The Accumulated Effects of Repeated Systemic or Local Injections of Low Doses of Corynebacterium parvum in Mice

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SUMMARY

The effects of 14 weekly injections, s.c. or i.v., of "human equivalent" doses (5.25 mg/sq m) of Corynebacterium parvum (CP) in mice have been compared. Both s.c. and i.v. CP caused significant splenomegaly and antibody to CP, but stimulation was considerably greater after i.v. CP. Delayed hypersensitivity levels to CP were similar after s.c. and i.v. injection. T-cell competence, as judged by phytohemagglutinin reactivity and delayed hypersensitivity to sheep cells, was unimpaired after s.c. CP and augmented by i.v. CP. Activated peritoneal macrophages capable of nonspecifically inhibiting tumor growth *in vitro* were detected only after i.v. CP, and *in vivo* resistance to tumor cell challenge was greater after CP administered i.v. than s.c.

INTRODUCTION

Most of the data with regard to CP injection in animals have been obtained with high and single doses of CP, as compared with lower but repeated doses used clinically. The currently acceptable dose in humans is around 5 mg/sq m (8, 9, 14), whereas an average dose range for mouse studies has been around 75 to 100 mg/sq m (6) (reviewed in Ref. 19). The present study was undertaken to see whether repeated low (human equivalent) doses of CP resulted in functional alterations in immunity similar to those reported previously for single high doses. The effects of systemic and local CP injections have also been compared, since both routes are used clinically (8, 9, 14).

MATERIALS AND METHODS

Mice. Adult female CBAT\textsubscript{T6} mice were used throughout except for the carcinogen studies, in which C57BL/6 × DBA/2 F\textsubscript{1} (hereafter called B6D2F,) mice were substituted. The reactivities of both strains to CP were similar as judged by natural and acquired, humoral, and cell-mediated responses.

Immunization. Mice received injections once a week for 14 weeks, either i.v. or s.c., of 35 μg CP (5.25 mg/sq m) (Coparvax; Wellcome Research Laboratories, Beckenham, Kent, England). The i.v. injections were via the lateral tail vein. The site of s.c. injection was varied because of skin thickening at the site of previous injections, but avoided the right footpad and thigh which were to be the sites of subsequent tumor and carcinogen challenge, respectively. Control mice received no CP.

**CP Agglutination.** Sera, doubly diluted in isotonic borate-buffered saline, pH 7.2, were dispensed into plastic microtiter trays (Scientific Supplies, Vine Hill, London, England). Where appropriate, mercaptoethanol was then added (0.1 M to a final dilution of 10%), and trays were allowed to stand at room temperature for 30 min. To each well was then added an equal volume of a 1:10 dilution of washed CP. Following brief agitation, trays were incubated for 30 min at 56° and then overnight at 4°. End points were recorded as the reciprocal of the final serum dilution causing agglutination. Duplicate determinations were made on each serum.

**DTH to CP.** Characteristics of DTH to CP in mice have been described previously (18). Seventy μg CP were injected s.c. into a hind footpad and footpad thickness was measured 24 hr later. Results are expressed as percentage increase in footpad thickness over uninjected feet.

**DTH to SRBC.** Mice were sensitized by injecting 10⁶ SRBC into a hind footpad, and 5 days later DTH was elicited in the ear by s.c. injection of 0.01 ml 50% SRBC. Ear thickness was measured 24 hr later, using the electrical modification of a micrometer screw gauge, as described in Ref. 4.

**PHA Response.** Spleen cells were prepared and their responsiveness to PHA was assayed as described previously (15). Results are expressed as stimulation indices, (cpm/stimulated culture)/(cpm/control culture).

**In Vitro Assay for Nonspecific Inhibition of Tumor Growth.** The technique was as described in Ref. 17. Peritoneal cells were mixed *in vitro* at a ratio of 10:1 with CBA-RI leukemia cells (13). Tritiated thymidine was added after 24 hr of culture, and its uptake by tumor cells was assessed 24 hr later.

**Tumor Challenge.** Mice received s.c. injections into the right hind footpad either s.c. with 2 × 10⁶ syngeneic M4 fibrosarcoma cells (2) or i.p. with 10⁶ RI leukemia cells (13). Tumor growth and survival times, respectively, were monitored.

**Carcinogen Challenge.** Both 3-methylcholanthrene and benzo(a)pyrene were suspended in trioctanoin (Eastman Organic Chemicals, Rochester, N. Y.); 1 mg of carcinogen was injected s.c. into the right thigh.
RESULTS

**Organ Weights.** Eight days after the final CP injection, the livers and spleens of 8 mice from each group were weighed. The mean spleen weight of untreated control mice was $0.064 \pm 0.002$ (S.E.) g, and those of groups treated with CP s.c. and i.v. were $0.127 \pm 0.006$ and $0.466 \pm 0.05$ g, respectively. All differences between groups are highly significant ($p < 0.001$). The same relationship was found with liver weights: controls, $1.09 \pm 0.04$ g, CP given s.c., $1.89 \pm 0.02$ g; and CP given i.v., $2.112 \pm 0.099$ g. Macroscopically visible granulomatous lesions were found in 7 of 8 livers from the CP i.v. group but none was found in either the CP s.c. or control mice.

**Immunity to CP.** Eight days after the final CP injection, mice were tested for both cellular and humoral immunity to CP (Chart 1). Both i.v. and s.c. CP-treated groups showed marked DTH reactivity to CP compared with untreated controls. Differences between the i.v. and s.c. CP groups were not significantly different. Both s.c. and i.v. CP-treated groups developed agglutinating antibodies against CP, but levels in the CP s.c. group were significantly less ($p < 0.01$) than in the CP i.v. group. Mercaptoethanol treatment did not reduce the antibody activity of any group.

**PHA Reactivity.** Ten days after the final CP injection, equal numbers of spleen cells from the groups were assayed for PHA reactivity in *in vitro* cultures (Chart 2). Spleen weights at this time were not significantly different from those at 8 days. There was no significant difference in PHA reactivity between untreated control spleen cells and those from CP s.c.-treated mice. Cells from mice pretreated with CP i.v. showed significantly increased reactivity ($p < 0.01$).

**DTH to SRBC.** Nine days after the final CP injection, mice were sensitized by s.c. injection of SRBC and DTH elicited 5 days later (Chart 3). DTH levels in non-CP-treated mice and mice treated with CP s.c. were not significantly different. The level of DTH expressed in mice pretreated with CP i.v. was significantly higher ($p < 0.01$).

**Nonspecific Antitumor Activity of CP-activated Macrophages.** After injection of either i.v. or i.p. CP, peritoneal cells have been demonstrated to be capable of nonspecifically inhibiting the growth of a variety of tumor cells *in vitro*. The phenomenon is attributable exclusively to the action of CP-activated macrophages (13, 17). In the present study, peritoneal cells from CP i.v.-treated mice markedly inhibited the growth of RI leukemia cells (97% inhibition), whereas cells from CP s.c.-treated and untreated mice were without effect (Chart 4).

**Resistance to Tumor Cell Challenge.** Ten days after final CP injection, mice were challenged with either $2 \times 10^4$ M4 fibrosarcoma cells in the right hind footpad or $10^2$ RI leukemia cells i.p. (Table 1). The relative effects of s.c. and i.v. CP against M4 were apparent from the tumor incidence. The tumor grew consistently in CP s.c.-treated and control mice whereas, with the CP i.v.-treated group, some mice did not...
develop tumors. With RI leukemia, CP s.c. pretreatment significantly prolonged survival time ($p < 0.01$), but the strongest protection was following treatment with CP i.v. ($p < 0.001$).

Resistance to Carcinogen Challenge. Eight days after the final CP injection, groups of 20 B6D2F1 mice were challenged with either methylylcholanthrene or benzo(a)pyrene. They were checked regularly for tumor growth over a period of 200 days. The pattern of development and final incidence of benzo(a)pyrene-induced tumors was similar for both CP-treated groups and control mice. With methylcholanthrene, however, CP i.v.-treated mice responded as normal, i.e., all mice developing tumors, but 20% of CP s.c.-treated mice remained tumor free at 200 days.

DISCUSSION

The repeated injections of low doses of CP used in this study were designed to simulate its clinical use more closely than previous animal studies. Mice received 14 weekly injections of 0.035 mg (5.25 mg/sq m) (6) i.e., total dose, 0.49 mg (73.5 mg/sq m). Both s.c. and i.v. injections were well tolerated with no signs of toxicity other than the occurrence of granulomatous lesions in the liver following i.v. CP, and skin thickening at the injection sites of s.c. CP. Liver granulomas have been reported following single i.v. injections of high doses (0.23 to 0.35 mg) of CP in mice (7, 21). The skin thickening at the s.c. injection site may represent the acquired DTH reactivity to CP discussed below.

Previous work has shown that the degree of systemic stimulation achieved by single doses of CP may be assessed by increase in spleen weight (1). Significant splenomegaly resulted from both repeated s.c. and i.v. CP, but i.v. stimulation was considerably greater (7.3 times that of control) than s.c. CP (2 times that of control). This most probably reflects the relative amounts of CP reaching the spleen following systemic and local injection.

In keeping with the splenomegaly results, high and intermediate levels of anti-CP-agglutinating antibody were found after repeated treatment with CP, i.v. and s.c., respectively. Some differences between single injections of high doses of CP and the present repeated low-dose regimen are apparent. A previous study found no significant levels of CP agglutinins after a single high dose (0.7 to 1.4 mg) of s.c. CP in mice (22), whereas, in this study, an overall lower dose (0.49 mg) given as repeated injections was effective. A qualitative difference between primary and secondary responsiveness to CP is indicated by the fact that after multiple i.v. CP doses, we found the antibody to be completely 2-mercaptoethanol resistant but, 2 to 3 weeks after a single high dose (0.7 to 1.4 mg) of i.v. CP, a 2-mercaptoethanol-sensitive component has been reported (22).

Levels of DTH to CP were similar following both repeated i.v. and s.c. doses of CP. DTH to CP has been reported in mice after a wide range of single s.c. doses (0.007 to 0.35 mg) of CP but was not detected after single high (0.35 to 0.7 mg) i.v. doses (18). This production of DTH by low but not high i.v. doses of CP recalls the phenomenon described for another particulate antigen, SRBC, where a high i.v. dose resulted in antibody formation, but low i.v. doses sensitized to give DTH reactivity (10). The development of DTH to CP is a relevant consideration in tumor therapy using CP, since recent data suggest that cell-mediated immunity to CP may be the basis for some components of its antitumor activity (3, 5, 18).

CP has been reported to depress various parameters of T-cell immunity in animals (reviewed in Ref. 19). Previous studies have shown marked depression of both PHA responsiveness (15) and DTH to SRBC (16) in mice following single high doses of CP i.v. (0.7 to 1.4 mg). In the present study, however, neither repeated i.v. nor s.c. low-dose CP impaired T-cell reactivity, as judged by both PHA responsiveness and DTH reactivity to SRBC. Indeed, in both assays there was significantly increased reactivity following i.v. CP.

The major component of the antitumor activity of CP is immunologically nonspecific and mediated by activated macrophages (reviewed in Ref. 19). Peritoneal macrophages from repeated i.v. CP-treated mice were highly activated, as evidenced by their ability to nonspecifically inhibit tumor cell growth in vitro, whereas cells from both normal and s.c. CP-treated mice were inactive. These in vitro findings were paralleled by the expression of tumor resistance in vivo; i.v. CP conferred significantly more protection than s.c. CP against both systemic and solid tumor challenge. These in vivo findings agree with previous studies using single, or few, high doses of CP where systemic CP has invariably been more effective than local injections in mice (12, 17, 18, 22). Interestingly, a recent report has shown that the antitumor protection afforded by repeated low doses of i.v. CP in mice was greater than the total dose given as a single injection (11).

A point of concern about the clinical use of systemic CP has been the reports of depressed T-cell immunity following high doses in mice (19). That no such impairment resulted from similar amounts given as repeated low doses is the...
most prominent difference between the 2 injection regimens. It is interesting to note that the present finding of unimpaired and augmented T-cell responsiveness to mitogens, after repeated doses of CP s.c. and i.v., respectively, accords with the preliminary clinical data from patients receiving repeated equivalent doses of CP by these routes (14). T-cell competence is clearly an important consideration in CP therapy, since recent animal data show that a component of the antitumor activity of CP is mediated by potentiating specific cell-mediated antitumor immunity (2, 20).

With regard to the choice of route of injection of CP; although it is apparent from recent animal studies that, under some conditions, local injections of CP may be highly effective therapeutically, e.g., intralesional injection (18) or injection near to the tumor site (18, 20), and specific immunotherapy by s.c. injection of CP mixed with irradiated tumor cells (2, 20), given an inaccessible or widely disseminated cancer, the present study suggests that systemic CP may be more effective. The degree of systemic stimulation, especially macrophage activation, achieved by repeated systemic injections of CP was considerably greater than the same doses given locally. Current studies using ^125^I-labeled CP (M. T. Scott, in preparation) also show a wider distribution of the organisms after systemic injection increasing the probability of CP reaching the vicinity of the tumor and initiating local CP-mediated antitumor effects mentioned above.

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