Comparison of Adjuvant Chemotherapeutic Activity against Primary and Metastatic Spontaneous Murine Tumors

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SUMMARY

Metastatic tumor incidence in BALB/c × DBA/8 F₁ female mice was examined in the presence and absence of adjuvant chemotherapy. Following surgical removal of spontaneous mammary adenocarcinomas, phenylalanine mustard, adriamycin, and 5-fluorouracil (PAF) were administered at 4, 2, and 50 mg/kg, respectively, once a week for six injections. Recurring tumors and new tumors developing in other breasts over the next 6 months were noted and surgically removed to allow time for originally undetectable pulmonary metastases to develop or to regress completely.

This regimen of PAF significantly decreased original tumor recurrences from 58% in controls to 36% in treated mice. New tumor development also was significantly reduced during the 5 weeks of PAF therapy and for 8 weeks thereafter. However, the incidence of pulmonary metastasis was unaffected by the chemotherapy, being 42% in controls and 37% in PAF-treated mice. About 30% of these metastases would have been undetectable at the time of original surgery. The findings stress the importance of developing agents and/or schedules that will specifically affect metastatic cells when administered early to minimal numbers of tumor cells. This system represents a stringent clinicalmimetic model for evaluating adjuvant chemotherapy in this regard.

INTRODUCTION

Mammary adenocarcinomas that appear spontaneously in BALB/c × DBA/8 F₁ (hereafter called CD8F₁) female mice have been shown to give rise to secondary pulmonary tumors (1). A lung bioassay method has been utilized to document a direct correlation between the incidence of secondary lung metastases and the size of the primary breast tumor; i.e., as the primary tumors increased in size, the incidence of metastasis also increased.

The primary spontaneous breast tumor growing in its autochthonous host mouse is an experimental model for clinical breast cancer because of its resemblance to, and correlation with, the human disease (7, 12, 18). The metastatic findings (1) have enhanced its experimental utility and have led us to examine the effect of surgical adjuvant chemotherapy on the incidence and/or development of lung metastases simultaneously with its effect on the primary neoplasm.

MATERIALS AND METHODS

Mice. All animals used in this study were CD8F₁ mice bred and maintained in our laboratories. Females of the strain develop mammary tumors spontaneously at an average of 10 months at an incidence of about 80% and are culled from the colony weekly. Male mice 2 to 3 months old were utilized for the lung bioassay.

Bioassay for Metastasis. The lungs from female mice were tested for the presence of metastases by bioassay. The animals were sacrificed by cervical disruption. The lungs from each mouse were removed aseptically through a midline chest incision, placed into a sterile Petri dish with sterile 0.15 M NaCl containing penicillin and streptomycin, 200 units/ml each, and coarsely minced with scissors. The mince was loaded into a sterile 3-ml syringe fitted with a 1-inch, 21 gauge needle and extruded s.c. between the right inguinal and axillary regions of a normal CD8F₁ male mouse. The inoculated male mice were observed for up to 5 months for s.c. tumor development resulting from metastatic lesions in the implanted lungs.

Enucleative Surgery. Female mice bearing single spontaneous tumors were anesthetized with sodium pentobarbital at a dose at 85 mg/kg i.p. Original surgery consisted of careful stripping out of the s.c. neoplasm by blunt dissection to deliberately leave microfoci of tumor cells behind. This technique results in a 60 to 80% recurrence rate and provides a tumor target for adjuvant therapy.

Radical Surgery. Recurring tumors and new primary tumors developing in other breasts over the next 6 months were noted and very carefully excised along with adjacent skin in an attempt to cure the tumor so as to allow time for originally undetectable metastases to develop or to regress completely. An overall cure rate of 75% has been attained by this method. Those tumors in the head and neck area and in the area of the hindquarters are particularly difficult to cure surgically and account for most of the recurrences. Only 9% of tumors in the other breasts have recurred following this more radical surgical procedure.

Chemotherapy. On the day after original surgery, the mice were separated randomly into 2 groups. One group remained as a surgical control; the other group received...
Therapy of Primary and Metastatic Murine Tumors

PAF* once a week for 6 injections, beginning the day after surgery. The 3 chemical agents comprising PAF, prepared as a single solution, were slowly soluble in 0.015 M NaCl when mixed on a magnetic stirrer for about 15 min. The solution contained each agent in an amount appropriate to administer 0.01 ml of solution per g of body weight. This regimen of chemotherapy never produced host weight loss in excess of 4%. At no time was there a body weight difference in excess of 6% between control and treated mice.

RESULTS

Earlier studies in which primary spontaneous breast tumors were removed and weighed at the same time as the lungs were removed and bioassayed showed the incidence of lung metastasis to be 2, 24, 48, and 68% in animals bearing tumors of less than 0.9, 0.9 to 2, 2 to 4, and 4 to 15 g, respectively (1). For the present investigation of the effects of surgical adjuvant PAF chemotherapy, single spontaneous tumors were enucleated from 140 female CD8F, mice. The mean and median weights and the range of weights of the removed tumors are shown in Table 1. All tumors weighed under 2 g, a range in which fewer than 24% of the animals might be expected to show metastasis.

All mice were palpated weekly for recurring tumors and for new primary tumors developing in other breasts in order that both could be removed while very small. By this means, we attempted to maintain the hosts as free from breast tumors as possible so as better to evaluate the therapy on lung metastases.

Effect of Adjuvant Chemotherapy on the Original Primary Tumor. Chart 1 graphically represents the effect of PAF chemotherapy on the original breast tumor by showing the percentage of mice that remained free of original tumor (i.e., without recurrence) as a function of time in days. The animals were followed for 6 months after original surgery, i.e., for slightly less than 5 months after cessation of therapy. Very few tumors have been found to recur beyond 4 or 5 months after their surgical removal and these have exerted little, if any, effect on the final experimental results. At 6 months, there was a difference in original tumor recurrence: 64% (44 of 69) of the PAF-treated mice remained free of original tumor as compared with only 42% (27 of 64) of the surgical controls. This difference is statistically significant by $\chi^2$ test ($p = 0.02$).

Effect of Adjuvant Chemotherapy on the Development of New Primary Tumors. Chart 2 shows that the percentage of mice that developed new primary tumors at mammary sites other than the original site also was significantly reduced during the period of PAF administration and for about 8 weeks thereafter ($p < 0.001$ by $\chi^2$ test). However, the difference between the 2 groups lost statistical significance by about 13 to 14 weeks after original surgery and remained insignificant for the remainder of the 6 months observation period.

In addition to comparing the numbers of mice developing new primary tumors, we also wished to compare the number of new tumors (often more than 1/mouse) that appeared in the groups. Chart 3 shows a cumulative plot of all new tumors that appeared in the controls and in the PAF-treated mice with time. The straight lines indicate that the appearance of new tumors among the mice was relatively constant, but the flatter slope of the line representing the PAF-treated mice shows that new tumors in these animals appeared at a lower rate during the 1st 13 weeks. This inhibition of new tumor development among the PAF-treated animals rapidly disappeared at about 13 to 14 weeks (as also was evident in Chart 2), and new tumors then began to appear at the same rate as in the controls, as indicated by the parallel slopes of the lines after 13 to 14 weeks.

Effect of Adjuvant Chemotherapy on Pulmonary Metastases. Lungs from all animals were examined for metas-

* The abbreviation used is: PAF, phenylalanine mustard (4 mg/kg)-adriamycin (2 mg/kg)-5-fluorouracil (50 mg/kg).
tasis. Those lungs with grossly obvious metastatic lesions were not bioassayed, whereas all others were. It was necessary to sacrifice about one-half of the animals before the end of the experiment owing to eventual inoperability of the tumors or because of obvious symptoms of metastasis. However, the number of animals and the time of their sacrifice were virtually identical in the control and PAF-treated groups and did not represent a variable between them. A comparison of the incidence of metastasis in the two groups is presented in Table 2. As noted above, the number of mice sacrificed during the course of the experiment was about the same in the control and PAF-treated groups (35 and 37, respectively), as was the percentage of those animals showing metastasis (54 and 46%, respectively). The final evaluation including all of the mice also showed essentially no difference in that 42% (27 of 64) of the control animals, and 37% (25 of 67) of the PAF-treated animals evidenced pulmonary metastasis. Of the 27 control mice and of the 25 treated mice that ultimately showed metastasis, 20 in each group were detectable grossly. This comparable finding in control and treated autochthonous female hosts minimized the possibility that any hormonally sensitive tumor cell growth may have been missed as a consequence of using male animals for bioassay. There was no correlation between recurrence of the original tumor and the presence of metastasis.

A comparison of the observed incidence of metastasis in this study in which lungs were examined 6 months after tumor removal with the expected incidence of metastasis in animals in which the lungs were examined simultaneously with tumor removal (1) is presented in Table 3. Again it may be seen that an increased incidence of metastasis occurred in animals bearing larger tumors when the lungs were examined either 6 months after tumor removal (observed) or at the time of tumor removal (expected). However, it is obvious that the incidence of metastasis observed 6 months after tumor removal was approximately 30% greater than the incidence expected from animals examined at the time of tumor removal. In other words, 30% of the observed metastases would not have been detectable at the time of original surgery. The data show that PAF was ineffective against incipient metastases in mice bearing smaller tumors, as well as ineffective against more established metastatic foci in mice bearing larger tumors.

**DISCUSSION**

The data show that a regimen of surgical adjuvant combination chemotherapy that exerted significant curative activity against a primary spontaneous breast adenocarcinoma as well as transient activity against new primary tumors developing in other breasts during the course of the experiment, had no effect on the incidence of pulmonary metastasis in autochthonous host mice. An earlier study in which lungs were bioassayed for metastasis at the same time as the spontaneous tumor was removed and weighed showed that tumors size was directly related to the incidence of metastasis and that animals bearing tumors of less than 0.9 g had a metastatic incidence of only 2% (1). It is apparent from the present data, however, that small tumors can and do metastasize (Table 3). The results underscore the fact that tumor cells can be seeded in the lung very early at undetectable levels and multiply, in time, to levels detectable grossly and by bioassay. (In this system, that is about 10^4 cells.)

The therapeutic advantages of administering chemical agents in the presence of minimal quantities of tumor tissue have long been documented in animals (3, 6, 8-13, 16) and, more recently, have been evidenced against clinical breast cancer (2, 5). However, the present findings show that a
regimen of adjuvant chemotherapy that cured minimal primary tumor did not cure minimal metastatic tumor.

The reason(s) for this difference in susceptibility of small numbers of primary tumor cells and small numbers of metastatic tumor cells is not known but is not without precedent. One mechanism suggested to explain such an effect is host immunosuppression. Sugarbaker et al. (19) showed that cyclophosphamide retarded the growth of primary Walker 256 tumors in rats, but its administration resulted in an increase in the number of pulmonary metastases. They attributed the difference in effect to the immunosuppressive activity of the drug. Similarly, Sakakibara and Oota (14) were able to induce metastases in hamsters implanted with Yoshida sarcoma cells by immunosuppressing the animals with antithymocyte serum, and Yuhas et al. (21) increased metastases in line 1 lung carcinoma in BALB/c mice by irradiation-induced immunosuppression. We have found (unpublished data) that the regimen of PAF used here temporarily suppresses both humoral and cellular immunity. However, it is difficult to accept a differential effect on minimal primary and secondary tumors simply as a result of immunosuppression, particularly if antigenically identical cells are involved.

Another mechanism postulated to explain the difference in therapeutic response of primary and secondary tumors is that the preponderant cell types comprising the 2 tumor types are antigenically different. From the results of an investigation of the capacity of i.v.-inoculated B16 melanoma tumor cells to form pulmonary nodules in C57BL/6 mice, Fidler (4) postulated that the survival of invasive and/or circulating malignant tumor cells is not a random phenomenon but, rather, that the malignant cells possess unique surface qualities which allow them to survive. A difference in cell type also may be inferred from the data of Wexler et al. (20) in which Lewis T241 tumor cell suspensions prepared from primary tumors were compared with tumor cells found in the venous heart blood of mice bearing T241 fibrosarcomas for their relative capacities to produce pulmonary tumors upon i.v. inoculation. It was found that an approximate 100-fold greater number of viable cells was required from the primary tumor than from the tumorous blood in order to initiate pulmonary tumors.

A 3rd possible explanation for a differential response of primary and metastatic tumors to chemotherapy is related to the site of the metastatic growth. In a study of data collected from the Eastern Clinical Drug Evaluation Program from September 1961, to December 1965, Slack and Bross (17) determined that the response to chemotherapy depended more strongly on the site of the metastasis than on the site of the primary from which it was derived. With a few exceptions, the superficial lesions (e.g., those in lymph nodes and in the integumentary system) tended to be responders, whereas the deep lesions (e.g., those in skeletal and respiratory systems) tended to be nonresponders. Furthermore, the metastatic lesions that did not respond did not give a reliable indication of drug action against parent tumor types. Metastases from breast tumors did not respond as well as the advanced primaries from which they were derived.

Whether the above possibilities are causative for the observed differential chemotherapeutic effect remains to be determined. However, it seems clear that agents and/or schedules should be developed to act specifically on the primary and specifically on the metastatic tumors within a given host. That this type of treatment may well result in the use of different chemical agents with different cytotoxic characteristics has been suggested by others (15, 17).

We believe that the CDBF, spontaneous tumor system as used here represents a clinimimetic animal breast tumor model in which simultaneously to evaluate therapy against both spontaneous primary tumors and spontaneous metastatic tumors in their autochthonous hosts. We suggest that it be used to test therapeutic regimens developed against primary and/or metastatic tumors in more artificial, transplanted animal systems as a more predictive indication of their clinical candidacy (12).

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