STANDARDIZATION OF PROCEDURES FOR CANCER
CHEMOTHERAPY SCREENING WITH EHRlich
ASCITES TUMOR CELLS*

YASUYUKI EGASHIRA, KOUICHI TAKANO, MASA-ATSU YAMADA,
YASUKO HIROKAWA,

Department of Pathology, National Institute of Health, Tokyo

DEN'ICHI MIZUNO, MIHOKO ABE AND YUKITO MASAMUNE

Department of Chemistry, National Institute of Health, Tokyo

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While there have appeared many reports on screening of synthetic drugs for cancer chemotherapy, not a few chemical compounds are being synthesized throughout Japan with no chance to be subjected to anti-cancer screening. To fulfill the demand to establish a screening system for such new compounds, a plan of "screening service" has recently been realized in the National Institute of Health, Tokyo. Approximately 2000 synthetic chemical compounds of various kinds have been collected and examined for their anti-cancer effects. The activity of this service system will probably be expanded so that it could cover test samples from all over the country in the future. There should be several problems to be considered from the methodological viewpoint. At the present, however, within the limit of utilities with which to start the investigation, it was unavoidably required to simplify the screening procedure so as to deal with as many samples as possible.

In the present paper, the process of standardization of the procedure for our anti-cancer screening system will be described. The following paper (Takano *et al.*, 1959) will present the result of screening obtained with some naphthoquinone derivatives.

MATERIALS AND METHODS

Cancer cells: The Ehrlich ascites cells have been maintained for a long period by serial passages through the peritoneal cavity of dd/Y strain of mice. Transfer was done regularly at a 7 days' interval by the intraperitoneal injection with 2×10^6 cells.

Mice: All experiments were performed using dd/Y strain of mice. It originally was raised in Department of Veterinary Sciences of the Institute and has been maintained at an animal farm in Shizuoka Prefecture. Five or 10 mice, 6 weeks old and weighing 18-22 g, made one group.

Inoculation of cells: The Ehrlich cells were inoculated into mice intraperitoneally or subcutaneously. As the site of subcutaneous injection, the inguinal region was selected.

Drugs known to have anti-cancer effects: Some known synthetic anti-cancer drugs such as 6-mercaptopurine (6-MP), mercury hematoporphyrin (MH) and nitromin ($\text{HN}_2\text{-N-oxide}$) as well as

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江頭靖之・高野宏一・山田正篤・広川康子(国立予防衛生研究所病理部)
水野伝一・阿部美穂子・正宗行人(国立予防衛生研究所化学部)

an antibiotic, mitomycin (MM), were used for the standardization of the procedure. They were administered by the intraperitoneal route.

RESULTS

Observations on Mice Inoculated with Tumor Cells Intraperitoneally

Inoculum size and longevity: Mice were divided into 3 groups, each consisting of 10 animals. Each mouse of the 1st group received intraperitoneally 1×10^7 cells in 0.2 cc of saline, the 2nd group 2×10^6 cells, and the 3rd 4×10^5 cells. Changes in body weight increase and in viability following inoculation are illustrated in Fig. 1.

Fig. 1. Changes in viability and body weight of ddY mice received i.p. different numbers of Ehrlich cells.

More than half of animals in the 3rd group inoculated with 4×10^5 cells survived over 30 days, the range of death occurrence being rather wide. In 2 other groups, all deaths occurred within a smaller range and body weight increased smoothly. Considering the practical easiness, an inoculum size of 2×10^6 cells was decided to be employed in the following experiments.

Comparison of ascites development between male and female mice: Body weight change and longevity were compared between male and female, mice each of them receiving 2×10^6 cells. As Fig. 2 shows, there was no significant difference between both sexes in the behavior after inoculation.

Fig. 2. Ascites development in male and female mice received i.p. 2×10^6 Ehrlich cells.

Distribution of tumor deaths among inoculated mice: Totally 105 mice were inoculated at different times with 2×10^6 Ehrlich cells and their survival times were plotted against probit values of the cumulative frequency. As shown in Fig. 3, the distribution of death occurrence made a straight line, with a 50% longevity of 14.3 days. It can be said from the data that, if the mean longevity of a group is almost 30 days or more, it will be significantly longer than others for some reason or other. Then the observation should be continued for at least 30 days after inoculation.

Anti-cancer effects of known drugs: Among four which had been known as anti-cancer drugs, 6-MP and MM demonstrated high effectiveness in the screening system. Administration was performed intraperitoneally once a day for 7 days, being started from the day following the inoculation with the Ehrlich cells. Various amounts of a drug, ranging around the level usually used, were first tried and, after the most suitable dose

Fig. 3. Distribution of longevity of mice after i.p. inoculation with 2×10^6 Ehrlich cells.

Fig. 4. Effect of 6-MP on i.p. inoculation with 2×10^6 Ehrlich cells.

was determined, the test was repeated with a certain amount which was pertinent for each drug. Fig. 4 illustrates the results of 6-MP administration, $625 \mu\text{g}$ per mouse per day, compared with normal mice and untreated control. MM showed comparative results with a smaller amount of $10\text{--}40 \mu\text{g}$, though direct comparison might not be reasonable. In the present screening, 6-MP was selected to serve as the positive control because of being a synthetic chemical.

Fig. 5. Weight distribution mode of solid Ehrlich tumor 14 days after s.c. inoculation with 2×10^6 cells.

Observation on Subcutaneous Group

A mouse which had received subcutaneously 2×10^6 Ehrlich cells began to exhibit a detectable tumor at the injection site about 7 days after inoculation. Following other workers' procedures (Moore *et al.*, 1949; Sugiura, 1955; and Yamamoto *et al.*, 1956), subcutaneous solid tumors were taken and weighed on the 14th day of inoculation. Weight distribution mode of 739 tumors is presented in Fig. 5. It showed a linear relation against the probit values of cumulative frequency, as was the case with the viability in the intraperitoneal group, with a 50% value of 435 mg (Fig. 6).

Fig. 6. Distribution of tumor weights 14 days after s.c. inoculation with 2×10^6 Ehrlich cells.

Table 1. Standard procedure of screening

Mouse	dd/Y strain, 6 weeks old, 18-22 g, 5 mice per group.
Tumor	Ehrlich ascites tumor.
Inoculation	7-day-old ascites, 2 × 10 ⁶ cells/0.2 cc saline/mouse, i. p. or s. c. (inguinal region).
Drug administration	7 i. p. injections starting from day after inoculation, 1200 µg/0.25 cc saline/mouse/day.

Standardization of Anti-Cancer Screening

According to the results of the present experiments, a standard procedure for screening was decided to follow the way summarized in Table 1, and results should be evaluated on several criteria described in Table 2.

Concerning the description in Table 1, administration of test samples was performed following the way employed by Stock (1955) with a modified standard dose of 1200 µg per mouse per day instead of 2500 µg, for the sake of convenience in practical use.

In Table 2, the increase in mean longevity is calculated by the following formula :

$$L = \frac{l_t - l_c}{l_c} \times 100$$

in which L represents longevity increase, l_t mean longevity of a treated group, and l_c that of the control. If the value of L of an experimental group is less than 25%, the effect of the drug is evaluated as -, the value between 25-50% is evaluated as ±, that between 50-75% as +, and that of 75% or more as ++.

Increase in body weight represents the ascites accumulation in addition to the physiological increase. The curve can give an approximate basis for analyzing toxic and curative effects of drugs.

Tumor weight ratio is determined as follows :

$$W = \frac{w_t}{w_c} \times 100$$

where W is tumor weight ratio, w_t mean tumor weight of a treated group, and w_c that of the control. The results are evaluated in the same manner as Sugiura (1955) dealt with tumor diameters; that is, the value of W more than 75% is -, that 50-75% ±, that 25-50% +, and that less than 25% ++.

Following the above standard, the screening results of 4 known anti-cancer drugs are

Table 2. Standard evaluation of result

Toxicity	Deaths occurring before 9th day of tumor inoculation are considered due to toxicity or accident and excluded from evaluation of effect.
In ascitic form	Viability after 30 days. Increase in mean longevity. Body weight increase curve.
In solid form	Tumor weight ratio after 14 days.

Table 3. Screening results on known anti-cancer drugs

Drug	Dose (per mouse per day)	Toxicity	Tumor type			
			Ascites		Solid	
			Viability	Longevity increase effect	No. of tumors weighed	Tumor weight decrease effect
	μg	(1)	(2)	(3)		(4)
6-MP	300	0/10	5/5	++	5	++
	625	0/10	5/5	++	5	++
	1250	1/10	3/4	++	2	++
MH	10	0/10	0/5	—	5	—
	40	0/10	0/5	—	5	±
HN ₂ -N-oxide	10	0/10	0/5	±	5	—
	40	0/10	0/5	±	5	—
MM	10	0/10	5/5	++	5	±
	40	0/10	3/5	++	5	+

(1) No. of deaths within 9 days/No. of mice used.

(2) No. of survivals after 30 days/that over 9 days.

(3) Evaluated by increase ratio of mean longevity to control.

(4) Evaluated by ratio of mean tumor weight to control.

tabulated in Table 3. So far as the present system is concerned, 6-MP in 625 μg was considered to be best as the positive control.

DISCUSSION

A wide tumor spectrum for a cancer chemotherapy screening was emphasized to be necessary and applied to practical use by Sugiura *et al.* (1952a and b). Besides transplantable animal tumors, human malignant tumor strains, established by Toolan (1954; 1957) in experimental animals, will serve as other important materials. In the present plan, only one kind of tumor, the Ehrlich ascites tumor, was used. Under the present situation, the procedure should be simplified so as to screen as many new samples as possible.

Standardization on the basis of the observations on tumor-inoculated mice, intra-peritoneally or subcutaneously, was considered reliable from several viewpoints. Furthermore, some known anti-cancer drugs were tried by this system. Each showed a different but constant effect in repeated tests, suggesting a high reproducibility of the results by the system. Among them, 6-MP was usually most effective and decided to serve as the positive control in the following examinations.

SUMMARY

A "screening service" system, using the Ehrlich ascites cells in dd/Y strain of mice, for anti-cancer effects of newly synthesized chemical compounds was opened in the National Institute of Health. The screening procedures and the evaluation of results were standardized on the basis of observations on several biological behaviors of mice intraperitoneally or subcutaneously inoculated with a certain number of tumor cells.

Some known anti-cancer drugs were tried by this system to certify the reliability of the method.

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