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Comparative Study on the Effects of Surgery, Chemotherapy, and Immunotherapy, Alone and in Combination, on Metastases of the 13762 Mammary Adenocarcinoma[1]

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SUMMARY

The effectiveness of a methanol-soluble fraction of *Mycobacterium butyricum* (MSF-MB) as a nonspecific immunological adjuvant was tested as a therapeutic modality in various combinations of surgery and chemotherapy in two confirmatory studies using Fischer 344/CRBL female rats bearing the naturally metastasizing, syngeneic 13762 mammary adenocarcinoma. Animals given s.c. tumor grafts and left untreated developed large tumor masses and had a mean survival of 47.8 (S.D., ±6.9) days. Excision of progressively growing tumors on Day 18 postimplantation prolonged survival to 65.5 ± 8 days, all animals dying from lung and organ metastases. Chemotherapy alone, *i.e.*, treatment of established tumors with the 17β-estradiol diester of *p* = [*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid (NSC 112259) at 5 mg/kg/day p.o. for 28 days, induced marked oncolysis and produced 25% cures. Following a short period of remission in the remaining animals and despite continuing chemotherapy, there was regrowth of drug-resistant tumors with a prolongation of survival to 87.3 ± 10.5 days. A short, nonimmunosuppressive, but oncolytic course (10 days) of chemotherapy, either before or after surgery, averaged 75.5% cures against metastases. Chemotherapy that was prolonged for 28 days, whether initiated before or after surgery, was immunosuppressive and reduced cures against metastases to 61%. Surgery followed by a single i.p. injection of 10 mg MSF-MB induced 10 to 20% cures. A 10-day period of chemotherapy either before or after surgery, followed by immunotherapy, was most effective and produced 90 to 100% cures. It would appear that chemotherapy alone was most effective against metastases when administered in a short, nonimmunosuppressive regimen. Thus, when chemotherapy produced a degree of oncolysis and tumor resorption with minimal or no immunosuppression and the primary source of metastases was excised, the possibility of an effective sensitization to tumor antigens was increased and nonspecific stimulation of the immune responses by MSF-MB was able to induce host resistance to cope with the remaining, possibly drug-resistant metastases.

INTRODUCTION

Among the many sites in which solid tumors occur in the body, those of mammary origin are among the most accessible to surgical excision. Surgery, therefore, is the procedure of choice for operable breast cancer and is most effective if carried out early when cancerous cells are still localized in the primary growth. However, there appears to be little difference, in terms of ultimate survival, between radical and simple mastectomy, once the cancer cells have metastasized to the regional lymph nodes. Clinically, therefore, the problem in treatment of breast cancer is the eradication of the metastases that have occurred before or during surgical manipulation of the primary growth.

Considerable experimental evidence has accumulated during recent years to show that the emergence and progressive proliferation of neoplastic cells are the result of an impairment in the immunological monitoring system (7). The decreased efficiency of immunological responses with aging coincident with increasing cancer incidence, the unresponsiveness to skin test antigens and impaired homograft rejection in patients with advanced and widespread cancer, the immunosuppressive effects of tumor growth *per se*, as well as the immunosuppression by chemotherapeutic agents, have been amply demonstrated. On the other hand, there is considerable experimental evidence to show that neoplastic cells of many types can be immunogenic in their autochthonous hosts and that, under certain conditions, the host is capable of mobilizing immunological defense mechanisms of varying strength and efficiency (5, 6). Therapeutically, therefore, to develop effective antitumor defenses, the immunological response capability of a cancerous host must be enhanced within a very critical time frame.

The inclusion of immunotherapy as an additional modality in a specific treatment regimen requires a careful evaluation of (*a*) the overall physiological viability of the tumor-bearing host, (*b*) the estimated total tumor mass remaining after a primary therapy such as surgery, chemotherapy, or irradiation, and (*c*) the systemic toxicity of the therapeutic agent or treatment regimen and the coinci-

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dent immunosuppression. In mammary cancer, therefore, immunotherapy would be most effective in combination with or in sequence to surgery, chemotherapy, and/or irradiation, with the objective of stimulating host defenses against metastases susceptible to the cytotoxic activity of immunologically competent lymphocytes. Immunotherapy in terms of the effective use of mycobacterial fractions such as MER[2] or MSF-MB in the immunopotentiation of resistance to transplants of syngeneic tumors (13, 14) and in the counteraction of immunosuppression resulting from tumor growth (3) has been demonstrated.

The purpose of this report is to demonstrate (*a*) the use of the 13762 mammary adenocarcinoma as an experimental mammary tumor model for in-depth evaluation of combinations of therapeutic modalities, (*b*) the effectiveness of MSF-MB as a nonspecific immunological adjuvant for controlling mammary tumor metastases, and (*c*) that, when used as adjunct therapy, immunotherapy is most effective when applied after a short regimen of chemotherapy that induces some oncolysis and tumor resorption, plus surgical extirpation of the primary mass.

MATERIALS AND METHODS

Animals. Female Fischer 344/CRBL rats, 35 days of age, were used throughout this investigation following a week of isolation at this laboratory and were maintained on Purina laboratory chow and tap water *ad libitum*.

Mammary Tumor Model. The 13762 mammary adenocarcinoma (13762MT), originally induced with 7,12-dimethylbenzanthracene by Segaloff (10), is 100% transplantable and lethal to female rats of the syngeneic Fischer CRBL strain. Histologically, it maintains an epithelial/stromal ratio of approximately 85/15%, with definite acinar formations of moderate size, arranged as papillary extensions of the tumor growth. The acini, which under hormonal stimulation will show milk-like secretion, are found in clumps and divided from each other by thin bands of supporting tissue. This tumor has been shown to compete with the uterus for endogenous estrogens (1) and with the normal mammae for prolactin.[3] Studies on metastases of the 13762MT have revealed that, by Day 18 after s.c. implantation, almost 100% of the implanted animals already have metastases to the lungs and/or organs. Therefore, excision of the tumor on Day 18 significantly extends survival and permits metastasized cells to develop into macroscopically discernible tumor foci. In this study, 40- to 50-day-old Fischer 344/CRBL females served as graft recipients. A single 2- to 3-cu mm piece of 13762MT was implanted s.c., via a 13-gauge trochar, on the right side midway between inguinal and axillary areas. Care was taken not to involve deeper muscular layers so as to facilitate surgical extirpation. Excision was a relatively simple enucleation of the tumor mass under ether anesthesia and closure of the wound with Michel clamps.

Chemotherapeutic Agent. The 17β-estradiol diester of *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid (NSC 112259) was selected because of its marked oncolytic activity in mammary tumor systems (11, 12) and because, at oncolytic dose levels, it is not immunosuppressive unless treatment is prolonged.

The immunosuppressive effects of the 17β-estradiol alkylating agent have been evaluated in rats by the primary hemagglutinin response to a single injection of sheep erythrocytes in classical-pretreatment, simultaneous, and delayed-treatment protocols (2). Evaluation of drug effects on the reticuloendothelial system has included the parameters of complete peripheral hematology and changes in thymus and spleen weights. Drug administration, 5 mg/kg/day or 11.7 mg/kg/treatment, 3 times weekly p.o., ranged from 6 to 21 consecutive treatments.

Treatment for 21 days prior to antigenic stimulus induced significant thymolysis and splenic atrophy, a leukopenia directed primarily against the lymphocyte, and a significantly lowered hemagglutinin response. Treatment for 6 or 8 days produced no significant effects on thymus or spleen weights, only a slight lymphopenia, and no suppression of the hemagglutinin response. Peripheral leukopenia increased with continued daily drug administration, and significant suppression of the humoral response was detectable after about 14 treatments. In this study, short-term therapy refers to 10 successive daily treatments, and prolonged therapy refers to daily treatment over a period of 28 days or longer. Compound formulations were prepared daily, in a sesame oil vehicle (Beacon Chemical Company, New York, N. Y.), and administered p.o. at a dose level of 5 mg/kg/day.

Immunological Adjuvant. Our interest in the mycobacteria for nonspecific enhancement of immune responses stems from the work of Old *et al.* (8, 9) and Weiss *et al.* (13, 14) with BCG. However, our studies have deviated in several major aspects from those of Weiss *et al.* In their studies, the preferred adjuvant fraction was MER, *i.e.*, the residue of bacterial bodies remaining after extensive extraction with methanol. The adjuvant used in our studies was MSF-MB, *i.e.*, the methanol-extractable material, and was administered i.p. as a 0.9% NaCl solution suspension at 10 mg/dose. Procedure for the preparation of MSF-MB has been reported in detail elsewhere (4). MSF-MB was chosen as the immunological adjuvant because it is relatively nontoxic, and i.p. injection does not result in multiple nodular (tubercle-like) formations throughout the peritoneal cavity as seen with BCG or MER. Furthermore, its immunopotentiating activity had been demonstrated (3, 4).

Experimental Design. This report presents the results of 2 separate but confirmatory studies. Study 1 (Table 1) involved 10 experimental groups, 10 rats/group, whereas Study 2 (Table 2), with slight variations, involved 9

[2] The abbreviations used are: MER, methanol-extracted (insoluble) residue of phenol-killed, acetone-washed *Bacillus Calmette-Guérin;* MSF-MB, methanol-soluble fraction of phenol-killed, acetone-washed *M. butyricum;* BCG, *Bacillus Calmette-Guérin.*

[3] A. E. Bogden, D. J. Taylor, E. Y. H. Kuo, M. M. Mason, and A. Speropoulos. The Effect of Perphenazine-induced Prolactin Response on Estrogen-primed Mammary Tumor-Host Systems, submitted for publication to CANCER RESEARCH.

experimental groups of 10 rats/group. In both studies, tumor grafts were implanted on Day 1, tumor growth was monitored by caliper measurements 3 times weekly, and surgery was performed on Day 18 or 28. Metastases to the lungs and organs were generally macroscopically discernible at necropsy, and questionable metastases were verified by histological examination. Both studies were terminated by sacrifice and autopsy on Day 211. "Cure" represents survival of animals to Day 211 without any evidence of tumor regrowth or metastases.

RESULTS

The results of the 1st study are summarized in Table 1 and illustrated in Charts 1 and 2. Chart 1 shows the tumor-growth curve for the untreated control group (Group I), as well as the effect of long-term chemotherapy, initiated on Day 18 when the tumor was established in early log phase of measurable growth (Group VI). Oncolysis was induced and tumor resorption took place and was progressive to Day 36. Thereafter, despite continuing therapy, tumor growth reoccurred. Although histologically still an adenocarcinoma, the regrowing tumor was now drug resistant. Chart 2 shows relative tumor sizes on Day 18 when chemotherapy was initiated for Groups VII, VIII, and IX; the tumors of these rats were excised on Day 28. The short course of chemotherapy was oncolytic, so that there was some tumor resorption prior to surgery.

In reference to Table 1, all untreated control animals (Group I) died from progressively growing s.c. tumors, with a mean survival of 41.8 days. Excision of tumors on Day 18 (Group II) significantly prolonged survival, but all eventually died, primarily from metastases to the lungs. When surgery on Day 18 was followed by immunotherapy on Day 20 (Group III), survival was also significantly prolonged; in addition, 2 cures were obtained. The same treatment sequence (Group IV), with an additional injection of adjuvant on Day 30, produced no improvement in cures or survival. Surgery on Day 18, followed immediately by chemotherapy for 28 days (Group V), cured 8 of 10 animals and significantly prolonged the survival of 2 animals that subsequently died with s.c. regrowths. Chemotherapy without surgery, initiated on Day 18 and continued to survival

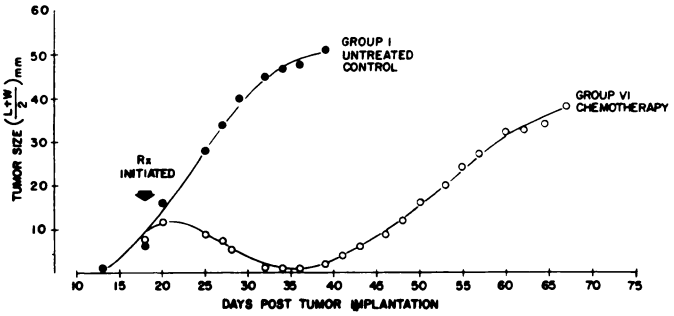

Chart 1. Regrowth of drug-resistant tumors following long-term chemotherapy initiated when tumors were well established in early log phase of measurable growth. *Rx*, treatment.

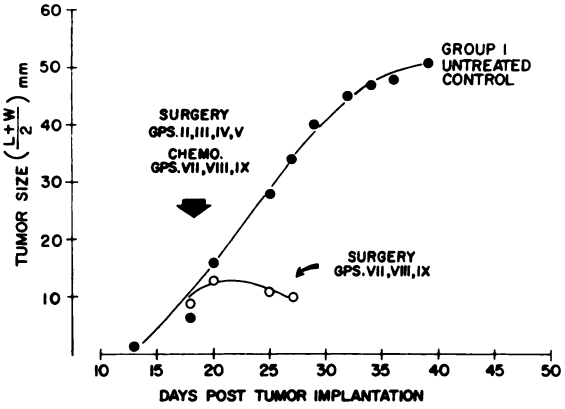

Chart 2. The oncolytic effect of a short course of chemotherapy (*CHEMO.*) (10 days) initiated prior to surgery. *GPS.*, groups.

Table 1

Study 1: Comparative effects of surgery, chemotherapy, and immunotherapy on metastases of the 13762 mammary adenocarcinoma

Group	Therapy	No. of rats surviving/ initial no.	% "cured"[a]	Survival (days)	Tumor regrowths[b]	Metastases: Lungs	Liver	Kidneys
I	Untreated controls	0/10	0	41.8 ± 5[d]	0	10	1	0
II	Surg.[c] D18	0/10	0	71.4 ± 11	8	10	0	2
III	Surg. D18 + Immuno. D20	2/10	20	76.1 ± 15	6	8	0	1
IV	Surg. D18 + Immuno. D20, 30	1/10	10	66.6 ± 14	9	9	1	3
V	Surg. D18 + Chemo. D18 (28 days)	8/10	80	114.5	2	0	0	0
VI	Chemo. D18 (28 days)	0/10	0	79.0 ± 12	10	10	1	1
VII	Chemo. D18 (10 days) + Surg. D28	7/10	70	104.3	3	3	0	0
VIII	Chemo. D18 (10 days) + Surg. D28 + Chemo. D28 (18 days)	3/10	30	93.3 ± 13	7	7	0	0
IX	Chemo. D18 (10 days) + Surg. D28 + Immuno. D30	9/9[e]	100		0	0	0	0
X	Chemo. D18 + Surg. D28 + Immuno. D40	5/10	50	106.6 ± 63.0	3	3	0	0

[a] Animals surviving in apparent good health with no indication of tumor regrowth or metastases on sacrifice Day 211.
[b] At implant site.
[c] Surg., surgery; D, Day; Immuno., immunotherapy; Chemo., chemotherapy.
[d] Mean ± S.D.
[e] One anesthetic death.

(Group VI), although producing oncolysis and significantly prolonging survival, was no better than surgery alone. When a short 10-day course of chemotherapy, initiated on Day 18, preceded surgery on Day 28 (Group VII), 7 of 10 animals were cured, and the survival of the remaining 3 animals was significantly prolonged. However, when a short course of chemotherapy preceded surgery and chemotherapy was continued postsurgery for a prolonged period (Group VIII), only 3 of 10 animals were cured. The most effective regimen, producing 100% cures, was obtained with a short course of chemotherapy initiated on Day 18, followed by surgery on Day 28 and by immunotherapy on Day 30 (Group IX). In the same regimen, when immunotherapy was delayed for 12 days the number of cures dropped to 50% (Group X).

The experimental design for the 2nd study (Chart 3) represents the tumor growth and regression curves of the various experimental groups as well as the relative tumor sizes at time of surgery, the periods of chemotherapy, and the times of immunological stimuli. The results are summarized in Table 2. Surgery on Day 18 (Group II) prolonged survival but produced no cures. Surgery followed by immunotherapy on Day 20 (Group III) produced 1 cure.

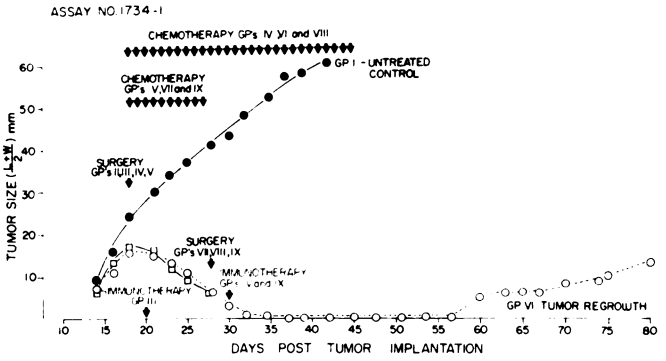

Chart 3. Experimental design for the 2nd study illustrating the applications of surgery and immunotherapy in relation to the oncolytic effects of chemotherapy. *GP.'s*, groups.

Surgery followed by prolonged chemotherapy (Group IV) produced 67% cures. However, surgery followed by a short course of chemotherapy and then by immunotherapy (Group V) produced 90% cures.

A prolonged course of chemotherapy alone (Group VI) produced 50% cures, whereas surgery preceded by a short course of chemotherapy (Group VII) produced 89% cures. However, when surgery was preceded by a short course of chemotherapy and was then followed by a prolonged course of chemotherapy (Group VIII), the cure rate dropped to 67%. The most effective combination was a short course of chemotherapy preceding surgery, followed by immunotherapy (Group IX), which produced 90% cures and significantly prolonged the survival of the remaining animal.

DISCUSSION

In analyzing the results of these studies, 2 factors must be considered: (*a*) the 17β-estradiol alkylating agent (NSC 112259), administered at 5 mg/kg/day over a 10-day period, is effectively oncolytic and produces no significant immunosuppression but, administered at the same dose level for 14 days or longer, is progressively immunosuppressive; and (*b*) the 13762 mammary adenocarcinoma, although responding initially and dramatically to chemotherapy, becomes drug resistant during prolonged drug administration. Induction of drug resistance is illustrated by tumor regrowth at the implant site despite continuing therapy (Charts 1 and 3). It is significant, therefore, that when surgery was preceded by a short course of chemotherapy and then followed by a prolonged course of drug treatment, the cure rate averaged only 49% in the 2 studies, compared with 80% when there was only a short course of chemotherapy preceding surgery. It would appear that, when chemotherapy becomes immunosuppressive, drug-resistant metastases are permitted to grow unchecked and the possibility for a cure is reduced. That host resistance (immunological factors) plays a significant role in the

Table 2

Study 2: Comparative effects of surgery, chemotherapy, and immunotherapy on metastases of the 13762 mammary adenocarcinoma

Group	Therapy	No. of rats surviving/ initial no.	% "cured"[a]	Survival (days)	Tumor regrowth[b]	Metastases		
						Lungs	Liver	Kidneys
I	Untreated controls	0/10	0	53.8 ± 8.7[d]	0	10	1	1
II	Surg.[c] D18	0/10	0	59.6 ± 5.0	4	10	8	0
III	Surg. D18 + Immuno. D20	1/10	10	61.1 ± 6.5	4	9	0	2
IV	Surg. D18 + Chemo. D18 (28 days)	6/9	67	64.7 ± 2.1	0	3	0	0
V	Surg. D18 + Chemo. D18 (10 days) + Immuno. D30	9/10	90	65	0	1	1	0
VI	Chemo. D18 (28 days)	5/10	50	95.6 ± 9.1	0	5	0	0
VII	Chemo. D18 (10 days) + Surg. D28	8/9	89	97	1	0	0	0
VIII	Chemo. D18 (10 days) + Surg. D28 + Chemo. D28 (18 days)	6/9	67	92.0 ± 12.1	3	1	0	1
IX	Chemo. D18 (10 days) + Surg. D28 + Immuno. D30	9/10	90	104	1	1	0	0

[a] Animals surviving in apparent good health with no indication of tumor regrowth or metastases on sacrifice Day 211. Groups IV, VII, and VIII each had 1 surgical death.

[b] At implant site.

[c] Surg., surgery; D, Day; Immuno., immunotherapy; Chemo., chemotherapy.

[d] Mean ± SD.

resultant responses to therapy is supported by the results obtained in those experimental groups in which immunotherapy was included in the combinations of surgery and chemotherapy. Whether the short course of chemotherapy preceded or followed surgery, nonspecific stimulation of the host's immunological mechanisms by MSF-MB produced the highest cure rate of all therapy combinations in both studies (90 to 100%).

Immunotherapy alone was unable to cope with a large s.c. tumor, and surgery only prolonged survival. The possibility that the cancerous hosts were being stimulated to cope with metastases when immunotherapy was used in combination with surgery (Table 1, Groups III and IV; Table 2, Group III) was indicated by the 10 to 20% cures obtained with this combination of therapies. That the combination of surgery and immunotherapy did not significantly prolong survival of the remaining animals would indicate that survival may well have been dependent upon a critical ratio of metastases to immune response capability, stressing the need to reduce the systemic tumor burden by chemotherapy.

An important factor pertinent to the absence of effective immune responses in cancer is the inadequacy of the antigenic stimulus provided by the neoplasm, either in terms of weak antigenicity of the tumor antigen (6) or in terms of the necessary antigen mass available to the lymphoid tissues at any one time, for effective sensitization.

The timing and sequence in which therapies are applied in combination with immunotherapy are important, therefore, and have an immunological rationale which is supported by the experimental results. Surgery removed the primary mass and source of metastasizing cells. Chemotherapy, when oncolytic and used in a short, nonimmunosuppressive regimen, resulted in tumor resorption, and where the immune response capability remained intact or was enhanced, increased the possibility of sensitization to tumor antigens. Thus, the oncolytic effect of the 17β-estradiol alkylating agent induced by a short treatment regimen was adequate for sensitization, providing the MSF-MB adjuvant was administered within 1 or 2 days after the last treatment. Delaying immunotherapy markedly reduced the number of cures. Therefore, it would appear that immunotherapy was most effective when the cell-mediated immune responses were stimulated at a time when tumor antigens were being released, or when they were being released in greater concentration as a result of an effective chemotherapy. Thus, where chemotherapy produced a degree of oncolysis without immunosuppression and the primary source of metastases was excised, nonspecific stimulation of the immune responses by MSF-MB was able to induce host resistance to cope with the remaining, possibly drug-resistant metastases. The results of these studies would indicate that the application of nonspecific enhancement of immune response capability as a therapeutic modality in combination with surgery and chemotherapy requires not only a thorough understanding of the timing required for adjuvant effects, time factors that are peculiar to each immunological adjuvant, but also a careful assessment of both the oncolytic and immunosuppressive activities of chemotherapeutic agents, as well as an appreciation of the optimal sequence in which therapies are to be used. It is also evident that the 13762 mammary adenocarcinoma has the essential metastasizing and therapy-response characteristics for mimicking the clinical situation and can be effectively used as an animal tumor model for evaluating therapy combinations and therapy sequences.

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