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Effects of Combination Drug Therapy on the Subcutaneous and Pulmonary Growth of a Slow and a Fast-growing C3H/He Mammary Carcinoma[1]

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ABSTRACT

Changes in susceptibility to treatment with Cytoxan, methotrexate, 5-fluorouracil, and Adriamycin, single or in combination, have been studied during the initial and progressive stages of s.c. and pulmonary (via tail vein injection) growth of two transplanted syngeneic C3H/He mammary carcinomas. One tumor was fast growing, reaching a size of 3 mm from a 1-mm s.c. implant in 7 days; the second tumor would grow to the same size in 30 days. The tumor with the slower growth rate was more susceptible to drug treatment, manifested by delayed growth as well as by prevented growth. The slower-growing tumor also remained susceptible longer, when treatment was delayed, than did the faster-growing tumor. Pulmonary growth was more often prevented by drug treatment than was s.c. growth. Tumor implants s.c. which had reached palpable size could be reduced temporarily to impalpable size by effective drug treatment but were rarely cured. The importance of early treatment relative to the time of tumor implantation was indicated when early treatment with a single drug proved more effective than did delayed treatment with a more potent combination of drugs.

INTRODUCTION

The fundamental rationale for adjuvant or prophylactic treatment of cancer derives from *in vivo* and *in vitro* studies of tumor cell kinetics which have demonstrated the following: (*a*) growth curves for most experimental neoplastic cells have a Gompertzian function; (*b*) the highest mitotic index is associated with the least-crowded conditions; (*c*) small tumor masses with high mitotic indexes are most sensitive to S-phase-specific drugs; and (*d*) the smaller the experimental tumor, the greater will be its potential curability by drug treatment (10, 12–14). The results of clinical studies (1, 4) have indicated that subsets of postmastectomy patients differ significantly with respect to the benefits derived from adjuvant chemotherapy and invite guarded optimism.

A variety of drugs effective against mouse mammary tumors have been described (9). Many of these are also active against human breast cancer when used against clinically established metastases (2). Some experimental studies have reported that surgical removal of the primary tumor followed by drug treatment reduced the number of expected metastases (6, 11). In other studies, systemic chemotherapy following excision of the primary tumor reduced the growth of local recurrence without affecting the incidence of metastases (5). Several investigators have also reported that drug therapy may facilitate metastatic growth (7, 14, 15). Fefer (3) found the net result of drug treatment to be a prolonged survival of murine tumor hosts. Apparently, it is not yet safe to predict the outcome of drug treatments even in experimental tumor systems.

Because the effectiveness of drug treatment (8), like the effectiveness of immune resistance (16, 17), appears to be reduced by increasing mass of malignant tissue (but is less affected by its dissemination), both the growth rate of the tumor and the timing of the initiation of drug treatment may be variables in the effectiveness of adjuvant chemotherapy. The study reported here has used a fast-growing and a slow-growing mouse mammary carcinoma to investigate the relationship of the time of s.c. and i.v. tumor implantation to the effectiveness of drug treatment.

MATERIALS AND METHODS

Mice. The tumor donors were pedigree breeding C3H/He mice, bred from the age of 8 weeks and removed from breeding at the appearance of the first mammary tumor or after the fifth litter. The experimental mice were 10- to 12-week-old females produced in full sib-mated line breeding of C3H/He mice. All the mice were from the defined-flora, pathogen-free breeding colony (mice carrying only the following nonpathogenic enteric bacteria: *Clostridium* sp., *Peptostreptococcus* sp., *Bacillus* sp., and *Bacteroides* sp.) maintained by the Department of Cancer Therapy Development at Pondville Hospital.

Tumors. The mammary carcinomas had developed spontaneously in multiparous C3H/He mice and had been serially transplanted in syngeneic virgin female mice. Tumor tissue from the first transplant generation had been stored in liquid N_2, and the tumors were used to the sixth transplant generation before being started again from the frozen generation 1. Tumor tissue was removed from freshly killed or live anesthetized donor animals, and one 1-cu mm piece of living tumor tissue was implanted s.c. in the right flank of each host animal to initiate new growth. Tumors from treated animals were never used.

Tumor 38-5 was the fifth spontaneous mammary carcinoma removed from C3H/He mouse 38. It had been shown in repeated tests (19) to be fast growing s.c. (from a 1-mm implant to 3 mm in 7 days) and to be immunologically neutral in transplantation tests; *i.e.*, the cured hosts of Tumor 38-5 displayed neither resistance against nor stimulation of the growth of reimplants of the same tumor. Tumor 93-2 was slow growing s.c. (from 1-mm implant to 3 mm in 30 days) and was neutral in transplantation tests (19).

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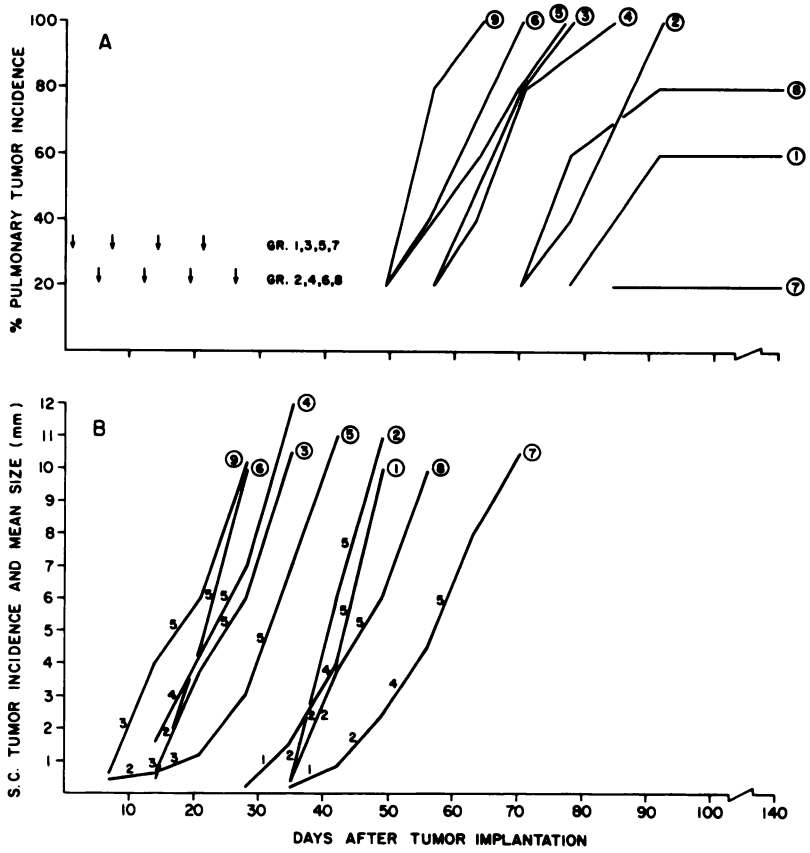

Chart 1. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and s.c. growth (*B*) of the fast-growing Tumor 38-5. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 and 2, Cytoxan, 100 mg/kg/week for 4 weeks; Groups 3 and 4, methotrexate, 50 mg/kg/week for 4 weeks; Groups 5 and 6, 5-FUra, 50 mg/kg/week for 4 weeks; Groups 7 and 8, all 3 drugs combined for 4 weeks; Group 9, placebo for 4 weeks. *Arrows*, injection schedules. Each group contained 5 mice. *Numbers along the curves* in *B*, number of mice with s.c. growth.

Tumor Implantation. Disruption of tumor tissue to obtain suspensions of dispersed cells was accomplished with the use of 105 mesh polyester cloth (HC-1-105 screen cloth; TETKO, Inc., Elmsford, N. Y.) by a mechanical procedure described in previous publications (17, 18). The s.c. (10^5 viable cells) and i.v. (3×10^4 viable cells) challenge implantation of treated and control mice were by injection of viable (trypan blue-negative) tumor cells suspended in culture medium (Roswell Park Memorial Institute Tissue Culture Medium 1640; Associated Biomedics, Buffalo, N. Y.) and consisting predominantly of single cells. The proportion of trypan blue-negative cells in the tumor cell suspensions was usually about 20%. The challenges were completed as soon as possible with cells from the same suspension, which was kept on ice to delay cell clumping.

In each of the experiments, the mice were given injections (the day of tumor challenge designated as Day 0) of 10^5 living tumor cells s.c. at the left shoulder and, at the same time, given i.v. injections via the tail vein of 3×10^4 cells. Following injection of tumor cells into the tail vein, tumor growth was found only in the lungs. The incidence of tumors at the s.c. injection site was checked twice weekly on the same day in all groups of each experiment. By measuring 2 bisecting diameters of each tumor and using only the lesser to express tumor size, the possible error of measuring the added diameters of more than one focus of growth along the needle path of injection was avoided. The tumors were excised with wide margins when they were close to 10 mm in diameter. Tumors that had reached this size were recognized as treatment-failures and had to be removed to keep the mice alive and healthy for the anticipated but slower development of tumors in the lungs. Frequent examination of the mice for signs of dyspnea decided the termination time for each animal. The mice were killed by asphyxiation in 100% CO_2 gas, and the lungs were examined for extent of tumor growth. The mice which had not developed pulmonary symptoms were killed and examined 140 days after the challenge. Because every untreated mouse in these experiments developed both s.c. and pulmonary tumor growth, treated mice which were negative at 140 days were considered to have been successfully treated.

Treatment. The experimental animals were given 4 weekly i.p. injections of the following drugs, individually or in combination: Cytoxan (cyclophosphamide monohydrate, courtesy of Mead Johnson Research Center, Evansville, Ind.), 100 mg/kg; methotrexate, RNX-0396 (courtesy of Lederle Laboratories, Pearl River, N. Y.), 25 or 50 mg/kg; Adriamycin (doxorubicin hydrochloride; courtesy of Adria Laboratories, Inc., Wilmington, Del.), 5 mg/kg; 5-FUra,[4] (Ro2-9757; courtesy of Hoffman-La Roche Inc., Nutley, N. J.), 25 or 50 mg/kg.

RESULTS

Drug Treatment of a Fast-growing Tumor. Chart 1 shows the results of a test with the fast-growing Tumor 38-5 in which drugs were given singly or in combination. The results show that the growth of s.c. implants was not prevented by treatments with Cytoxan, methotrexate, or 5-FUra, used singly or in combination. Each drug tested produced only a delay in the growth of the s.c. tumors, particularly when given in combina-

[4] The abbreviation used is: 5-FUra, 5-fluorouracil.

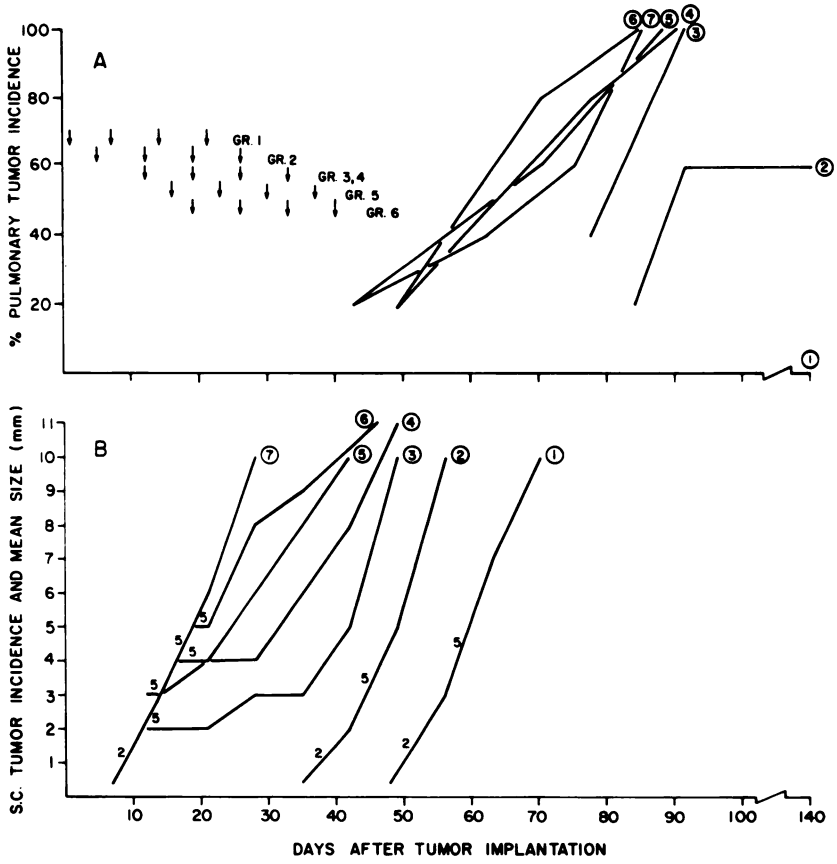

Chart 2. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and the s.c. growth (*B*) of the fast-growing Tumor 38-5. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 to 6, 3 drugs in combination (Cytoxan, 100 mg/kg/week for 4 weeks, plus methotrexate, 25 mg/kg/week for 4 weeks, plus 5-FUra, 25 mg/kg/week for 4 weeks); Group 7, placebo for 4 weeks. *Arrows*, injection schedules. Each group contained 5 mice. *Numbers along the curves* in *B*, number of mice with s.c. growth.

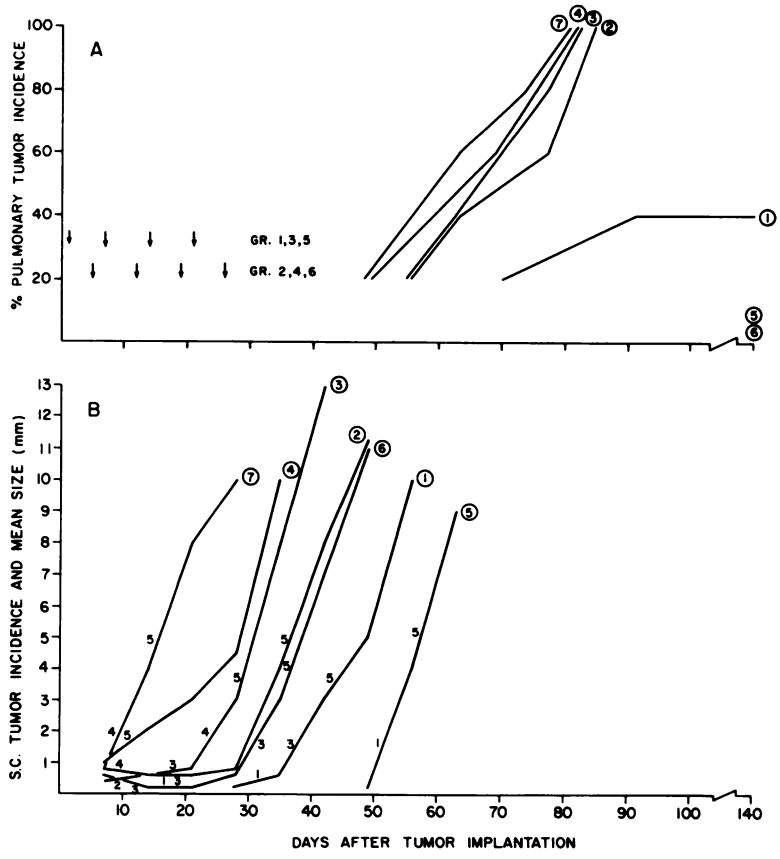

Chart 3. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and the s.c. growth (*B*) of the fast-growing Tumor 38-5. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 and 2, Cytoxan, 100 mg/kg/week for 4 weeks; Groups 3 and 4, Adriamycin, 5 mg/kg/week for 4 weeks; Groups 5 and 6, both drugs combined for 4 weeks; Group 7, placebo for 4 weeks. *Arrows*, injection schedules. Each group contained 5 mice. *Numbers along the curves* in *B*, number of mice with s.c. growth.

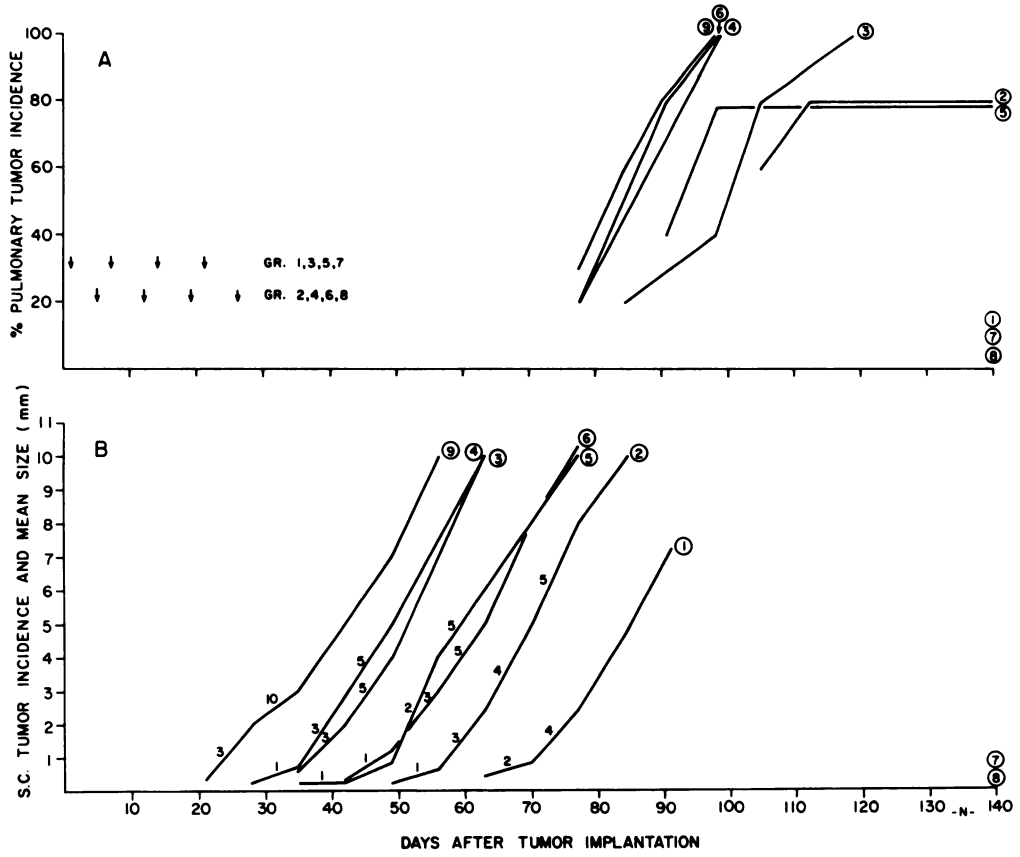

Chart 4. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and the s.c. growth (*B*) of the slow-growing Tumor 93-2. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 and 2, Cytoxan, 100 mg/kg/week for 4 weeks; Groups 3 and 4, methotrexate, 50 mg/kg/week for 4 weeks; Groups 5 and 6, 5-FUra, 50 mg/kg/week for 4 weeks; Groups 7 and 8, all 3 drugs combined for 4 weeks; Group 9, placebo for 4 weeks. Groups 1 to 8 contained 5 mice each; Group 9 contained 10 mice. *Numbers along the curves* in *B*, numbers of mice with s.c. growth.

tion, and particularly when given from the day of challenge. The results of i.v. tumor challenge show that treatment with all 3 drugs from the day of challenge prevented pulmonary growth in 4 of 5 mice in Group 7. The same treatment, delayed 5 days in Group 8, prevented pulmonary growth in only 1 of 5 mice. Cytoxan alone, given from the day of challenge, prevented pulmonary growth in 2 of 5 mice.

Chart 2 shows the results of a test with Tumor 38-5 in which the treatment with Cytoxan, methotrexate, and 5-FUra was started at progressively later times following s.c. and i.v. challenge. Treatments were started: (*a*) on the day of challenge; (*b*) 5 days after challenge; (*c*) on day 12 with s.c. tumors at a size of 2 or 3 mm; (*d*) on Day 16 with tumors at a size of 4 mm; and (*e*) on Day 19 with s.c. tumors at a size of 5 mm. The results show that the treatment succeeded only in delaying the s.c. tumor growth, with the greatest effect when treatment was started on the day of challenge. The greatest effect against pulmonary growth was also achieved when the treatment was started on the day of challenge; a delay of 5 days increased pulmonary growth from 0 of 5 mice to 3 of 5 mice. Pulmonary growth was not prevented when treatment was started 12 or more days after challenge.

Chart 3 shows the results of a test with Tumor 38-5 in which the treatment with Cytoxan and Adriamycin, singly or combined, was started on the day of i.v. and s.c. challenge or 5 days after challenge. The results show that early combined treatment with Cytoxan and Adriamycin was most effective both in delaying s.c. growth and in preventing pulmonary growth. The figures also show that early treatment with Cytoxan alone gave better results against both s.c. and pulmonary growth than did delayed treatment with the more potent combination of Cytoxan and Adriamycin.

Drug Treatment of a Slow-growing Tumor. The slow-growing Tumor 93-2 was tested in the same way as the fast-growing Tumor 38-5 (see Chart 1), using the same drug treatments and following the same schedule. Chart 4 shows that combined treatment from Day 0 or from Day 5 with Cytoxan, methotrexate, and 5-FUra, prevented both s.c. and pulmonary tumor growth in all the mice. Early treatment with Cytoxan alone also prevented pulmonary tumor growth, but prevented s.c. growth in only 1 of 5 mice. Delayed treatment with Cytoxan was as effective as early treatment with 5-FUra, pulmonary tumor growth being prevented in 1 of 5 mice in each group.

Chart 5 shows the results of a test with Tumor 93-2, similar to the test presented in Chart 2. The results show that combined treatment with Cytoxan, methotrexate, and 5-FUra was completely effective against the growth of pulmonary tumors even if treatment was started as late as Day 26 after challenge and that it could still prevent pulmonary tumors in 3 of 5 mice when treatment was started on Day 28. Growth s.c. was less readily

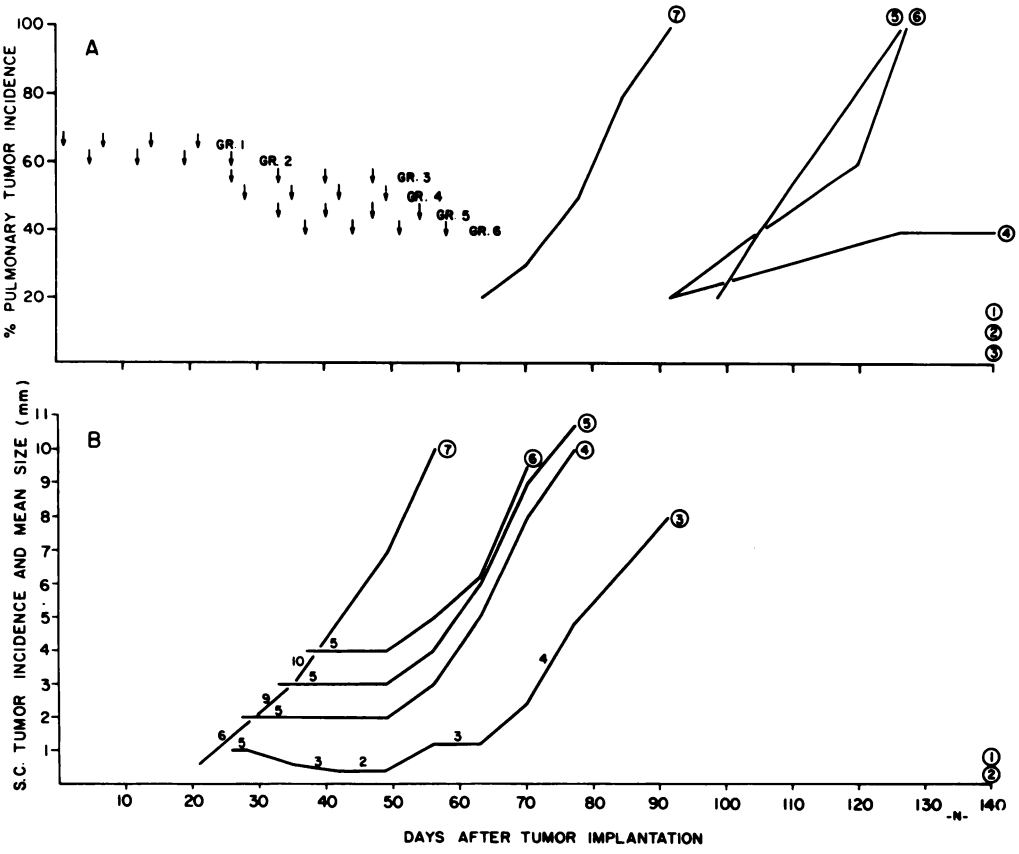

Chart 5. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and the s.c. growth (*B*) of the slow-growing Tumor 93-2. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 to 6, 3 drugs in combination (Cytoxan, 100 mg/kg/week for 4 weeks, plus methotrexate, 25 mg/kg/week for 4 weeks, plus 5-FUra, 25 mg/kg/week for 4 weeks); Group 7, placebo for 4 weeks. *Arrows*, injection schedules. Groups 1 to 6 contained 5 mice each; Group 7 contained 10 mice. *Numbers along the curves* in *B*, number of mice with s.c. growth.

controlled; only 1 of 5 palpable tumors was cured after treatment was started on Day 26 (Chart 5*B*, Group 3). Treatments started on Days 0 and 5 were completely effective in preventing s.c. as well as pulmonary growth.

Chart 6 shows the results of a test with Tumor 93-2, in which the treatment with Cytoxan and Adriamycin, singly or combined, was started (like the test shown in Chart 3) on the day of i.v. and s.c. challenge or 5 days after challenge. The results show that early combined treatment with Cytoxan and Adriamycin was more effective in delaying and preventing pulmonary and s.c. tumor growth than either drug given alone. The combined drug treatment was very effective against this slow-growing tumor, giving better results even when delayed to Day 5 than did treatment with Cytoxan started on Day 0.

DISCUSSION

The results of the investigation reported here suggest that there was a period of relatively greater susceptibility to drug treatment during the early phase of tumor establishment when cures could be achieved. Of 2 mammary carcinomas with different growth rates, 38-5 and 93-2, it was Tumor 93-2, with the slowest growth rate, which was more controllable, presumably because it remained longer in a susceptible growth phase. Once either tumor had reached palpable s.c. growth, they could be reduced temporarily by effective drug treatment to impalpable size (Chart 3*B*, Group 6 Chart 5*B*, Group 3) but, with the exception of one case (Chart 5*B*, Group 3) could not be cured. However, the longer latency of tumors in treated mice measured from the last injection of drugs compared to the latency of tumors in control mice measured from Day 0 [*e.g.* Group 1 *versus* Group 7 (Chart 2*B*)] suggests that the treatment had reduced the original s.c. inoculum of 10^5 viable cells to a much lower number and may have come close to eliminating the implants.

For reasons that cannot yet be explained, the drug treatments more effectively prevented the growth of tumor cells implanted in the lungs than tumor cells implanted in the loose connective tissue of the skin. It may be that the drugs injected i.p. were more readily and more abundantly available where blood-borne tumor cells lodged and were, therefore, more effective. Since it has also been found that implanted C3H/He mammary carcinomas invariably grow slower in the lungs than s.c. (19), they could possibly have remained longer in an unestablished, susceptible growth phase. Whatever the reason for the observed effect, it is encouraging that malignant growth, under conditions that simulate venous dissemination, has a growth phase which is particularly susceptible to chemotherapy.

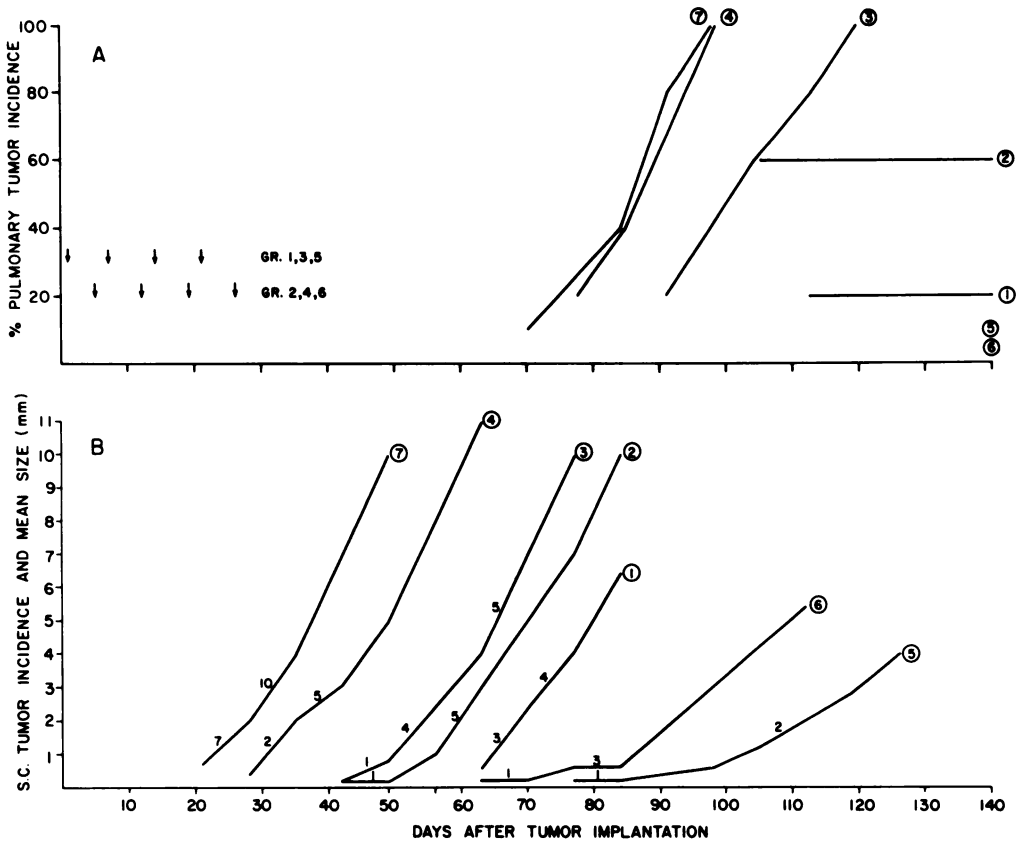

Chart 6. The effect of drug treatments on the pulmonary (via tail vein) growth (*A*) and s.c. growth (*B*) of the slow-growing Tumor 93-2. Each animal was implanted both i.v. and s.c. The mice received the following treatments: Groups 1 and 2, Cytoxan, 100 mg/kg/week for 4 weeks; Groups 3 and 4, Adriamycin, 5 mg/kg/week for 4 weeks; Groups 5 and 6, both drugs combined for 4 weeks; Group 7, placebo for 4 weeks. *Arrows*, injection schedules. Groups 1 to 6 contained 5 mice each; Group 7 contained 10 mice. *Numbers along the curves* in *B*, number of mice with s.c. growth.

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