (P< 0.2). Slight increases in pancreatic metastases were seen both at day 28 and at the terminal stage when animals were treated with CB at 150 mg/kg (data not shown). It should be noted that 150 mg/kg s.c. was an LD₄₀ in this experiment, so the effects on lung and pancreatic metastases may be secondary to the toxic effects of CB in the animals that survived drug treatment. Spleen and liver were also examined for metastatic nodules in all groups, and no effect of CB administered s.c., either decreasing or enhancing metastasis to these organs, was observed (data not shown).

![Fig. 6. Spontaneous pulmonary metastatic nodules in animals shown in Fig. 5B, determined by necropsy either at day 28 after tumor challenge or at the terminal stage (days 29-36 for vehicle controls; 28-50 for CB-treated). The difference in the number of nodules determined by simultaneous necropsy of treated animals versus vehicle controls on day 28 is significant (P < 0.05). The fraction above the bars represents the number of animals bearing pulmonary tumors over the number of animals in the group.](https://example.com/fig6.png)

The effects of peritumoral CB administered s.c. in the region of a s.c. M109 tumor challenge or growing tumor nodule have now been confirmed with an intradermal tumor model, where CB administered s.c. does not directly contact the growing tumor nodule. Single or multiple bolus doses (days 1, 3, and 5 after tumor nodule appearance) retard tumor growth, prolong survival, and produce a significant level of tumor regressions. The effects of CB on Tumor Cell Viability in Vivo. The cytostatic rather than cytotoxic effects of CB in vitro suggest that in vivo effects on tumor growth do not arise from cytotoxic effects. However, localized very high nominal concentrations of CB are

### Table 3 Cytochalasin B s.c. versus Madison 109 lung carcinoma s.c. (1 × 10⁴ viable cells)

<table>
<thead>
<tr>
<th>CB treatment*</th>
<th>Necropsy (day)</th>
<th>Median survival</th>
<th>Lifespan</th>
<th>Tumor weight at death (g)</th>
<th>Fraction with invasion</th>
<th>Tumor-free survivors, day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/kg</td>
<td>1</td>
<td>23</td>
<td></td>
<td>2.6 ± 0.5*</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>23</td>
<td></td>
<td>0.6 ± 0.2*</td>
<td>1/10</td>
<td></td>
</tr>
<tr>
<td>150 mg/kg</td>
<td>1</td>
<td>28</td>
<td></td>
<td>4.9 ± 1.1</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>28</td>
<td></td>
<td>4.4 ± 2.2</td>
<td>1/3</td>
<td></td>
</tr>
<tr>
<td>150 mg/kg</td>
<td>1</td>
<td>22-36*</td>
<td>30</td>
<td>100</td>
<td>6.4 ± 0.3</td>
<td>0/5</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>28</td>
<td></td>
<td>4.9 ± 1.1</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>1</td>
<td>36-38*</td>
<td>37*</td>
<td>123</td>
<td>3.8 ± 1.0</td>
<td>0/3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>1</td>
<td>28</td>
<td></td>
<td>4.9 ± 1.1</td>
<td>4/5</td>
<td></td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>TAD</td>
<td>28</td>
<td></td>
<td>3.8 ± 1.0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>TAD</td>
<td>29-36*</td>
<td>34</td>
<td>100</td>
<td>6.8 ± 1.0</td>
<td>0/4</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>TAD</td>
<td>28-50*</td>
<td>40</td>
<td>118</td>
<td>3.7 ± 1.3</td>
<td>2/6</td>
</tr>
</tbody>
</table>

* Vehicle, CM-cellulose, 1%/Tween 20, 2%.
* Mean ± SE.
* P = 0.001 compared to vehicle controls.
* Terminal.
* P < 0.05 compared to vehicle controls.
* TAD, tumor appearance day.
conceivable following s.c. treatment under the tumor site, so it was necessary to determine more directly whether CB could function as a localized cytotoxic agent in vivo. Animals were challenged with B16F10 tumor s.c. and the tumors were allowed to grow to measurable size (8 days), at which time they were treated with CB at either 100 or 10 mg/kg s.c. under the tumor site or with CM-cellulose/Tween 20 vehicle. Four or 6 days subsequent to drug treatment, at which time growth inhibition was apparent, tumors were excised and the viability of the cells was determined by plating efficiency, dye exclusion, and in vivo bioassay in recipient mice. Table 4 presents the results of this study. It is apparent that cell viability is not compromised by CB treatment, in comparison with vehicle control-treated cells. In vivo bioassay of the cells recovered from treated tumors shows that the latency period prior to tumor nodule appearance in the recipients is not significantly affected in the CB-treated donor tumors, nor is the survival of the recipient mice significant different when they received tumor from groups treated either with high or low dose CB, as compared to those challenged with vehicle-treated control tumors (P > 0.05).

The absence of cytotoxic effect of CB in vivo is further supported by experiments utilizing multiple bolus doses of CB administered i.p. at 50 mg/kg to animals bearing s.c. B16F10 tumors. Systemic CB administered on days 1, 4, and 7 after s.c. tumor challenge produces a small but significant inhibition of tumor growth rate compared to controls, suggesting that high, localized, potentially cytotoxic concentrations of CB are not necessary for inhibition of growth of s.c. tumor nodules.

Effects of CB i.p. on B16F10 or M109 i.p. The absence of direct cytotoxicity of CB on B16F10 melanoma cells in vivo is also apparent when CB at the maximum tolerated i.p. dose of 50 mg/kg is administered to animals bearing an i.p. tumor challenge (Table 5). With CB treatment given either as a single bolus dose 1 day after tumor challenge or as two bolus doses on days 1 and 7, increase in survival compared to vehicle controls was less than 20%. CB under these conditions also produced a marked enhancement of metastasis to the spleen at the terminal stage of the disease and appeared to accelerate the seeding of metastases to the liver and pancreas as well as to the spleen when necropsy was performed on day 12 after tumor challenge.

As shown in Table 5, CB administered i.p. against a M109 i.p. challenge produced a marked increase in the proportion of animals exhibiting splenic metastases and a significant increase in the mean number of nodules in the spleens, when compared both to vehicle controls and to untreated controls. A significant increase in the mean number of nodules in the liver was observed in the animals treated with a single dose of CB on day 1, when compared to the controls, but in animals given CB treatment i.p. on days 1, 4, and 7 the increase in liver metastases versus vehicle controls was not statistically significant. This multiple-dose group showed a slight increase in survival (17%) compared to vehicle controls. These effects of i.p. CB at 50 mg/kg are consistent with transient immunosuppression produced under these conditions and are not indicative of cytotoxicity against the tumor challenge.

Effect of CB i.v. on M109 i.v. When CB was administered i.v. at 5 mg/kg in 48% ethanol either 1 hour prior to i.v. tumor challenge (1 x 10⁶ viable cells) or 24 h subsequent to i.v. tumor challenge, there was no increase in lifespan compared to vehicle-treated controls (7 or 8 animals/group; data not shown). All animals had multiple lung nodules when examined either at day 20 or at the terminal stage of the disease, and there was no significant difference in the number of nodules in the treated compared to the control groups. No metastases were visible in the spleen, liver, or pancreas in any of the mice, indicating that CB administered i.v. does not enhance splenic metastases.

**DISCUSSION**

More than 3000 papers dealing with the effects of CB in vitro have been published, and some work has appeared on the in vivo toxicity of other cytochalasin congeners (27–29). However, the work presented here is the first detailed in vivo evaluation of the toxicity of CB administered i.p., s.c., or i.v. and is the first in vivo evaluation of CB against a tumor model system. It is apparent that against a s.c. tumor challenge a single dose of CB administered s.c. at 100 mg/kg, which is below its LD₅₀, is able to delay the time of tumor appearance, inhibit tumor growth rate, and prolong host survival in B16F10 melanoma and M109 lung carcinoma. The growth inhibition and extended survival is true whether drug treatment is given 1 day after tumor challenge or treatment is withheld until palpable tumor nodules have appeared. In the M109 model system a single dose of CB also inhibits tumor invasion and spontaneous lung metastases. The effects on the B16F10 model persist at a 10-fold lower dose, and at either dose it does not appear that effects are due to measurable gross cytotoxicity. As shown in Table 4, clonogenicity, tumorigenicity, and vital dye exclusion are not significantly altered by CB treatment in vivo, at times (4 and 6 days after CB treatment) when tumor growth is significantly inhibited in the treated animals. Evidence on CB clearance in vivo (29) indicates that CB would be cleared from the injection site and from the animals long before the 4- to 6-day post-injection point, especially at the 10 mg/kg low dose treatment. Work with M109 lung carcinoma intradermal tumors treated with CB administered s.c. or i.v. confirms this delayed effect of CB on tumor growth, suggesting a noncytotoxic basis for the action of CB.

The mechanistic basis for the inhibition of growth and metastasis remains to be established, but the effects of CB on cytoskeletal structure and function suggest that alterations of either tumor or host cell movement could lead to measurable effects on local tumor growth, invasion, and metastasis. CB and other cytochalasin congeners have been shown to be noncytotoxic against a variety of cell types in culture at concentrations up to 100 μg/ml (200 μM) for short term exposures in the range

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**Table 4 In vivo cytotoxicity of cytochalasin B against B16F10 melanoma**

<table>
<thead>
<tr>
<th>Group</th>
<th>Analysis day</th>
<th>Cell viability (%)</th>
<th>Second TAD</th>
<th>Recipient survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TBE</td>
<td>PE</td>
<td>PE</td>
</tr>
<tr>
<td>Vehicle</td>
<td>12</td>
<td>23</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>17</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>CB, 10 mg/kg</td>
<td>12</td>
<td>20</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>13</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>CB, 100 mg/kg</td>
<td>12</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>9</td>
</tr>
</tbody>
</table>

* TBE, Trypan blue exclusion; PE, plating efficiency.

---

*McQuiggan and Fondy, unpublished observations.*
challenges are underway to address this point. Of actin depolymerization and microfilament alteration increased deformability (42, 43) to a greater extent than it inhibited locomotion (44), potentially facilitating tumor invasion. Alternatively or additionally, deleterious effects that cytochalasins may exert exploitable effects on inherent and utility-dependent functions such as tumor invasion, spontaneous metastasis, and angiogenesis. If any such in vitro effects can be accessed.

Table 5 Effect of cytochalasin B i.p. on B16F10 melanoma and on M109 lung carcinoma i.p.

<table>
<thead>
<tr>
<th>CB dose</th>
<th>Median survival (days)</th>
<th>Fraction with terminal metastases (nodules)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>M109, 1 x 10^6 viable cells i.p.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>20</td>
<td>5/6 (10 ± 3)*</td>
</tr>
<tr>
<td>Vehicle</td>
<td>20</td>
<td>5/6 (10 ± 2)</td>
</tr>
<tr>
<td>50</td>
<td>22</td>
<td>5/6 (53 ± 17)</td>
</tr>
<tr>
<td>50</td>
<td>24</td>
<td>6/6 (21 ± 5)</td>
</tr>
<tr>
<td>B16F10, 1 x 10^6 viable cells i.p.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>16</td>
<td>3/5 (2 ± 1)</td>
</tr>
<tr>
<td>50</td>
<td>16</td>
<td>4/4 (100 ± 0)</td>
</tr>
<tr>
<td>50</td>
<td>14</td>
<td>4/4 (87 ± 7.5)</td>
</tr>
</tbody>
</table>

* Mean ± SE.

The results should be of value in the study of the effects of cytochalasin B and other cytochalasin congeners on tumor model systems in vitro, particularly in determining the potential utility of cytochalasins as chemotherapeutic amplifiers in combination chemotherapy in vivo. In vitro studies indicate to us that cytochalasins may exert exploitable effects on inherent and induced drug resistance in tumor models and might alter motility-dependent functions such as tumor invasion, spontaneous metastasis, and angiogenesis. If any such in vitro effects can be extended to in vivo models, the results could be of considerable value, particularly in view of the fact that more than 24 natural and semisynthetic congeners are available for structure-activity evaluation and for the optimization of cytochalasin-based effects. Beyond these 24 congeners, a large family of additional semisynthetic modifications of natural congeners is readily accessible.

REFERENCES

22. Somers, K. D., and Murphey, M. M. Multicell in the presence of
Effects of Cytochalasin B in Culture and *in Vivo* on Murine Madison 109 Lung Carcinoma and on B16 Melanoma

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