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# Combination Chemotherapy and Surgical Adjuvant Chemotherapy on MS-2 Sarcoma and Lung Metastases in Mice

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**Abstract**—*The MS-2 tumor behaved like human spontaneous tumor: slow and progressive growth, extensive dissemination, high capacity to metastasize to the lung and low antigenicity. The chemotherapeutic sensitivity of MS-2 sarcoma and the antimetastatic activity of some clinically active antineoplastic drugs administered alone or in combination, or in surgical-adjuvant chemotherapy studies were investigated. Significant inhibition of tumor growth was obtained with: melphalan (ME), cyclophosphamide (CY), BCNU, adriamycin (AD), daunorubicin (DR) and bleomycin (BL). ME and BCNU showed activity in reducing the percentage of mice with metastatic activity of some clinically active antineoplastic drugs administered alone or tumors in conjunction with surgery drastically reduced the number of mice with lung foci. This treatment was found to be more effective on pulmonary metastases than was surgery and single-drug therapy. The antimetastatic activity of adjuvant chemotherapy with AD plus ICRF-159 supplied at different times with respect to surgery was also examined. A synergistic effect was observed on lung metastases when the treatment was performed before the surgery; when the combined treatment was performed after the surgery, a lower synergistic effect was found.*

## INTRODUCTION

INTRAVENOUS implantation to induce artificial metastases cannot be regarded in a strict sense as an appropriate model for lung metastases [1, 2]. Some spontaneously metastasizing tumors have been used in a variety of experiments designed to study the antimetastatic properties of antitumor drugs applied as single agents, or in combination chemotherapy with or without surgery: Lewis lung carcinoma [3, 4], B16 melanocarcinoma [5], mammary adenocarcinoma [6] and other tumors [7].

We have described a cell line, referred to as MS-2, established *in vitro* in our laboratory [8, 9], and the biologic characterization of the MS-2 tumor produced in BALB/c mice by transplant of *in vitro*-cultivated MS-2 cells [10, 11]. The MS-2 tumor was histologically classified as a malignant sarcoma. The neoplasma behaved like human spontaneous tumors: slow and progressive growth, extensive dissemination, high capacity to metastasize to the lung [11], and low antigenicity [10].

The objectives of this study were to examine the chemotherapeutic sensitivity of MS-2 sarcoma and the antimetastatic activity of some clinically active antineoplastic drugs administered alone or in combination, or in surgical-adjuvant chemotherapy studies, in order to find out whether or not this experimental model could have any predictive value as a secondary screen for agents against solid tumors.

## MATERIALS AND METHODS

### *Animals*

All the animals used throughout these experiments were 6–8-week-old syngeneic BALB/c mice, obtained from Charles River Breeding Laboratories, Calco, Italy.

### *Tumor*

The MS-2 tumor was maintained by serial intramuscular (i.m.) passages of a tumor cell

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*Table 1. Single-drug chemotherapy of i.m. MS-2 sarcoma*

| Class | Drug | Days of treatment | Vehicle | Route | Dose (mg/kg/day) | % Inhibition of tumor growth* | | MST† (days) | % ILS |
|---|---|---|---|---|---|---|---|---|---|
| Antimetabolites | — | | | | | — | (24) | 38.7 | |
| | MTX | 3, 7, 10 | water | i.p. | 15 | 36.5* | | 40.0 | 3 |
| | | | | | 25 | — | | 11.6 | −70 |
| | — | | | | | — | (24) | 42.8 | |
| | 5-FU | 3, 7, 10 | saline | i.p. | 45 | 23.5* | | 48.3 | 12 |
| | | | | | 65 | — | | 23.6 | −44 |
| Alkylating agents | — | | | | | — | (21) | 37.6 | |
| | ME | 3, 7, 10 | 10% ethanol | i.p. | 7 | 66.6‖ | | 48.7 | 29 |
| | | | | | 10 | 83.3‖ | | 40.0 | 6 |
| | — | | | | | — | (33) | 45.5 | |
| | CY | 6, 10, 14, 18 | water | i.p. | 40 | 64.9* | | 54.5 | 10 |
| | | | | | 60 | 84.2‖ | | 60.5§ | 27 |
| | | | | i.v. | 20 | 47.8‖ | | 57.5 | 17 |
| | | | | | 40 | 79.7‖ | | 57.0 | 21 |
| | | | | | 50 | 83.0‖ | | 61.0§ | 27 |
| | — | | | | | — | (26) | 49.0 | |
| | CY | 14, 16 | water | i.p. | 100 | 86.2‖ | | 66.1§ | 34 |
| | | | | | 150 | 92.3‖ | | 67.8§ | 38 |
| | — | | | | | — | (24) | 45.7 | |
| | BCNU | 3, 7, 10 | 10% ethanol | i.p. | 10.0 | 59.5‖ | | 46.8 | 2 |
| | | | | | 17.0 | 74.5‖ | | 46.5 | — |
| | — | | | | | — | (24) | 38.7 | |
| | BL | 3, 7, 10 | saline | i.p. | 40.0 | 67.9‖ | | 47.0 | 21 |
| | | | | | 50.0 | 69.1‖ | | 47.4 | 22 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mitosis | — | | | | | — | (24) | 38.7 | |
| inhibitor | VCR | 3, 7, 10 | saline | i.p. | 0.5 | 46.3‖ | | 40.7 | 5 |
| | | | | | 1.0 | 46.0‖ | | 38.6 | — |
| Intercalating | — | | | | | — | (33) | 47.5 | |
| agents | AD | 6, 10, 14, 18 | water | i.v. | 2.5 | 42.3 | | 56.6 | 19 |
| | | | | | 3.5 | 68.0* | | 62.8¶ | 32 |
| | — | | | | | — | (24) | 48.0 | |
| | AD | 1, 8, 15, 22, 29 | water | i.v. | 4.4 | 42.0 | | 54.5 | 13 |
| | | | | | 6.6 | 65.0‖ | | 62.7¶ | 30 |
| | | | | | 10.0 | 76.0‖ | | 48.5 | 1 |
| | — | | | | | — | (25) | 33.6 | |
| | AD | 1, 4, 7, 10, 13 | water | i.v. | 4.0 | 69.0‖ | | 44.3¶ | 31 |
| | — | | | | | — | (33) | 47.5 | |
| | DR | 6, 10, 14, 18 | water | i.v. | 3.25 | 8.0 | | 51.3 | 8 |
| | | | | | 4.0 | 38.0 | | 51.1 | 7 |
| | — | | | | | — | (24) | 48.0 | |
| | DR | 1, 8, 15, 22, 29 | water | i.v. | 5.3 | 40.0 | | 59.8§ | 24 |
| | | | | | 8.0 | 39.0 | | 55.1 | 15 |
| | | | | | 12.0 | 65.0* | | 25.1 | −47 |
| | — | | | | | — | (25) | 33.6 | |
| | DR | 1, 4, 7, 10, 13 | water | i.v. | 6.0 | 61.0‖ | | 43.5¶ | 29 |
| Miscellaneous | — | | | | | — | (25) | 33.6 | |
| | ICRF-159 | 1, 4, 7, 10, 13 | 0.3% Klucel | i.p. | 30.0 | 17.0 | | 34.8 | 3 |
| | | | | | 50.0 | 9.0 | | 38.0 | 13 |

*In parenthesis, day of observation. ‖$P<0.01$; *$P<0.05$ as evaluated by Student $t$-test.
†Median survival time. ¶$P<0.01$; §$P<0.05$ as evaluated by Student $t$-test.
‡Percentage increase in host life span.

homogenate into the right hind leg. For chemotherapy experiments tumors from donor mice were removed under sterile conditions 10 days after implantation and placed into a Petri dish in 199 medium. The cell suspension obtained by Potter homogenization was passed through gauze. The cell number was counted in a hemocytometer after staining with Türk solution in order to eliminate red blood cells, then injected i.m. into the right hind leg ($10^5$ cells/mouse). Tumor growth and median survival times were calculated as described by Geran *et al.* [12]. For antimetastatic activity experiments, the suspension was injected into the foot-pad ($10^6$ cells/mouse).

*Surgical tumor removal*

The surgical removal of the tumor was carried out as previously described [11]. Animals were anesthetized with sodic thiopenthal (Farmotal, Farmitalia, Milano, Italy). The tumor-bearing foot and leg were washed with 95% alcohol. Surgical sterile natural thread was used to tie the femoral artery and adjacent blood vessels. The muscle and femur were cut out with sterile scissors, (the popliteal lymphnodes were included in the line of amputation) and the wound was closed with metal wound clips. Surgical removal resulted in less than 10% mortality; no primary tumor recurrence or bacterial infections were observed.

*Assay for metastasis detection*

Mice were killed by cervical dislocation, the lungs were removed aseptically, minced, inserted into a syringe with a small amount (0.5–0.6 ml) of 199 medium, and injected subcutaneously (s.c.) into syngeneic normal BALB/c mice. The lungs of each animal were bioassayed individually. Mice were observed for 100–120 days after tissue implantation. The tumor growth after s.c. implant of lungs was the criterion for the presence of lung metastatic foci.

*Drugs*

Drugs were prepared at a concentration such that the dose could be given in a volume of 10 ml/kg body weight. The vehicles employed are reported in the Results section. The following drugs were used: methotrexate (MTX), bleomycin (BL), vincristine (VCR), 5-flourouracil (5-FU), melphalan (ME), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), cyclophosphamide (CY), adriamycin (AD), daunorubicin (DR), and ICRF-159. MTX, BL, VCR, 5-FU, ME, BCNU and CY were supplied by Dr. H. Wood, Division of Cancer Treatment, National Cancer Institute, USA. AD and DR were supplied by Farmitalia, Milano, Italy. ICRF-159 was generously supplied by Dr. H. Hellmann, Imperial Cancer Research Fund, London, England. The drugs were administered intraperitoneally (i.p.) except for DR and AD, which were injected intravenously (i.v.) according to different schedules.

*Statistical analysis*

Statistical significance was evaluated by means of Student *t*-test or by the Mussett test [13].

## RESULTS

*Effectiveness of single-drug chemotherapy against i.m. MS-2 sarcoma*

The schedules of treatment and the results of the chemotherapy tests of single-drug chemotherapy against i.m. MS-2 sarcoma are summarized in Table 1. Significant inhibition of tumor growth was obtained with ME (which was probably toxic at the higher 10 mg/kg dose tested), BCNU, VCR and BL at all the doses tested. Of these drugs, only BL at the dose of 50 mg/kg also produced a significant increase of survival time. On mice bearing MS-2 sarcoma treated with CY administered i.v. or i.p., a marked therapeutic response was evident against both early disease or advanced tumor (in the group treated at 14th and 16th day after tumor implant). A statistically significant reduction of the tumor growth and an increase of life span were observed.

The antitumor activity of AD was dependent on the dose and the schedule of treatment adopted. When treatment was started at the 1st or at the 6th day after the tumor injection and given every 3rd day, AD at the doses of 3.5–4 mg/kg significantly reduced the tumor growth and increased the life span. When treatment was started at the first day and given on a weekly schedule, the dose of 4 mg/kg was inactive, the dose of 6.6 mg/kg was the optimal dose, and the dose of 10 mg/kg strongly reduced the tumor growth but did not enhance the mice life span, presumably because of toxic effects. DR administered according to these three different schedules was less active than AD. ICRF-159 did not show any therapeutic effect on tumor growth and on life span of tumor-bearing mice. MTX and 5-FU administered at very

high doses (one toxic and the lower probably subtoxic) slightly altered the pattern of tumor growth.

*Antimetastatic activity of single-drug chemotherapy*

Table 2 reports the data on antimetastatic activity of single-drug chemotherapy in mice bearing i.m. MS-2 sarcoma. The drugs were administered at days 3, 7 and 10 after the tumor implant; the lungs were bioassayed four days after the last treatment. At the dose of 17 mg/kg BCNU was active in reducing the percentage of mice with lung metastatic foci (9/21 = 42.8%; $P<0.01$). MTX, 5-FU, BL and VCR were inactive. ME at the dose of 10 mg/kg was also active in reducing the percentage of animals with pulmonary metastases (12/19 = 63.1%; $P<0.01$). ME and BCNU, which were previously shown to be active in reducing the tumor growth (Table 1), also significantly reduced the percentage of mice bearing lung metastases. The other compounds tested, which did not alter the tumor growth, did not show any effect against the metastasizing ability of this tumor.

*Table 2. Effectiveness of single-drug chemotherapy on pulmonary metastases in mice bearing i.m. MS-2 sarcoma*

| Drug* | Dose (mg/kg/day) | No. of animals with tumor / No. of mice inoculated† | % Mice with metastases‡ |
|---|---|---|---|
| — | | 22/22 | 100 |
| MTX | 15.0 | 15/15 | 100 |
| | 25.0 | 10/10 | 100 |
| — | | 20/20 | 100 |
| 5-FU | 45.0 | 20/21 | 95.2 |
| | 65.0 | N.D.§ | — |
| — | | 20/20 | 100 |
| ME | 7.0 | 19/20 | 95.0 |
| | 10.0 | 12/19 | 63.1‖ |
| — | | 20/20 | 100 |
| BCNU | 10.0 | 19/21 | 90.4 |
| | 17.0 | 9/21 | 42.8‖ |
| — | | 22/22 | 100 |
| BL | 40.0 | 17/18 | 94.4 |
| | 50.0 | 17/20 | 85.0 |
| — | | 22/22 | 100 |
| VCR | 0.5 | 18/19 | 94.7 |
| | 1.0 | 13/17 | 76.4 |

*The drugs were administered i.p. at days 3, 7 and 10 after the tumor implant and dissolved in the vehicles reported in Table 1.
†Bioassay carried out at the 14th day after tumor cell implant into the foot pad.
‡‖$P<0.01$ as evaluated by Mussett test.
§Not done.

*Effect of surgical–adjuvant two-drug combination chemotherapy against lung metastases of MS-2 sarcoma*

Response data from surgical–adjuvant chemotherapy experiments done with AD plus CY are summarized in Table 3. The experiments were carried out on MS-2 tumor-bearing mice in which the surgical amputation of the tumor-bearing leg was performed 13 days after implant, when 100% of control mice had already developed pulmonary metastases [11]. The drugs were administered i.v. or i.p. at 7, 11, 14 and 19 days after the footpad injection of MS-2 tumor cells. At start of treatment the tumor was palpable. The lung bioassay was carried out 7 days after the last treatment. AD (4 mg/kg i.v.) slightly inhibited the percentage of mice with lung metastases. At the dose of 60 mg/kg, CY administered i.p. was more active than AD (7/14 = 50%; $P<0.01$). In mice treated with two-drug combination chemotherapy, the percentage of animals with metastatic foci in the lung was strongly reduced (1/15 = 6.6%; $P<0.01$).

Table 4 shows the data of the antimetas-

*Table 3. Effect of surgery plus adriamycin and cyclophosphamide combination chemotherapy on lung metastases of MS-2 sarcoma in BALB/c mice**

| Drug† | Dose (mg/kg/day) | Lung metastases evaluation† No. of animals with tumor / No. of mice inoculated | % |
|---|---|---|---|
| — | — | 15/15 | 100 |
| AD | 4 | 11/15 | 73.3 |
| CY | 60 | 7/14 | 50.0§ |
| AD+CY | 4+60 | 1/15 | 6.6§ |

*Surgery carried out at 13th day after tumor implant.
†Treatment at days 7, 11, 14, 19; AD was administered i.v.; CY was administered i.p. a few minutes after AD.
‡Bioassay evaluation carried out at the 23rd day after tumor cell implant into foot pad.
§$P<0.01$ as evaluated by Mussett test.

*Table 4. Effectiveness of surgical-adjuvant two-drug combination chemotherapy against lung metastases of MS-2 sarcoma. Different activity with respect to time of surgery**

| Drug | Days of treatment | Dose (mg/kg/day) | Lung metastases evaluation† No. of animals with tumor / No. of mice inoculated | % |
|---|---|---|---|---|
| — | | | 10/11 | 90.9 |
| ICRF-159 | 1, 4, 7 | 50 | 11/11 | 100.0 |
| AD | | 4 | 6/8 | 75.0‡ |
| AD+ICRF-159 | | 4+50 | 1/10 | 10.0§ |
| — | | | 11/11 | 100 |
| ICRF-159 | 15, 18, 21 | 50 | 7/7 | 100 |
| AD | | 4 | 11/12 | 91.6 |
| AD+ICRF-159 | | 4+50 | 4/10 | 40.0‖ |
| — | | | 11/11 | 100 |
| ICRF-159 | 1, 4, 7, 15, 18, 21 | 50 | 10/11 | 90.9 |
| AD | | 4 | 6/11 | 54.0 |
| AD+ICRF-159 | | 4+50 | 5/9 | 55.0 |

*Surgery carried out at the 13th day after tumor cell implant into the foot pad. ‖$P<0.05$ §$P<0.01$ as evaluated by Mussett test.
†By bioassay carried out at the 20th day after tumor cell implant in the group treated before the surgery and at the 26th day after tumor cell implant in the group treated after, or before and after tumor cell implant.
‡$P<0.05$ between group treated with AD and group treated with AD plus ICRF-159.

tatic activity of adjuvant chemotherapy, with AD plus ICRF-159 supplied at different times with respect to surgery. ICRF-159 did not show any antimetastatic activity given as a single agent at the dose of 50 mg/kg i.p. Treatment with AD alone after surgery was inactive; treatment before, or before and after surgery reduced the percentage of mice with lung metastases, but this reduction was not statistically significant. A synergistic effect of AD with ICRF-159 was observed on lung metastases when the treatment was performed before the surgery ($1/10=10\%$; $P<0.01$). Lower synergistic effect was found when the combined treatment was performed after surgery ($4/10=40\%$; $P<0.05$). When the combined treatment was performed before and after surgery, a lower activity was observed.

## DISCUSSION

Several types of systems have been employed in cancer research for the evaluation and detailed investigation of antitumor

agents: spontaneous tumors [14, 15], first-generation transplants of spontaneous tumors [16], transplanted tumors [7, 10, 17], carcinogen-induced tumors [18, 19] and primary virus-induced tumors [20, 21]. Although in the last 10 years considerable effort has been made to develop new tumor systems, there is a definite need to find animal tumors that might serve as biological models and chemotherapeutic predictors of solid metastasized tumors in man. A selection and development of new anticancer drugs, or investigation of different schedules and strategies against cancer could be better achieved by adopting such models.

The MS-2 sarcoma, on the basis of the properties previously described [9–11], could be considered interesting in this respect. We have examined the chemotherapeutic effect of some clinically active antineoplastic drugs administered alone or in combination against the MS-2 sarcoma. In addition, surgical–adjuvant chemotherapy experiments performed with one or two drugs were done to determine the relative effectiveness of clinically important drugs on spontaneous metastases of lung.

The MS-2 tumor, transplanted i.m., was sensitive to the alkylating agents ME, CY and BCNU, to the intercalating agents AD and DR, and to BL, which produces ruptures into DNA [22]. However, only CY, BL, AD, and DR gave a significant increase of the life span of tumor-bearing mice. AD and CY were markedly effective; treatment of mice with CY resulted in a marked therapeutic response against both early disease and advanced tumor. The activity of CY against MS-2 tumor, similarly to that observed in the K-MSV non-producing tumor described by Ikawa *et al.* [23], was greater than that of BCNU. In parallel, Carter [24] suggested that many human solid tumors may be more responsive to the treatment with CY than to treatment with BCNU. The MS-2 tumor proved to be less sensitive to the treatment with the phase-specific drugs MTX and VCR, the cycle-specific 5-FU, and refractory to the chelating agent ICRF-159. The low activity of cell cycle non-specific agents can be attributed to the slow growth of MS-2 sarcoma; it is possible that drugs active also on non-dividing cells can affect the cell population of such a tumor. In particular, AD activity is in accordance with the *in vitro* effect observed on plateau-phase cells [25]. Our experience with MTX, 5-FU and VCR was too limited to make a definite assessment of low effectiveness of these agents against MS-2 tumor. ICRF-159 was certainly inactive, since we have tested it on various schedules, utilizing various doses (Giuliani, unpublished data).

The effectiveness against pulmonary metastases of some drugs was also examined. In this regard, of the seven drugs tested ME and BCNU showed activity in reducing the percentage of mice with metastatic lung foci. These two alkylating agents administered to mice bearing MS-2 sarcoma reduced the tumor growth as well. It can be therefore supposed that the reduction of the primary tumor mass decreased the metastases by minimizing the shedding of tumor cells [26].

The relative success of surgical–adjuvant chemotherapy in the treatment of cancer has increased the efforts to use drugs in combination with surgery. It is of great interest to have data on the surgical–adjuvant chemotherapy from many animal tumor-systems as discussed by Gottlieb *et al.* [27]. The effectiveness of surgical–adjuvant chemotherapy against pulmonary metastases must be evaluated in animal models where each aspect of metastases formation is physiological; in this respect the i.v. implantation of tumor cells can be regarded as a model for primary lung cancer [28].

Many authors have already demonstrated a therapeutic synergism between AD and CY both in animal tumors [29, 30], and in human tumors [31, 32]. Using the MS-2 solid tumor model, combination chemotherapy with AD–CY on advanced tumors in conjunction with surgery drastically reduced the number of mice with lung foci. This combination was found to be more effective on pulmonary metastases than was surgery and single-drug therapy.

We have also examined the effectiveness of surgical adjuvant two-drug combination chemotherapy with adriamycin plus ICRF-159. The adjuvant chemotherapy performed early in the course of the disease and before surgery resulted in a lower number of mice with lung metastases. This observation is compatible with the antimetastatic effect of ICRF-159 due not to direct killing of metastatic cells, but rather to the changes in the vasculature that prevents the entry of the tumor cells from the primary tumor into the circulation [33], or to inhibition of blood coagulation, or an antiproteolytic effect. This rational supports an explantation for the observed effect. We can therefore say that AD and ICRF-159 combination treatment leads to "explained therapeutic synergism" [34].

The higher activity of AD when administered before rather than after surgery was also observed against a highly metastasizing mammary carcinoma [35]. Adjuvant chemotherapy with AD plus ICRF-159 performed after the surgery was slightly effective. This probably occurred because of the high number of metastatic foci at the start of chemotherapeutic treatment. It cannot be excluded that AD reduced the size of the lung metastases; further titration studies should be carried out in order to answer this question. A reduction in the size of lung metastases could be, in fact, of clinical relevance.

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