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Antitumor Activity of Indomethacin in Mice Bearing Advanced Colon 26 Carcinoma Compared with Those with Early Transplants

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ABSTRACT

The antitumor activity of indomethacin (IND) was investigated in mice bearing advanced colon 26 adenocarcinoma compared with its early transplants. Treatment with 0.001% IND in drinking water retarded the growth of tumor when commenced at Day 1 after the tumor inoculation. The suppression of the tumor growth by IND continued up to 4 to 5 weeks as long as the size of the tumor remained small. On the other hand, IND given to mice bearing a large burden of the tumor at 2 weeks after the inoculation had facilitated the tumor growth. IND reduced tumor-associated PGE_2 production in mice bearing either small or large burdens of the tumors. These results indicate that the antitumor activity of IND depends on tumor size and therefore is not simply associated with the reduction of PGE_2 levels. We found that colon 26 caused changes in parameters reported for tumor cachexia, such as weight loss, wasting of muscle and adipose tissues and hypoglycemia, when it grew to around 1 g at 2–3 weeks after the tumor inoculation. IND given to the mice with large burdens of colon 26 alleviated the cachexia of the mice, resulting in the increase of the survival time even though the growth of the tumor had been facilitated. It is possible that IND affects the tumor growth and survival by reversing tumor-induced disorders in homeostasis.

INTRODUCTION

PGs[1] are known to be one of the regulators of tumor growth and its spread (1–3). PGs facilitate tumor growth by suppressing immune systems against tumors (4–6). Increased PG production by tumors has been associated with aggressive tumor progression (2, 3). IND, an inhibitor of PG synthesis, has impeded the growth of tumors in experimental animals (1–3, 7–12). However, tumor response to IND treatment has been variable. In some tumor models it did not inhibit tumor growth or inversely facilitated tumor growth (13–16) while significantly reducing PGE_2 levels. Consequently, the antitumor activity of inhibitors of prostaglandin synthesis on tumor growth is still controversial.

PGs are produced by tumor cells and macrophages, which are one of stroma elements. PGs are known to affect not only tumor cell growth but also various systems such as those for angiogenesis (17, 18), coagulation (19, 20), and defense against tumor cells (4, 6), which are seen in tumor stroma and interact with tumor growth. Therefore, it is possible that IND, an inhibitor of PG production, exerts a different effect on the growth of tumors when tumor stroma elements differ.

The experiments presented here were designed to determine whether IND exerts various effects on the growth of tumors because of the presence of different elements in tumor stroma in mice, *i.e.*, those with early transplant and advanced stage colon 26 adenocarcinoma. The study shows that the antitumor activity of IND depends on the stage of growth of the tumors treated. IND inhibited the growth of an early transplant of colon 26 adenocarcinoma without well-developed stroma, but it facilitated the growth of the tumor when at an advanced stage.

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[1] The abbreviations used are: PG, prostaglandin; IND, indomethacin; PGE_2, prostaglandin E_2; NK, natural killer.

MATERIALS AND METHODS

Mice. Four-week-old male CDF_1 (BALB/c × DBA/2)F_1 mice were obtained from Shizuoka Agricultural Corporation for Laboratory Animals, Hamamatsu, Japan. The mice were used at 5 weeks of age. Six mice per cage were kept at 22 ± 2°C and 55 ± 5% of humidity. In experiments for measuring food and drinking water consumption, each mouse was kept in a small cage.

Tumors. Murine colon 26 adenocarcinoma cells, kindly supplied by Dr. T. Kataoka at the Cancer Chemotherapy Center, Cancer Research Foundation, Tokyo, Japan, were cultured *in vitro* with RPMI 1640 containing 10% fetal calf serum. A single cell suspension of these cells, obtained by treatment of trypsin (10^6 cells), was s.c. inoculated into the CDF_1 mice.

Drug Treatment. Indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in ethyl alcohol at a concentration of 10 mg/ml, and 1 or 2 ml was added to 1000 ml of drinking water (0.001 or 0.002%). Drug treatment commenced on the days stated in the text. The drinking water was changed twice a week without showing any decomposition detected by thin-layer chromatography. The mice demonstrated no gross evidence of toxicity and no weight loss as a result of this drug for a period of 6 weeks.

Measurement of Tumor, Body, and Tissue Weight. Body weight and tumor size were measured twice a week, and carcass weight, the difference in weight between whole body and tumor tissues, was calculated. The tumor weight (mg) was estimated by using the following equation, $ab^2/2$, where a and b are tumor length and width in mm, respectively. The left epididymal adipose tissue and the gastrocnemius muscle of the left hindleg were removed and weighed.

Determination of PGE_2 and Glucose Levels. The concentration of PGE_2 in the plasma was determined by using a commercially available radioimmunoassay kit (New England Nuclear, Boston, MA) as described elsewhere (11). Plasma samples were immediately frozen and kept at −70°C until the assay. In order to avoid degradation of PGE_2 during the assay, PGE_2 in the plasma was first extracted with organic solvent (21) and then measured for its content. Briefly the plasma samples were treated with petroleum ether, and then PGE_2 was extracted with ethyl acetate-isopropyl alcohol in an acidic condition. Glucose concentrations in plasma samples were determined by a color reaction method with *o*-toluidine (22).

Data Statistics. Differences in tumor size and PGE_2 concentration in plasma were compared by using the Mann-Whitney U test, while the survival time differences were compared by using Cox's test. Differences were considered to be significant when the probability value was less than 0.05 ($P < 0.05$).

RESULTS

Effect of IND on Tumor Growth. The effect of IND on the growth of colon 26 adenocarcinoma is shown in Fig. 1. In these experiments treatment with IND at 0.001 and 0.002% was commenced at 1, 8, 15, and 22 days following the tumor inoculation and the treatment was continued until the mice died. When the treatment was started at Day 1, IND suppressed the growth of tumor during the first 4 weeks of the treatment and thereafter facilitated the tumor growth to some extent. On the other hand, IND treatment which was given beginning on Day 8 showed no significant inhibition of the tumor growth during the first 4 weeks and thereafter facilitated the growth. Furthermore, IND clearly facilitated the tumor growth, when

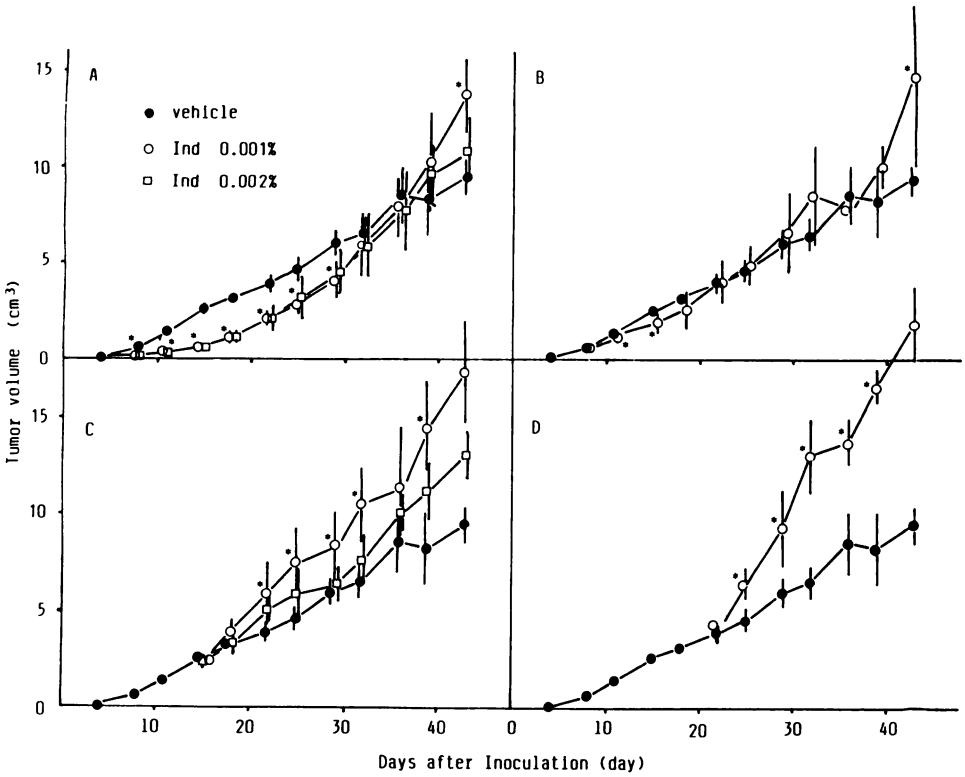

Fig. 1. The effect of IND on the growth of colon 26 in mice with early transplants compared with those bearing advanced tumor. Mice were inoculated s.c. with colon 26 (1×10^6 cells) at Day 0 and were then given 0.001 and 0.002% IND in drinking water beginning on Days 1 (*A*), 8 (*B*), 15 (*C*), or 22 (*D*) after the tumor inoculation until they had died. *, significant difference ($P < 0.05$) in tumor volume between mice administered with IND (0.001%) and vehicle. ●, vehicle; ○, IND 0.001%; □, 0.002%. *Bars*, SD.

the treatment commenced from either 15 or 22 days after tumor inoculation.

In these experiments we observed no significant change in water consumption among mice bearing colon 26 in early and late phase of the tumor growth and age-matched normal mice (4.2 ± 0.7, 4.7 ± 1.3 *versus* 4.5 ± 1.1 ml/day/mouse). In addition, IND significantly suppressed the tumor growth at doses over 0.000125% when it was begun on Day 1, whereas it facilitated the growth at the same dose range when it was begun on Day 22. Therefore, the contradictory effects of IND on the tumor growth is not dose related, and IND appears to suppress the growth of colon 26 as long as the tumor burden is small (early transplant), whereas it facilitates the tumor growth when the tumor burden is large (advanced tumor).

Increase of Survival by IND. IND was then examined for its antitumor activity in terms of the increase of survival of mice bearing colon 26. As Fig. 2*A* shows, IND treatment beginning on Day 1 after the tumor inoculation delayed the growth of the tumor and consequently increased the survival time. On the other hand, IND treatment beginning on Day 22, when the tumor burden was large, again enhanced the tumor growth (Fig. 2*B*). However, IND significantly increased the median survival time by 17% ($P < 0.05$) even though the growth of the tumor had been facilitated.

Inhibition of PGE_2 Production. It has been reported that the increased production of PG by tumors is associated with tumor growth and that IND inhibits the growth of tumors by reducing PG production. Therefore, we decided to investigate the antitumor activity of IND towards early and advanced stages of colon 26 adenocarcinoma in mice to determine whether this antitumor activity is related to a difference in its ability to inhibit PG production during the growth of the tumor. We measured plasma PGE_2 levels in mice bearing colon 26 after IND administration. As Fig. 3 shows, the PGE_2 concentration in the plasma increased with the growth of colon 26. An increased concentration of PGE_2 was first observed at 10 days after the tumor inoculation, which reached the maximum at 18 days (280 pg/ml). Administration of IND for 8 days or 11 days reduced PGE_2 concentrations in mice bearing either small or large burdens of the tumor (Table 1), indicating that the different effect that IND has on small tumors compared to that on more advanced tumors is not simply associated with the decrease in PGE_2 concentration by IND.

Effects of IND on Mice Bearing Advanced Colon 26. Actions of IND on mice bearing colon 26 at its advanced stages, namely those which had grown for 3 and 4 weeks after the implantation, were further investigated to clarify the effect of this drug on a large tumor burden compared with that on a small one. One of characteristic of mice bearing advanced colon 26 is that they are cachectic. In another study we have also observed that colon 26 adenocarcinoma causes severe cachexia when the tumor grows up to the size of 1 g at around 2 to 3 weeks after the tumor inoculation (23). Fig. 4 shows carcass weight change (whole body weight minus tumor weight) of CDF_1 mice inoculated with colon 26 adenocarcinoma. Carcass weight loss up to 33% of the whole body was the most typical at 3 weeks after the tumor inoculation. IND given to the cachectic mice had an improved general appearance and their weight loss had significantly reversed in a few days, while at the same time the tumor had continued to grow even faster (Fig. 1). On the other hand, IND treatment had no influence on the body weight of normal mice (data were not shown). In these experiments we observed no significant change in food consumption among tumor bearers, those treated with IND and age-matched normal mice (4–5 g/mouse/day).

Colon 26 adenocarcinoma also caused tissue wasting of muscle and adipose tissues and hypoglycemia in the mice (Table 2). The most characteristic change was the wasting of the epididymal adipose tissue. The tissue weight was greatly reduced to 7.7% of that in normal mice at 4 weeks after the tumor inoculation. Administration of IND improved the physical condition of the mice within a week; serum glucose concentrations

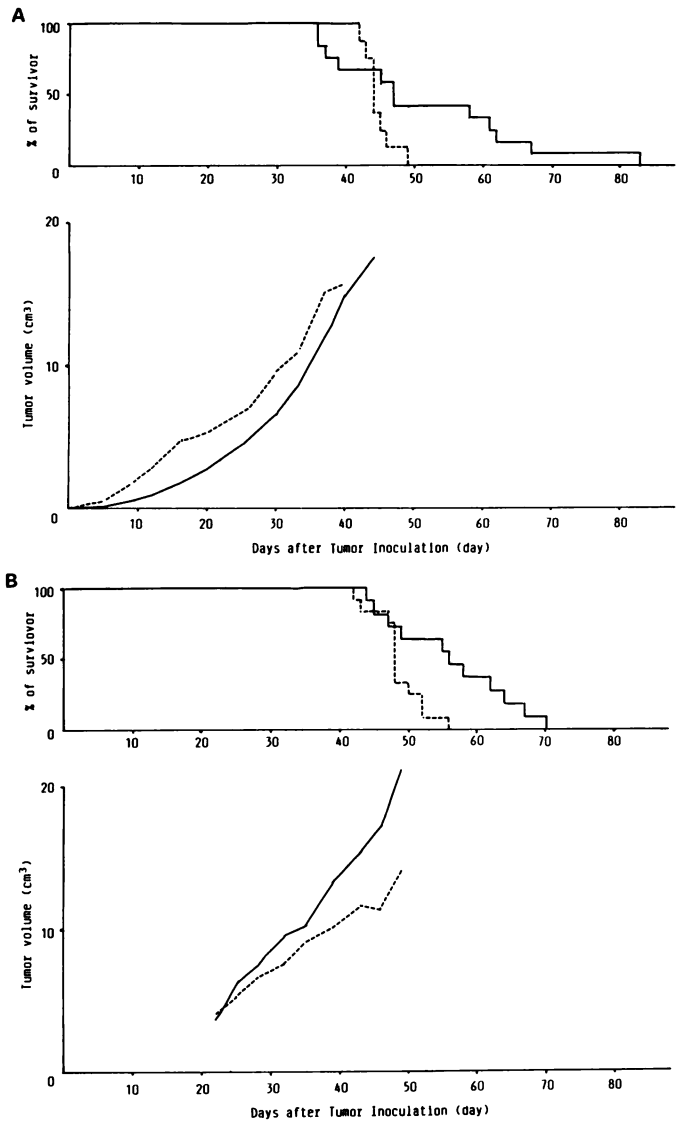

Fig. 2. Effect of IND on tumor growth and survival time of mice bearing colon 26 adenocarcinoma. Tumor-bearing mice were given IND in drinking water (0.001%) beginning on Day 1 (*A*) or 22 (*B*) after the tumor inoculation until they died. The survival time (*top*) and tumor volume (*bottom*) are shown thereafter. - - -, vehicle; ——, IND 0.001%.

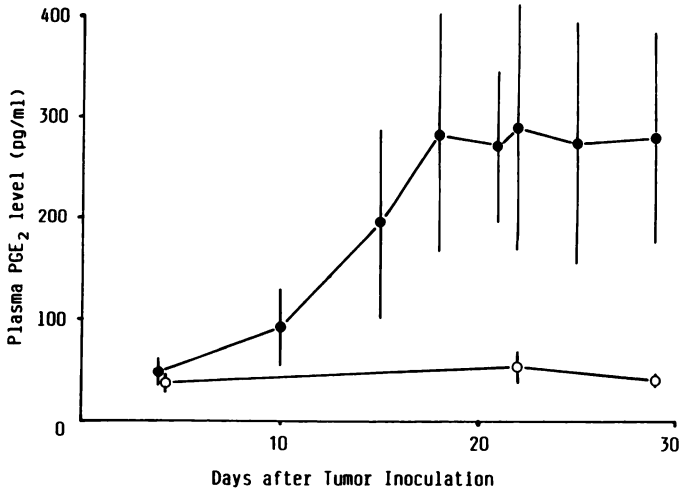

Fig. 3. Elevation of blood PGE_2 levels in mice bearing colon 26 adenocarcinoma. Tumor-bearing mice (●) and age-matched normal mice (○) were killed at various time after the tumor inoculation, and PGE_2 concentrations in plasma samples were measured. *Bars*, SD.

Table 1 *Effect of indomethacin on PGE_2 concentrations in plasma*

IND treatment[a]	PGE_2 concentration[b] in plasma ± SD (pg/ml)
Exp. 1	
Vehicle (Day 1–11)	71.3 ± 24.7
IND 0.001% (Day 1–11)	25.7 ± 2.9[c]
Exp. 2	
Vehicle (Day 22–29)	380.5 ± 58.8
IND 0.001% (Day 22–29)	90.8 ± 41.0[c]
Tumor-free	
Vehicle (daily for 8 days)	53.1 ± 8.0
IND 0.001% (daily for 8 days)	51.6 ± 8.8

[a] IND or vehicle was administered for 11 (Exp. 1) and 8 (Exp. 2) days beginning on Day 1 and 22, respectively, after inoculation of colon 26 adenocarcinoma. Normal mice were used as control (tumor free).

[b] PGE_2 concentrations were measured on Day 11 (Exp. 1) and Day 29 (Exp. 2).

[c] $P < 0.01$ as compared with the group that was administered vehicle.

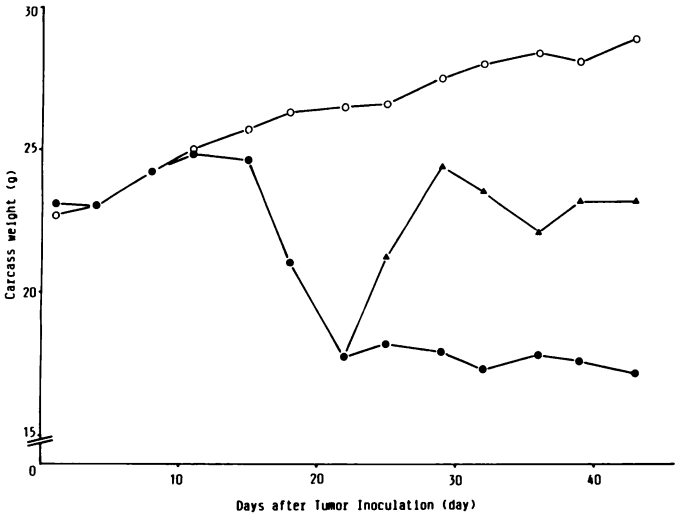

Fig. 4. The effect of IND on tumor-induced weight loss. The mean body weight of either normal mice or tumor-bearing mice (carcass weight) were measured twice a week. The tumor-bearing mice were divided into two groups at 22 days after the tumor inoculation, and IND in drinking water (0.001%) was given to one of the two groups. Normal mice (○), tumor-bearing mice given vehicle only (●), and given IND (▲).

and gastrocnemius muscle weights reached that of normal mice and adipose tissue weight had improved substantially. Thus, IND was capable of curing the disorders of homeostasis caused by large burdens of colon 26 adenocarcinoma. This must have resulted in an increase in the survival time and at the same time it facilitated the growth of the tumor.

DISCUSSION

The antitumor efficacy of IND, an inhibitor of PG production, remains controversial. IND has been reported to suppress tumor growth in some animal tumor models (1–3, 7–12), while it facilitates tumor growth in other tumor models (13–16). The present study shows that IND exhibits a different effect on the growth of small and large burdens of colon 26 adenocarcinoma. IND suppressed tumor growth as long as the tumor burden was small, whereas it facilitated the growth of a large burden of the tumor. These contradictory effects of IND were related to the phase of the tumor growth but not to dosages of IND, since it was observed at the same dose ranges tested (0.000125–0.002%). These results provide further insight into explanations of the controversial effects of IND on tumor growth. Young *et al.* (11) made a similar observation that IND augmented NK

Table 2 *Improvement of the cachectic condition in tumor-bearing mice by IND*

Mice	Treatment[a]	Tumor[b] weight (g)	Adipose tissue[b] weight (mg)	Muscle[b] weight (mg)	Serum glucose[b] (mg/dl)
Normal mice	Vehicle		285 ± 75	156 ± 13	150 ± 8
Tumor bearer	Vehicle	3.17 ± 0.61	22 ± 7	103 ± 13	88 ± 18
Tumor bearer	IND 0.001%	5.09 ± 0.94[c]	123 ± 39[c]	141 ± 8[c]	141 ± 19[c]

[a] Normal mice and mice bearing colon 26 adenocarcinoma (22 days after tumor inoculation) were administered for 8 days.
[b] Weight of tumor, left epididymal adipose tissue or gastrocnemius muscle of the left hind leg and levels of serum glucose were measured on Day 29.
[c] $P < 0.01$ as compared with tumor bearer (Day 29) that was administered with vehicle.

cytotoxicity of splenocytes and showed a significant *in vivo* antitumor activity in mice bearing small burdens of Lewis lung carcinoma though such effects were not significant in mice with large burdens (>1 g) of the tumor (11).

The difference in the response of advanced tumors to IND compared with that of small tumors, particularly early tumor transplants, can be attributed to the fact that each contains different tumor tissue elements. Tumors contain normal tissue stroma elements such as blood vessels, fibroblasts, lymphocytes, macrophages, and other inflammatory cells and endothelial cells. In small tumor tissues or early transplanted tumors, tumor stroma may not be fully developed. On the other hand, in large tumor tissues, the tumor stroma is well developed, and tumor necrosis is often observed. In addition, different quantity and type of immune or inflammatory cells may be infiltrated into tumor tissues at early and advanced stages of the tumor growth. This is possibly true for colon 26. Our preliminary experiments have shown, that in the advanced tumor, necrosis was seen extensively and neutrophils were infiltrated into tumor tissues outside the necrotic areas, while in the early stage of growth, tumor necrosis occurred to only a slight extent and neutrophils were infiltrated into the necrotic portion (data were not shown).

PGE_2 is known to suppress a variety of immune responses including T-cell functions (24, 25), cytokine production (26), and macrophage/NK cell-mediated cytotoxicity (5, 11). Therefore, the antitumor activity of IND has been reported to be attributed to its ability to strengthen these immune resistance mechanisms by inhibiting the production of immunosuppressive PGs (1, 2, 8, 27), particularly PGE_2. However, the present study does not confirm the relationship between the inhibition of the production of PGE_2 and the antitumor activity of IND. IND facilitated the growth of colon 26 adenocarcinoma when it was large, though it inhibited production of PGE_2. These results indicate that the antitumor activity of IND is not simply dependent on the ability of the drug to restore immune functions by inhibiting PGE_2 production.

Although the present study could not clarify the definite mode of the antitumor action of IND, the ability of IND to reverse cachexia should be considered as one of various actions of this drug on tumor growth. When colon 26 adenocarcinoma was large, it caused cachexia; weight loss, wasting of adipose tissues and muscle, and reduction of blood glucose levels (23). IND improved the cachectic condition and the physical appearance. Cachexy is disorders of homeostasis which are caused in consequence of response by the host to tumors. Factors produced by macrophages and monocytes as the result of responses to tumor growth, such as tumor necrosis factor (cachectin) and interleukin 1, are known to cause cachexia-like symptoms (28, 29) and enhance PGE production (30, 31). Although the role of PGs in causing the cachexia-like symptoms by tumor necrosis factor and interleukin 1 is not yet known, it is likely that PGE-related disorders facilitated the cachexia of mice bearing colon 26 adenocarcinoma and that IND reversed the cachexia by suppressing the production of PGs. The precise mechanism by which IND improves the cachectic condition of the mice remains to be elucidated.

Tumor cachexia is known to be associated with the poor prognosis of cancer patients, shorter survival irrespective of objective responses to therapies (32), and deterioration of the quality of life. The results of the present study on the antitumor activity of IND in which the increase of the survival time is accompanied by an improvement in cachectic condition but is not accompanied by the inhibition of tumor growth gives us some insight about cancer therapies; therefore, inhibitors of PG production are warranted to be pursued as therapeutic agents for the treatment of cachexia, although they may facilitate tumor growth.

Antitumor agents have generally been assessed by using mice bearing small burdens of transplantable tumors, particularly those just implanted with tumor cell suspension. However, tumors in the early stages of growth are different from those in advanced stages regarding their tumor stroma elements. In early transplanted tumors, tumor stromas are not well developed. This difference in tumor stroma elements may reflect the efficacy of drugs to be assessed and often leads to a wrong assessment of the efficacy of these drugs. In separate experiments using three different murine transplantable tumors, colon 26 adenocarcinoma, Ehrlich carcinoma, and UV2237 fibrosarcoma, we have also observed that tumors at different stages of growth had various responses to cytostatics (33). Many of the cytostatics tested were less effective when the tumor burdens became large, but a few were almost equally effective on small (early transplants) and large tumors. Establishment of animal tumor models relevant to the clinical situations is needed for definitive drug assessments.

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