Comparison of the Inhibition of Tumor Growth following Local or Systemic Administration of Corynebacterium parvum or Other Immunostimulating Agents with or without Cyclophosphamamide

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ABSTRACT

The present investigations have compared the effect of systemic to local administration of various nonspecific immunostimulating agents (NSSA's), with and without chemotherapy, on the growth of a primary tumor and have evaluated the relative effectiveness of the systemic and local use of those agents combined with chemotherapy on the growth of a distant tumor focus. The intratumor (i.t.) inoculation of Corynebacterium parvum more effectively inhibited primary tumor growth than did its use by any other route tested. Neither Bacillus Calmette-Guérin nor Bru-Pel displayed such a property when administered either i.t. or systemically. Strikingly better results were obtained when i.t. C. parvum or Bru-Pel were given with cyclophosphamide (CY) than did their systemic use either alone or following chemotherapy. This occurred to a lesser degree with Glucan. I.t. C. parvum used in conjunction with CY was more effective than any of the other immunomodulators similarly used. Such findings are analogous to those obtained with systemic NSSA administration. Since the combination of i.t. C. parvum with systemic CY was highly effective in controlling the growth of a distant tumor focus of lesser size than the treated primary tumor, there is justification for further evaluation of the worth of treatment of a primary tumor with NSSA prior to its removal for the control of the metastatic disease.

Other findings in these investigations, worthy of comment, are those indicating (a) that despite the putative uniformity of animals, tumors, and treatment regimens, in any group of mice, there was a wide range of response of individual tumors to the therapy; (b) that regrowth of totally regressed tumors occasionally occurred during continued and prolonged immunotherapy; and (c) that administration of certain NSSA's resulted in death when inoculated i.t., but the mortality could be prevented by a dose of CY given 4 days prior to the NSSA.

INTRODUCTION

There has been widespread use of intra- or paralesional (local) injection of nonspecific immunostimulating agents (NSSA's), particularly BCG, for the treatment of certain human tumors (10, 12, 17, 21). Such routes of administration in a variety of experimental animal systems have been evaluated not only relative to their antitumor effect (3, 4, 9, 13-16, 18-20, 22-31, 33, 34) but also in regard to host responses elicited (15, 16, 22, 23, 26, 28). Only a small number of investigators, however, have compared the effects of different immunostimulating agents inoculated locally in the same model (15, 19, 22). Moreover, relatively few investigators have reported results of studies designed to compare the effect of local with systemic administration of immunopotentiators when used as the only treatment (13, 15, 16, 20, 25, 26, 28, 29), and they have noted divergent findings. No similar studies have been carried out by using chemotherapy with immunotherapy. Most importantly, specific comparison of the i.t. route with other routes of administration in the control of distant tumor foci has received little attention. Since the response of both primary and distant tumor foci to local immunotherapy could have significance relative to the development of clinical therapeutic strategies, a series of investigations has been carried out to obtain more information in that regard. Using the same model system, we have (a) compared the effect of systemic to local immunotherapy (CP, BCG, Bru-Pel, and Glucan), with and without chemotherapy, on the growth of a "primary" tumor and (b) evaluated the effectiveness of systemic and local administration of those agents with chemotherapy on the growth of a distant tumor. This report presents results from those studies.

MATERIALS AND METHODS

Mice. Mice used in this study were inbred C3HeB/FeJ females, 8 to 12 weeks of age.

Tumor and Experimental Design. The tumor used was a spontaneous mammary carcinoma arising in a C3H/HeJ female and was maintained by transfer in C3HeB mice. By 8 weeks of tumor growth, approximately 40 to 50% of animals demonstrated lung metastases. Tumor cell suspensions were prepared by mincing tumor fragments with scissors on an 80 mesh nylon screen and by washing the cells through the screen with Medium 199. The cells were counted by trypan blue exclusion as a test of viability. The suspension was then diluted in Medium 199 until it contained 200,000 viable cells in 0.1 ml. In all experiments,
tumors were transferred by inoculating $2 \times 10^6$ viable tumor cells s.c. into the left hind leg distal to the popliteal node, and the tumors that arose were designated as primary. A "distant" tumor was produced by injecting the right hind leg in the same manner at the same time with either $2 \times 10^6$ or $5 \times 10^6$ tumor cells. A tumor approximately 5 mm in diameter developed by 14 days in both legs after inoculation of $2 \times 10^6$ cells. Two diameters of the tumor at right angles were measured with a Vernier caliper and averaged. All observations in an experiment were made by the same person. Prior to the beginning of treatment, all tumors were measured. Mice were assigned to various groups in the experiment so that each contained primary tumors of equivalent size. All mice were weighed, and the tumor diameter and health of the animal were noted prior to each injection. Control mice received 0.85% NaCl solution by the appropriate route at the time when treatments were given to test animals. Since averages ± S.D. of tumor growth for a group of mice fail to portray adequately the variability of response to treatment, growth curves for individual animals in an experiment are presented. Since the primary objective of these studies was to determine the effect of the various treatment regimens on tumor growth inhibition and survival was not an end point, experiments were terminated at the times indicated on the charts. In those investigations in which all animals died by the time of sacrifice, survival data are presented. In others the proportion of survivors at the time of sacrifice may be obtained from the individual charts. In experiments with a single tumor, the NSSA's were administered either alone or with chemotherapy in each experiment. For clarity of presentation, all information concerning the various NSSA's without chemotherapy is separately presented from that obtained when chemotherapy was used with them.

CY. CY was prepared immediately before use and diluted so that the desired amount, 60 mg/kg body weight, was contained in 0.01 ml/g body weight. CY was administered i.p. every 7 days, beginning on Day zero.

CP. Wellcome Coparvax (supplied by Dr. J. K. Whisnant, Burroughs Wellcome and Co., Research Triangle Park, N. C.) was used in a dose of 1.4 mg dry weight of organisms every 7 days, beginning on Day 0. CP was administered either i.p., directly into the tumor (i.t.), or s.c. distal or proximal to the tumor, in the opposite hind leg, or in the back of the animal. Since we have previously shown that the i.p. and i.v. routes of administration of CP with (7) or without (8) CY are equivalent in effecting tumor growth inhibition, the i.p. route was used as systemic therapy because of its relative ease of administration.

BCG. BCG was purchased from Research Foundation, Chicago, Ill. Preparations of lyophilized live organisms (15 mg dry weight) were reconstituted in 1.5 ml sterile water and used immediately. The dose used was 1.0 mg equivalent wet weight administered every 7 days, beginning on Day 0, and was injected either i.p. or directly into the tumor.

Bru-Pel. Bru-Pel, a nonviable aqueous ether-extracted Brucella abortus preparation, was supplied as a lyophilized powder (by Dr. J. S. Youngner, University of Pittsburgh School of Medicine, Pittsburgh, Pa.) and suspended in phosphate-buffered saline [8.5 g NaCl, 3.54 g K$_2$HPO$_4$, and 7.24 g Na$_2$HPO$_4$ per liter (pH 7.0)] at a concentration of 7 mg/ml. The amount used was 1.4 mg in 0.2 ml volume injected i.p., i.v., or i.t. every 7 days, beginning on Day 4. Control mice received 0.2 ml phosphate-buffered saline by the appropriate route. The dose used was that recommended by the developer of this NSSA.

Glucan. Derived from Saccharomyces cerevisiae, Glucan was supplied (by Dr. N. R. DiLuzio, Tulane University School of Medicine, New Orleans, La.) as a 0.85% NaCl solution suspension and used in a dose of 0.45 mg in 0.2 ml volume i.p., i.t., or i.v. every 7 days, beginning on Day 4. The dose used was that recommended by the developer of this NSSA. When i.t. immunotherapy resulted in complete regression of a tumor, subsequent inoculations were made s.c. in the region of the regressed tumor.

Statistics. In comparisons relative to the number of completed regressions, Fisher's exact $t$ test was used and, for making comparisons of tumor growth, a $t$ test for paired comparisons was used.

RESULTS

Comparison of Systemic to Local Immunotherapy with and without Chemotherapy on the Growth of a Primary Tumor

In the Absence of a Distant Tumor. When CP was used either i.p., or s.c. in the opposite leg, or s.c. between the tumor and RLN's, there was little if any effect on tumor growth (Chart 1). When it was administered into the tumor, total regression occurred in approximately one-half of the animals surviving treatment ($p < 0.05$). Growth in the other animals was unaffected by therapy. Of 24 animals thus treated, 12 failed to survive the first injection; with 1 exception, those surviving remained alive following subsequent repetitive inoculations, i.e., until sacrifice at 6 weeks.

![Chart 1. Comparison of local to systemic CP on tumor growth. Effect on a single tumor.](https://example.com/chart1.png)
When given injections s.c. distal to the tumor, all animals survived. Temporary total regression of 2 tumors occurred, and the growth of several others was retarded.

When BCG was used, its i.p. administration was without effect, except for the inhibition of the growth of 1 tumor in the group (Chart 2). Similarly, injection into the tumor was ineffective. Whereas only 2 of 14 mice died following an initial i.t. BCG injection, a significant number died as a result of the third i.t. inoculation. The median survival for animals receiving i.p. BCG was 29 days [28.9 ± 6.03 (S.D.)]; for i.t. BCG, it was 24 days (19.9 ± 8.67); and for the 0.85% NaCl solution control, it was 28 days (28.9 ± 2.02).

Bru-Pel when administered either i.p. or i.t. without chemotherapy had no effect on tumor growth (Chart 3). As observed with CP, a large proportion (5 of 12) of mice died following the first i.t. injection. The median survival of animals receiving i.p. Bru-Pel was 27 days (26.7 ± 6.26); for those receiving i.t. Bru-Pel, it was 28 days (21.1 ± 15.85); and for the 0.85% NaCl solution control, it was 25 days (27.2 ± 8.47).

Glucan (0.45 mg) when administered either i.t. or i.v. without chemotherapy similarly failed to demonstrate an effect on tumor growth and survival. When Glucan (4.2 mg) was given i.p., it also had no effect.

When CP was administered with CY, a strikingly better result occurred when CP was injected into the tumor than when it was given by any other route (Chart 4). The use of CP with CY either i.p. or s.c. distal to the tumor resulted in tumor inhibition greater than that observed when CP was given proximal to the tumor or s.c. in the contralateral leg. Its injection i.t. resulted in the total regression of almost all tumors (17 of 18). Results from the concomitant control with i.t. CP alone are presented in Chart 1. In a second experiment (Chart 5) in which animals were followed for a longer period, 2 of 7 completely regressed tumors reappeared during therapy. When a first dose of CP was administered i.t. and subsequent injections were given i.p., more tumors completely regressed (3 of 8) than when all inoculations of CP were i.p. (1 of 12). In animals without complete regression, tumor growth inhibition was similar.

Little benefit was achieved by the use of BCG either i.p. or i.t. when given with CY (Chart 6). In contrast to findings with the use of i.t. BCG alone, all animals survived repeated BCG injections when given in combination with CY.

Tumor growth inhibition was greater with the i.t. rather than the i.p. route of Bru-Pel when given with CY. The former mode of administration resulted in total regression of one-half of the tumors (Chart 7). When used in combination with CY, Glucan injected i.t. inhibited tumor growth to a greater degree than when the Glucan was given i.v. (Chart 8), but the difference was not statistically significant. No complete regressions were observed during 13 weeks of treatment. Following cessation of therapy, 2 tumors completely disappeared (not shown).
In the Presence of a Distant Tumor (Table 1). The influence of treatment on the primary tumor was essentially similar to that observed when no second tumor focus was present. CP when administered i.t. or s.c. distal to the primary tumor in conjunction with systemic chemotherapy produced a better effect than when it was given by any other route tested. Similarly, Bru-Pel, administered with chemotherapy, produced more total regressions when
Local versus Systemic Administration of NSSA

Comparison of Systemic to Local Immunotherapy with Chemotherapy on the Growth of a Distant Tumor

When animals harbored a 5-mm tumor on each hind leg, inhibition of growth of the tumor in the right leg by chemoinmunotherapy was greater when CP was administered systemically than when the primary tumor in the left leg was the recipient of repeated CP inoculations (Chart 9). Whereas 7 of 24 tumors totally regressed with systemic therapy, local or regional treatment of the primary tumor resulted in fewer regressions. Regrowth of totally regressed tumors occasionally occurred during either treatment. In a second experiment with a 3-mm tumor in the left hind leg and a 1-mm tumor in the right, the weekly administration of CP (1.4 mg) into the left tumor resulted in a greater retardation of tumor growth in the right leg than when CP was administered systemically. In the former group, 3 tumors totally regressed, whereas in the latter none did so (Chart 10). The average survival in Group A (treatment with CY only) was 40.0 ± 6.4 days (median, 39 days); in those receiving CP i.p. (Group B), it was 48.9 ± 10.8 days (median, 52 days). When CP was given i.t. (Group C), the median survival was 70 days with 4 of 13 still alive after 10 weeks (p = 0.08). Both systemic and intratumor administration of BCG were completely ineffective in controlling growth of a distant tumor (Chart 11). When Bru-Pel was given, no advantage for i.t. inoculation was observed (Chart 12). Untreated tumors were slightly better controlled when Bru-Pel was given i.p. Little benefit occurred from i.v. Bru-Pel administration.

DISCUSSION

The observation that the i.t. inoculation of CP more effectively inhibited growth of a treated tumor than did its use by any other route used agrees with the findings of some investigators (16, 25, 26) but not those of others (20, 28, 29) who have compared local (i.t.) and systemic (i.p. or i.v.) administration of CP in the management of established palpable tumors. Only Likhite and Halpern (16), using a mammary carcinoma in DBA/2 mice, and Scott (25, 26), using a mastocytoma in C57BL/6 × DBA/2 F, mice, have reported that CP given i.t. was more effective in inhibiting tumor growth than was its systemic administration (i.p. in the former and i.v. in the latter). On the other hand Suit et al. (28, 29) reported that i.v. CP was superior to its i.t. use in restraining the growth of mammary and methylcholanthrene-induced sarcomas in C3H mice and Purnell et al. (20) similarly observed that the i.v. was better than the i.t. route in the control of CaD2 tumors in DBA mice.

A direct comparison of the relative effectiveness of i.t.-inoculated CP with other NSSA’s similarly administered for control of local tumor growth has rarely been carried out...
Chart 8. Comparison of local to systemic Glucan (G) with CY on tumor growth. Effect on a single tumor.

(3, 19). Paslin et al. (19) in 1974 compared i.t. Corynebacterium granulosum with i.t. BCG by using a melanoma in golden hamsters and reported that tumors regressed, survival was prolonged and lung metastases were decreased following the use of the C. granulosum, whereas such effects were not noted with BCG. Chassoux and Salomon (3) reported that living or heat-killed BCG, the methanol extraction residue of inactivated BCG organisms, and CP produced permanent cures of a portion of rat sarcomas treated i.t., but only living BCG cured all of the animals. In our investigations, only i.t. CP was effective in restraining tumor growth, whereas neither BCG nor Bru-Pel displayed such a property. The latter is a nonviable aqueous ether extract of B. abortus, is a potent interferon-inducing antivirus agent, and has significantly protected animals harboring an ascitic sarcoma (11, 32). CP in an equivalent dose was significantly less effective in the same tumor model (11). In the present studies, Bru-Pel in combination with CY did display some inhibition of tumor growth but not as much as did CP with CY.

The failure of i.t. BCG to produce an effect in these investigations is in keeping with our previous findings with that agent (5) but differs from those obtained by the few investigators who utilized i.t. BCG in different experimental systems (30, 34, 35). Since BCG is reputed to be more effective when administered to hosts with highly antigenic tumors, this may account for the different observations.

Table 1

<table>
<thead>
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<th>Treatment (wk)</th>
<th>0.85% NaCl solution</th>
<th>CY</th>
<th>CP i.p. + CY</th>
<th>BCG i.p. + CY</th>
<th>Br-Pell i.p. + CY</th>
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<tr>
<td>0</td>
<td>5.48 ± 1.49</td>
<td>5.22 ± 1.11</td>
<td>5.37 ± 0.96</td>
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<td>5.18 ± 0.92</td>
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<td>6.15 ± 2.47</td>
<td>6.32 ± 2.42</td>
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<td>6.29 ± 2.46</td>
</tr>
<tr>
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<td>7.15 ± 2.50</td>
<td>7.32 ± 2.51</td>
<td>7.37 ± 2.52</td>
<td>7.29 ± 2.55</td>
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<tr>
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<td>8.15 ± 2.55</td>
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<td>9.32 ± 2.66</td>
<td>9.37 ± 2.67</td>
<td>9.29 ± 2.69</td>
</tr>
</tbody>
</table>

Mean ± S.D.

ª First number in parentheses, number of mice; second number in parentheses, number of complete regressions in each group.

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Whereas our findings were obtained with a mammary tumor that is weakly antigenic, those by Zbar et al. (34, 35) and by Tokunaga et al. (30) were obtained with tumors that are more highly antigenic (guinea pig hepatoma in the former and methylcholanthrene-induced sarcoma in the latter). Since dose-response tests were not performed, it is possible that optimal doses of Bru-Pel and BCG were not tested for antitumor activity. Further evaluation of these agents in this system might demonstrate such antitumor activity.

The strikingly better results obtained with i.t. CP, with Bru-Pel, and to a lesser degree with Glucan, when given with CY, over that resulting from their administration alone or following the systemic administration of those agents in combination with chemotherapy further substantiates our previous observations (5). These results also support our contention that immunomodulators by themselves, even when optimally used, are likely to be ineffectual or less effective than when combined with chemotherapy. That tumors inoculated with CP in animals who did or did not receive systemic chemotherapy totally regressed is noteworthy since, in our model, prior extensive use of that NSSA by a variety of other routes has consistently failed to produce such an effect (7, 8). The findings indicating that i.t. CP is more effective than any other immunomodulator when used with CY also supports our prior findings obtained when the systemic route of NSSA administration was used (5).

The observation that a single i.t. injection of CP or Bru-Pel resulted in the rapid death of a large number of animals.
Our observation that the i.t. administration of NSSA's with or without chemotherapy resulted in maximal restraint of tumor growth supports the contention that such agents are more effective when they are in direct contact with tumor antigen (2). Several investigators (13, 14, 26) have reported that, similar to our experience, paratumor injection of NSSA's had some antitumor effect but that the results following i.t. inoculation were better. The present findings demonstrating the superiority of the i.t. route correlate with those previously reported by us (6), indicating that the i.t. inoculation of CP augmented RLN cell cytotoxicity to a greater extent than did its i.p. administration. Although injection of CP between the tumor and RLN's also increased RLN cell cytotoxicity, it failed to do so to the same extent as did the i.t. injection. That the i.t. injection of BCG is ineffective in inhibiting tumor growth as well as increasing RLN cell cytotoxicity is noteworthy. What role the sensitized RLN plays in the antitumor effect following i.t. injection remains unclear and is worthy of further study.

The observation that the i.t. administration of a NSSA is effective in controlling growth of a treated tumor has clinical significance. If a NSSA given by that route of administration could favorably affect the control of metastatic tumor, use of the i.t. route would have even greater importance. Such an observation would provide a rationale for the administration of immunotherapy into a primary tumor prior to its removal. The present findings that i.t. CP with CY was more effective than was systemic CP with CY in controlling the growth of a distant tumor, providing that the latter represented a tumor burden less than that of the primary tumor and that there was an inhibitory effect on the distant tumor whether or not the treated primary tumor totally regressed, lends encouragement to the concept that treatment of a primary tumor with NSSA may be particularly advantageous in impairing the growth of metastases.

That despite the putative uniformity of animals, tumors, and immunochemotherapeutic regimens used, in any group of mice, there resulted a wide range of response of the individual tumors is significant. Some tumors totally or partially regressed, others demonstrated zero growth, while still others continued to grow progressively at differing rates. Such findings suggest that the results of intralesion inoculation are more likely related to specific tumor host factors than to a nonspecific local phenomenon.

The regrowth of totally regressed tumors during continued and prolonged immunochemotherapy or subsequent to cessation of such therapy provokes speculation relative to the cause. If a clone of tumor cells resistant to the chemotherapeutic agent had arisen, it might have been anticipated that the susceptibility of the remaining cells to the host immune response to the tumor augmented by the NSSA would have resulted in their eradication. Whether (a) the remaining cells have developed "immunological resistance," (b) the host immune response is inadequate to destroy even a minimal tumor burden due to or irrespective of the prolonged use of chemotherapy, or (c) there is some other explanation remains entirely conjectural.

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