

Effect of Selected Tumor-inhibitory Agents on the Transplantability of Sarcoma 180 Ascites*

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SUMMARY

Several compounds known to inhibit the growth of Sarcoma 180 were compared for their cytotoxic activity *in vivo*. Standard inocula from donor mice which were treated with massive doses of the drugs were transplanted at 4 and 24 hours after treatment into untreated recipient animals. Viability was evaluated by cell counts and survival time. The effectiveness of triethylenethiophosphoramide and actinomycin D was in contrast to the lack of effect of amethopterin and 6-mercaptopurine. Azaserine, 6-diazo-5-oxo-L-norleucine, 6-methylpurine, N-methylformamide, 5-fluorouracil, and purine riboside had various degrees of activity. The viability test used can uncover some of the limitations of tumor-inhibitory compounds. Some of the reasons underlying such limitations are discussed. The importance of identifying compounds with cytotoxic potentiality is emphasized.

Cytotoxic compounds can be selected for growth-inhibitory activity following the exposure of cells in culture media to constant concentrations of inhibitors for several days (7). The toxicity of such potential anticancer agents *in vivo*, due to interference with the function of vital organs, frequently obscures their activity against neoplastic cells. For this reason, the distinction between cytostatic and cytotoxic effects of these compounds is often not provided by measuring their antineoplastic activity in tumor-bearing rodents. Evaluation of the effect *in vivo* of various compounds on the transplantability of tumor cells can provide information on the cytotoxic activity of candidate agents. Burchenal and co-workers (2, 3) have used this technique in studies of the "sterilizing" capacity of some chemotherapeutic agents on transplantable mouse tumors.

Ascitic tumors appear to be most suitable for this type of study, since a known number of cells can be transferred from one host to another.

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In addition, their growth can be estimated accurately by serial counts of the free cells present in the peritoneal cavity and can be correlated with observations on the survival time of the tumor-bearing animals.

In this investigation, the activity of several compounds on the transplantability of Sarcoma 180 (S-180) in ascitic form has been evaluated. The chemotherapeutic agents studied were 6-mercaptopurine (6-MP), purine riboside,¹ 6-methylpurine,¹ 5-fluorouracil (5-FU), azaserine (AZS),¹ 6-diazo-5-oxo-L-norleucine (DON),¹ triethylenethiophosphoramide (TSPA), N-methylformamide, amethopterin, and actinomycin D. Actinomycin D and TSPA proved to be the most effective agents; amethopterin, N-methylformamide, and 6-mercaptopurine were the least effective ones. Correlation of the observed effects of these drugs on the transplantability of S-180 cells with the available information on the activity of the same drugs on tumor growth has given rise to new questions concerning their activity *in vivo*.

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MATERIALS AND METHODS

Sarcoma 180 ascites was transplanted in HaICR Swiss mice by intraperitoneal inoculation of 10^6 cells. The weight of the animals used ranged between 20 and 25 gm. Each of the drugs was injected intraperitoneally into donor mice bearing 8-day-old tumors at a dose approximately 4 times the LD_{50} . At intervals of 4 and 24 hours after treatment these animals were sacrificed, and the cells (10^6) were implanted into untreated recipient mice. Occasional deviation from the above procedure is specified. Growth of the ascites tumors in the recipient mice was evaluated in each experiment by counting the total number of cells present in the peritoneal cavity of five mice per group on the 4th, 7th, and 13th days after transplantation. The cells were collected and handled according to procedures described previously (15). The average survival time of the tumor-bearing animals was evaluated in each experiment on a minimum of twenty mice per group.

RESULTS

The results obtained are summarized in Table 1. The number of free cells, which could be washed from the peritoneal cavity of the recipient mice 7 days after the inoculation of tumors previously exposed to actinomycin D or TSPA, was only 3-8 times larger than the original inoculum. During the same period the control tumors grew more than 400 times the original inoculum. The average survival time of the animals which succumbed to the inoculation of the exposed cells was 50-90 per cent longer than that of the controls. In addition, 30-80 per cent of the animals which had received these cells survived for more than 60 days, indicating that most of the one million cells inoculated had been irreversibly damaged by the treatment in the donor mice. The cytotoxic effect of TSPA appeared to be greater when the period of exposure of the tumor in the donor mice was 4 hours rather than 24 hours. Actinomy-

TABLE 1
EFFECT OF SELECTED TUMOR-INHIBITORY AGENTS ON THE TRANSPLANTABILITY OF SARCOMA 180 ASCITES

| COMPOUND | DOSE (MG/KG) | TIME* (HR.) | NO. OF EXP. | TOTAL CELL COUNTS (NUMBER $\times 10^6$) (TREATED/CONTROL) | | AV. SUR- VIVAL† TREATED/ CONTROL (DAYS) | NO. SURVIVING MICE‡/NO. TREATED MICE |
|-------------------|-----------------|----------------|----------------|--|----------|---|---|
| | | | | 7th day | 13th day | | |
| TSPA | 400 | 4 | 3 | 8/431 | 53/992 | 32/17 | 82/103 |
| " | 400 | 24 | 3 | 4/431 | 69/992 | 26/17 | 34/100 |
| Actinomycin D | 8.2 | 4 | 2 | 4/446 | 95/1218 | 32/19 | 30/67 |
| " | 8.2 | 24 | 2 | 3/446 | 35/1218 | 36/19 | 32/64 |
| AZS | 400 | 4 | 3 | 74/429 | 590/1281 | 23/18 | 15/103 |
| " | 400 | 22 | 3 | 42/429 | 500/1281 | 27/18 | 18/103 |
| DON | 100 | 4 | 1 | 181/337 | 762/865 | 17/19 | 0/35 |
| " | 100 | 24 | 1 | 49/337 | 581/865 | 23/19 | 2/32 |
| " | 1000 | 24 | 1 | 6/431 | 424/1065 | 27/18 | 3/35 |
| 6-Methylpurine | 80 | 4 | 3 | 63/412 | 543/1168 | 23/17 | 0/103 |
| " | 80 | 24 | 3 | 5/412 | 244/1168 | 25/17 | 8/114 |
| 5-FU | 4000 | 4 | 2 | 3/375 | 110/1038 | 30/17 | 6/38 |
| " | 4000 | 24 | 3 | 33/354 | 377/816 | 20/17 | 1/90 |
| Purine riboside | 710 | 4 | 4 | 215/450 | 764/1218 | 20/17 | 0/123 |
| " | 710 | 22 | 2 | 139/441 | 720/1148 | 21/18 | 1/70 |
| N-Methylformamide | 8000 | 4 | 1 | 264/328 | 1044/913 | 16/16 | 0/35 |
| " | 8000 | 24 | 2 | 222/355 | 856/845 | 18/18 | 0/78 |
| 6-MP | 1100 | 4 | 1 | 296/476 | 826/936 | 16/17 | 0/34 |
| " | 1100 | 24 | 2 | 302/411 | 743/1047 | 18/17 | 1/68 |
| Amethopterin | 400 | 4 | 5 | 233/416 | 867/897 | 19/18 | 2/107 |
| " | 400 | 24 | 5 | 105/483 | 661/874 | 20/16 | 2/130 |

* Time between treatment of the donor mouse and its sacrifice. Azaserine and purine riboside, at the doses used, were lethal for most of the animals within 24 hours.

† Average survival of mice dying within 60 days.

‡ Animals surviving longer than 60 days from the day of tumor inoculation.

cin D was equally effective with the two periods of exposure.

Azaserine, DON, 6-methylpurine, and 5-FU were about equally active in reducing the viability of the exposed cells, as can be noted from both the retardation of growth of the transplanted tumors and the prolonged survival of the inoculated animals. The effect of 5-FU was definitely greater when the period of exposure of the tumor cells was only 4 hours. Purine riboside, N-methylformamide, 6-MP, and amethopterin did not reduce viability significantly; the slight reduction in initial growth of the tumors was not reflected in any prolongation of the survival time of the inoculated mice.

With the exception of amethopterin, none of these compounds had a significant effect on the growth of the tumor in the donor mice during the 24-hour period of exposure to the drug as reflected by the total number of cells washed from the peritoneal cavity.

The lack of significant effects of amethopterin was evident even when only 100 exposed cells were inoculated into the recipient mice. In this

case, although growth of the tumor was retarded when the cells were exposed for 24 hours (Chart 1), the survival time of the recipient mice was not increased significantly (Table 2). The survival of seventeen out of 104 recipient mice for a period longer than 60 days does not seem to be significant in view of the fact that some control animals, inoculated with 100 untreated cells, also survived for the same period.

The effect of a single administration of 400 mg/kg of amethopterin was compared with that of the same total amount fractionated into twenty doses given every 3 hours over a period of 60 hours starting on the 5th day after tumor implantation. In this experiment, at the time of sacrifice of the donor mice, the average number of free cells of the untreated tumors was 410×10^6 ; that of the tumors exposed to the single dose of amethopterin was 133×10^6 cells, and that of the tumor exposed to the fractionated doses of the antimetabolite was 36×10^6 cells. From each of these groups, tumor inocula of 1,000,000 or 100 cells were administered intraperitoneally to untreated recipient mice. The growth of these tumors

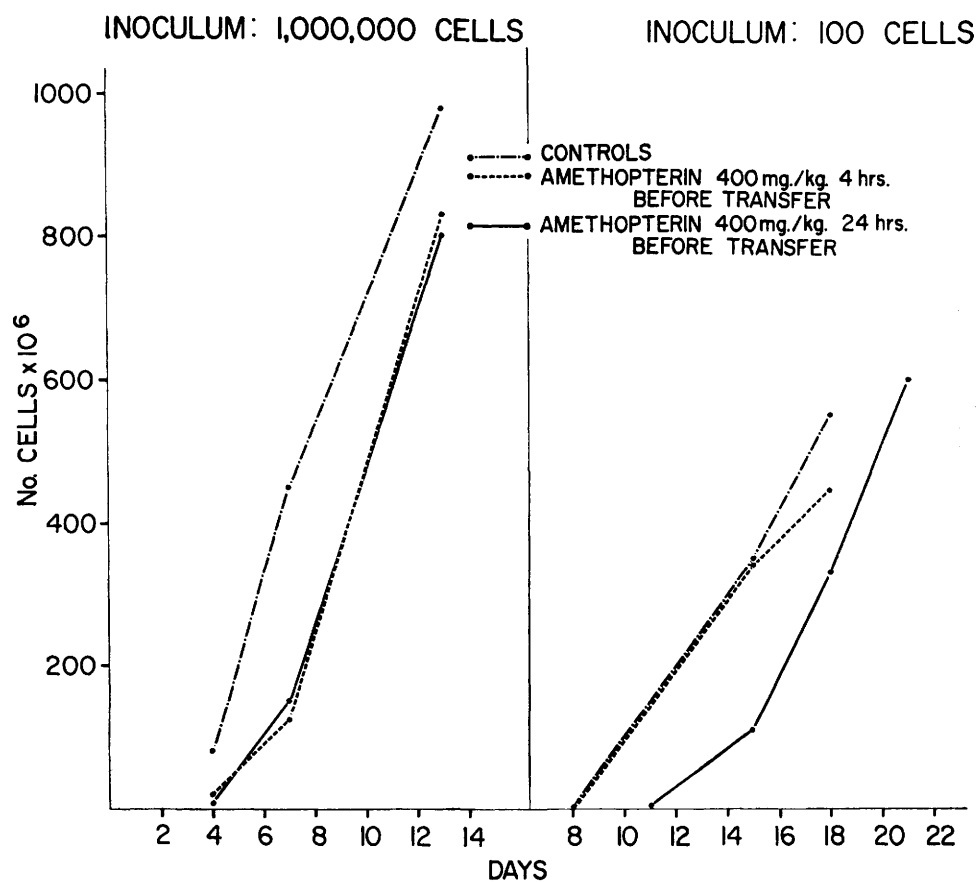


CHART 1.—Rate of growth of Sarcoma 180 ascites in untreated mice following inoculation with cells from donor

animals which had been treated 4 and 24 hours previously with a massive dose of amethopterin.

is shown in Chart 2. In this experiment, also, the single injection of amethopterin had only a slight effect on the viability of the exposed tumor cells. Repeated administration of the drug, however, was effective in reducing the viability of a large portion of the inoculated cells to the extent that no significant growth was seen up to 21 days after the inoculation of 100 exposed cells. The

greater effect of repeated doses of the antimetabolite cannot be attributed to treatment of cells during an earlier phase of development of the tumor, since, in a different experiment, a single injection of amethopterin had the same slight effect on the viability of a 5-day-old or an 8-day-old tumor.

In a few experiments the activity of amethop-

TABLE 2
EFFECT OF AMETHOPTERIN ON THE VIABILITY OF S-180 ASCITES CELLS

| TREATMENT (MG/KG) | PERIOD OF EXPOSURE* (HOURS) | INOCULUM (NO. CELLS) | AV. SURVIVAL EXPOSED/CONTROLS (DAYS) | NO. SURVIVING/NO. INOCULATED† | |
|-------------------|-----------------------------|----------------------|--------------------------------------|-------------------------------|----------|
| | | | | Treated | Controls |
| 400 | 4 | 1 × 10 ⁶ | 19/18 | 2/107 | 0/116 |
| 400 | 4 | 1 × 10 ² | 34/30 | 11/69 | 5/67 |
| 400 | 24 | 1 × 10 ⁶ | 20/16 | 2/130 | 0/133 |
| 400 | 24 | 1 × 10 ² | 31/27 | 17/104 | 3/89 |

* Period of time between treatment of the donor mice and their sacrifice.

† Animals surviving more than 60 days after the inoculation of the tumor.

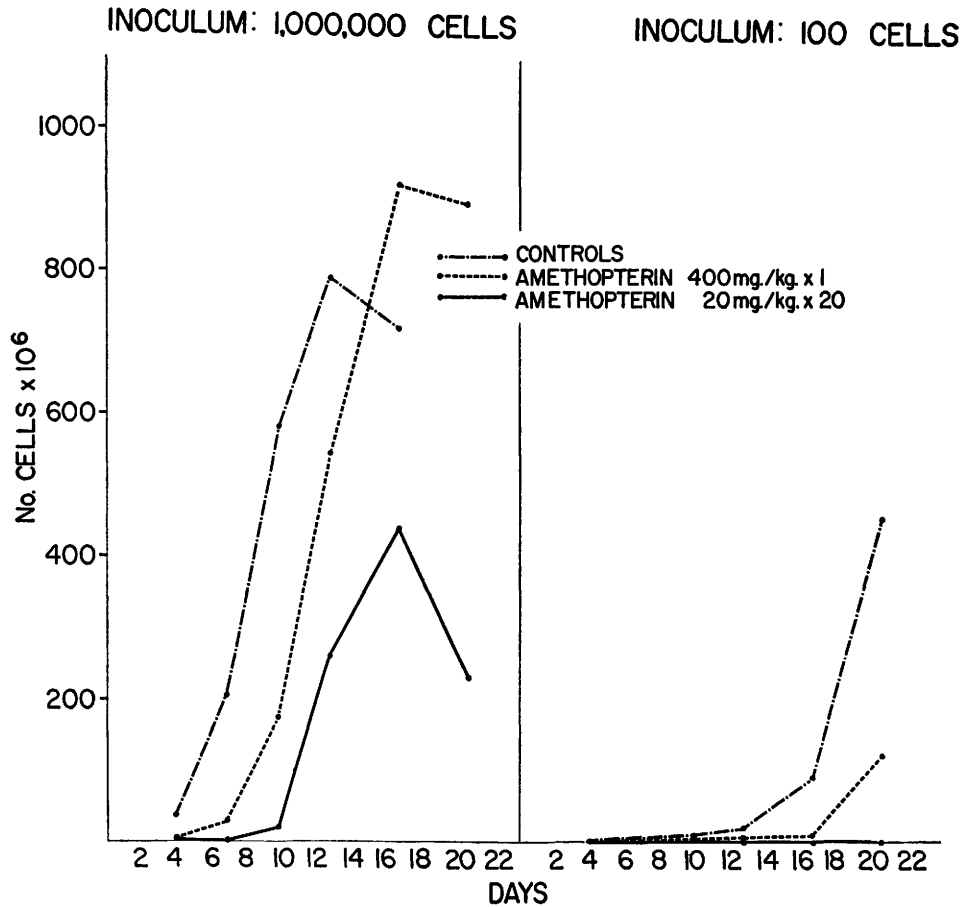


CHART 2.—Rate of growth of Sarcoma 180 ascites in untreated mice following inoculation with cells (a) from donor animals which had been treated 3 days previously with a single

massive dose of amethopterin and (b) from donor animals which received one-twentieth this dose during twenty injections administered at 3-hour intervals.

terin on the viability of leukemia L1210 ascites cells was also studied, because this tumor is known to be particularly sensitive to the effects of this drug (14). No significant effect on the viability of these leukemic cells could be detected when the antimetabolite was injected into the donor mice 4–6 hours prior to their sacrifice, and inocula of 1,000,000 or 100 cells were injected into the recipient animals.

DISCUSSION

By the technic used in this study, tissues of donor animals are flooded with amounts of each drug greatly in excess of that which could be tolerated during a chemotherapeutic test. The effectiveness of an alkylating agent (TSPA) and an antibiotic (actinomycin D) was in contrast to the lack of effect of two antimetabolites (amethopterin and 6-mercaptopurine) on the viability of Sarcoma 180. Tumor cells which had been exposed to very high concentrations of either actinomycin D or TSPA for only 4 hours were incapable of multiplying when transferred into untreated recipient mice. This cytotoxic effect of TSPA is similar to that of other alkylating agents on the transplantability of mouse tumors (2, 3).

In the test system studied, compounds classed as "active" by virtue of their capacity to inhibit the growth of a transplantable tumor in the usual screening procedures may be divided into two groups on the basis of their effects on the transplantability of cells. One type, represented by TSPA and actinomycin D, combines promptly with vital cellular constituents in a manner that reduces viability. In this case, the drug may act through chemical combination as described for the reaction between alkylating agents and nucleic acids (16). The second group includes the compounds which are inactive in this test, such as amethopterin and 6-mercaptopurine. These compounds act more slowly so that no effect on cell viability is seen within 24 hours.

Azaserine, DON, and 6-methylpurine were more active when the period between treatment and transplantation was 24 hours rather than 4 hours. These compounds impaired the viability of the treated tumors as shown by the significant retardation of tumor growth in the untreated recipient mice. Survival time of these animals, however, was only slightly longer than that of the controls. N-methylformamide, which was included in this study because of its known carcinostatic activity (5), was without effect in this test system. 5-Fluorouracil was more active when the tumor was exposed for 4 hours instead of for 24 hours. It is possible that, during the 24-hour interval,

materials from affected cells may become available to other neoplastic cells, thereby preventing the toxicity of the drug. A similar result could be expected if the proportion of nonviable cells counted at 4 hours was greater than at 24 hours.

Purine riboside, which has been found to be very toxic in mammals (9, 17) did not significantly affect the viability of the exposed tumors. Rapid removal of the drug from the peritoneal cavity does not seem to be a reasonable explanation for the lack of effect of the massive amounts of the compound administered. The possibility that metabolic transformation of purine riboside (10) did not take place during the exposure of the tumor cells in the peritoneal cavity of the donor mice seems unlikely in view of the prompt cytotoxic effect of this agent for mammalian cells *in vitro* (1).

The regression of established solid Sarcoma 180 following treatment with 6-mercaptopurine (6) expresses a profound influence of this antimetabolite on the vitality of the tumor. The lack of effect of 6-mercaptopurine on the viability of tumor cells may reflect an insufficient period of contact with the drug in this test for the occurrence of permanent metabolic damage or may be the result of a possible reversal of the effects of this antimetabolite by the purines available in the donor or recipient mice, since the cytotoxicity of this compound can be reversed competitively *in vitro* (12). A similar explanation is unlikely for purine riboside, however, since the cytotoxic effects of this agent at 2.5×10^{-6} M could not be reversed in cell culture by any of the physiological purines, their nucleosides or nucleotides, nor by related cofactors such as diphosphopyridine nucleotide, triphosphopyridine nucleotide, flavin-adenine dinucleotide, or Coenzyme A.² Whether other reversing factors are liberated *in vivo* from destroyed cells deserves further study.

Amethopterin was not effective in these experiments, although it is well known to be active in clinical and experimental studies. This finding was somewhat surprising in view of the inhibition of the growth of S-180 cells in culture by amethopterin (7, 11) and the inhibition of folic acid reductase associated with the remarkable binding of the drug by this enzyme (4, 8, 18, 19). The lack of effect of amethopterin might be attributed to rapid excretion of the drug following the single injection. However, about one-fifteenth of the estimated original concentration of the drug was still present in the ascitic fluid after 24 hours as measured by microbial assay. Also, the activity of aminopterin on the transplantability of two leukemias was not evident even in nephrectomized

² M. T. Hakala, personal communication.

mice (2). The slight effect of amethopterin may be explained, in part, by reversal of the metabolic inhibition by end-products of the inhibited reactions which may be available in the donor or recipient mice. This possibility would be consistent with the reversal of the effects of amethopterin observed *in vitro* (11, 13). The fact that repeated divided doses of the antimetabolite were more effective than the same total dose given at one time suggests, however, that factors other than those mentioned above may be partially responsible for the results obtained with this agent. Relatively slow uptake of the drug due to a limited active transport might result in the passage of a larger amount into the cells following frequent administration than could occur following any single massive dose.

The pharmacological characteristics of drugs which lack activity in this test system but which are capable of retarding the growth of the experimental tumor used deserve further study. The effects of such growth inhibitors may occur only after incorporation into some cellular constituents or after conversion to active forms. Also, the activity of such compounds may be limited by the rate of uptake into the cells or by the presence of metabolites capable of preventing their growth-inhibitory activity.

Compounds with rapid cytotoxic activity *in vivo* can be selected by the test described. This procedure seems to be more adequate than any test *in vitro* to uncover some of the limitations of a tumor-inhibitory compound. A test *in vitro* may not always distinguish between irreversible and reversible metabolic damage. Transplantation of cells from a treated donor limits the influence of the host on the antineoplastic effects of the compounds studied. The procedures discussed can be used only to evaluate the effects of the agents tested on the tumor cell per se and in this respect resemble a system *in vitro*. An irreversible metabolic alteration leading to the death of the exposed cells, or to a change of their "antigenic" characteristics becomes evident, however, upon transplantation of these cells into untreated recipient mice. Further work toward effective chemotherapy may be focused profitably on the identification of agents with cytotoxic potentiality and means of increasing their antitumor selectivity.

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