DEVELOPMENT OF RESISTANCE TO NITROGEN MUSTARD N-OXIDE IN THE YOSHIDA SARCOMA

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In recent years the problem of resistance of bacteria to various antimicrobial agents has become important with the progress of chemotherapy. On the other hand, search for cancer inhibiting agents which has made notable progress these several years, has resulted in the discovery of various useful compounds. Nevertheless, complete cure of human cancer is still an impossibility.

It has been frequently observed by most clinicians that tumor inhibiting agents used on certain patients become ineffective sooner of later, though they are efficacious at the beginning of use. Especially, in the treatment of leukemia this phenomenon has most frequently been encountered. Such a fact may be one of the important and fundamental problems in the chemotherapy of cancer, as in bacteria. The problem of tumor-resistance to agents, however, has been studied by few investigators. Burchenal[1-3] and Law[4-7] have reported the development of resistance to folic acid antagonists in a transplantable mouse leukemia and that to colchicine in a mouse ascites tumor has been studied by Lettré[8] and Klein.[9]

With the intention of making cytological and cytogenetical studies on the malignant tumor, in addition to therapeutic interest, I have carried out successive transplantations of the Yoshida sarcoma,[10] accompanied by continued injections of several tumor inhibiting agents and studied the development of tumor resistance to these compounds.

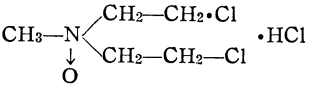
The experimental study reported here is concerned with the response of the Yoshida sarcoma in consecutive transplant generations to Methyl-bis-(β-chlorethyl)-amine N-oxide Hydrochloride (Nitrogen Mustard N-oxide) (MBAO).

I wish to express my sincere gratitude to Professor Dr. Fukuzo Oshima for valuable advice given during the course of this study. Thanks are also due to Mr. J. Aoki for his technical assistance.

PROCEDURE AND RESULTS

Treatment:

Methyl-bis-(β-chlorethyl)-amine N-oxide Hydrochloride (MBAO) used in this experiment is an alkylating agent having the formula as follows.



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The effect of MBAO upon the Yoshida sarcoma has been reported by Ishidate *et al.*,[11] Yoshida[12] and Kaziwara-and-Kobayashi.[13] This compound is used generally as a useful tumor inhibiting agent in Japan because of the wide therapeutic dose range and relative lack of bye-effects, in addition to its great therapeutic index.

In this study rats weighing about 100 g and of mixed breed, of which almost 100 per cent were susceptible to the tumor transplantation, were employed. The rats that received intraperitoneal inoculations of 0.02 ccm of undiluted ascitic fluid of the Yoshida sarcoma, containing about 2×10^6 tumor cells, were divided into two main groups; one group in which intraperitoneal successive transplantations were performed with no treatment as control, and one group which was treated with MBAO during all transfer generations. Experimental observations in the latter were made in 3 sublines as follows;

1) In all transfer generations of this subline intraperitoneal injection with MBAO was commenced 5 days after inoculation and continued daily until death of the animals (MBAO-i.p. resistant subline).

2) Only in the 1st transfer generation subcutaneous injection was commenced 5 days after inoculation, but in subsequent generations the injection was started right after inoculation and continued daily until death of the hosts (MBAO-s.c. resistant subline).

In other words, tumor cells in MBAO-i.p. resistant subline were in a condition free of the agent for 4 days after inoculation in each generation, and in the MBAO-s.c. resistant subline, tumor cells were maintained always under the existence of the agent throughout all transfer generations. Treatment with the agent was started with daily doses of 50 μg in the 1st generation of both resistant sublines, and when the treatment became ineffective for control of the tumor growth, namely, when the tumor resistance developed to that dose, daily dose of 250 μg was used in the next generation. In such a manner, the daily dosage was increased step by step from 50 μg to 250 μg, 500 μg and 1 mg. The daily dose of the agent was always dissolved in 0.5 ccm of physiological saline.

3) In successive transplant generations of this subline, intraperitoneal injection of 2 mg of MBAO was made, once a generation, on the 5th day after inoculation (MBAO-2 mg$\times$1 resistant subline)

Transfer of tumor ascites to the next generation:

Transfer was made by intraperitoneal inoculation of the tumor ascites obtained right before the death of animals. The inoculation was made on 2–7 animals in each generation, and each animal received about $1\text{–}2\times10^6$ tumor cells.

Criterion of the development of resistance:

The development of tumor resistance was judged by the following. 1) Change in total number of tumor cells in the ascites following injections. 2) Frequency of regular and abnormal mitotic figures of the tumor cells. 3) Survival time of host animals. 4) Intensity of the infiltrative growth of tumor. If both the tumor cells and their mitotic figures did not decrease in number and

the infiltration of tumor cells into the surrounding tissue was observed to the same degree as in untreated control animals, in spite of the continued administration of the agent, it was regarded that the tumor acquired a resistance to that treatment. In such cases the daily doses were increased in the next generation, as has already been mentioned.

On the contrary, if both tumor cells and their mitotic figures were markedly decreased in number following injections, it was concluded that the tumor has not yet acquired a complete resistance, though the survival time or infiltration was the same as in untreated control animals. As far as the tumor remained sensitive to that dose, injections were similaly repeated in the following generation. The calculation of tumor cells was carried out usually as follows; Whole nucleate cells were counted in unit volume (1 cmm) of tumor ascites obtained, then the percentage of tumor cells in nucleate cells was enumerated in Giemsa stained smear preparations of the same ascites. The number of tumor cells was calculated, based on these two values. Morphological changes of tumor cells and frequency of mitotic figures were observed mainly by giemsa staining.

MBAO-i.p. resistant subline

The genealogy and response to treatment in each transfer generation of this subline can be seen in fig. 1. Following the intraperitoneal injection of 50 μg of MBAO on the 5th day after inoculation in the 1st generation, tumor cells 24 hours after injection showed the so-called 1st period type,[14] which is seen usually 3-4 days after inoculation. The dimension of the cytoplasm of these cells was less than that of the nucleus, and both showed intensive basophility by giemsa staining. Furthermore these cells were relatively small in size. With consecutive injections 9 days after inoculation the majority of tumor cells became enlarged and occupied by the degenerative forms showing lighter nucleus and nucleoli. At the same time the basophility of cytoplasm decreased and largy azurophile granules were seen dispersed all over the cytoplasm (fig. 6). Intermingled with such degenerative cells only a few of the 1st period type were seen. 12 days after inoculation, however, the major part of the tumor cells con-

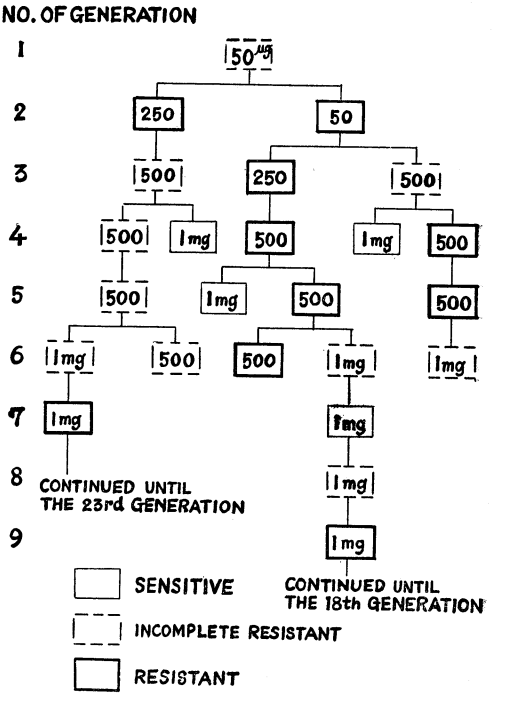

FIG. 1. Genealogy and response to the treatment in each transfer generation of MBAO-i.p. resistant subline.

sisted of the 1st period type, contrary to the previous findings, and degenerative large cells were not seen. On the other hand regular mitotic figures of tumor cells were observed abundantly and the tumor grew, unaffected by treatment at these stages. Survival times of these animals were twice that of untreated control animals and the infiltrative growth of tumor cells was of the same degree as the controls. Thus, injecting 50 μg of MBAO daily into the abdominal cavity tumor cells were temporarily destroyed with degenerations, but the remaining cells (the 1st period type) multiplied in spite of the continued injections resulting in death of the hosts. Therefore, it is evident that resistance to 50 μg of MBAO already developed in the 1st generation. Furthermore, in the 7th generation, about 100 days after the beginning of this subline, resistance developed to the same treatment with 1 mg, and henceforth this subline was observed continuously until the 23rd generation under the same treatment.

MBAO-s.c. resistant subline

The genealogy and response to treatment in each transfer generation of this subline are shown in fig. 2. In this subline, resistance to 1 mg was acquired at the 7th generation, about 60 days after the start of the first injection. At the 10th generation there was seen also a resistance to consecutive injection with a daily dose of 2 mg. Following the 7th transfer generation this resistant subline has been continued until now, for 63 generations with the same treatment with 1 mg of MBAO.

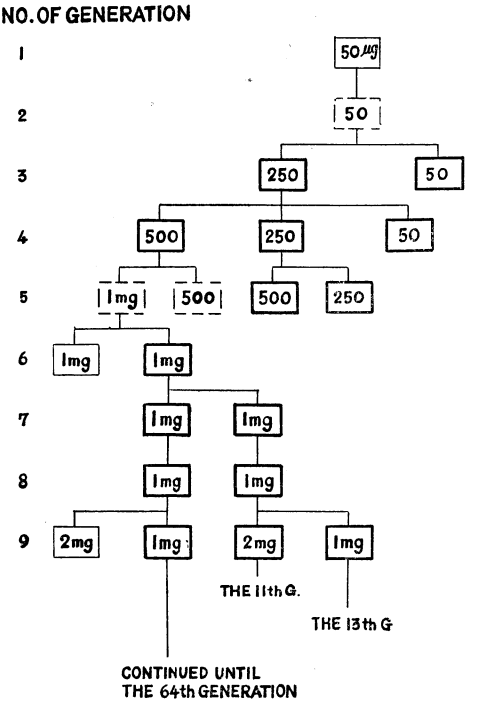

FIG. 2. Genealogy and response to the treatment in each transfer generation of MBAO-s.c. resistant subline.

MBAO-2 mg × 1 resistant subline

Serial transplantations of MBAO-2 mg × 1 resistant subline were carried out through 21 successive transfer generations during about 170 days. At the 1st generation, tumor cells were destroyed strikingly by the injection and the remaining tumor cells were very few in number. Host animals died by growth of these and survival times were about twice that of untreated control animals. Infiltrative growth of tumor was only slightly seen. Although the remarkable decrease of tumor cells after injection was seen in all transfer generations until the 6th generation, sensitivity of tumor cells to the agent was gradually decreased

following the 7th generation, namely, about 60 days after the start of this subline.

At the 12th transfer generation, about 100 days after the beginning, almost complete resistance was observed, and survival times and infiltrative growth of the tumor were also of the same degree as in untreated control groups. Resistance at this stage was seen not only to injections of 2 mg of MBAO a generation, but also to continued injections of 1 mg daily for 4 days following 2 mg on the 5th day after inoculation. In the 7th generation of this subline, complete resistance to consecutive subcutaneous injections with daily doses of 1 mg started from right after inoculation was already seen. Therefore even if complete resistance to an intraperitoneal injection of 2 mg once a generation was not yet acquired, it can be said that the 7th generation of this subline showed almost the same resistance as the MBAO-s.c. resistant subline.

Behavior of dividing cells in resistant subline

Behavior of dividing cells of these resistant sublines to MBAO was shown in fig. 3. In a control strain, 3 hours after intraperitoneal injection of 1 mg of MBAO the mitotic figures were decreased in number to one half or one third of those immediately before, and 9-12 hours after injection it reached a minimum. 24 hours after injection there was a slight increase in number, but very few in comparison with those immediately before injection. 24-48 hours after injection the major part of the mitotic figures consisted of characteristic abnormal divisions; adhesion, scattering, coagulation, and bridge formation of chromosomes (see figs. 7-14). Similar results were obtained also by subcutaneous injection. Thus, intraperitoneal or subcutaneous injection of 1 mg of MBAO in the original control strain resulted in a dramatic decrease in number of mitotic figures and occurrence of abnormal mitosis. On the other hand, in MBAO-i.p. and s.c. resistant subline, the decrease in mitotic figures was slightly observed and the majority of mitotic figures at 24 and 48 hours after injection was almost normal (figs. 15-17).

Generally speaking, the dividing cells are more sensitive to various tumor inhibiting agents than resting cells.[15] Therefore, from the fact mentioned above,

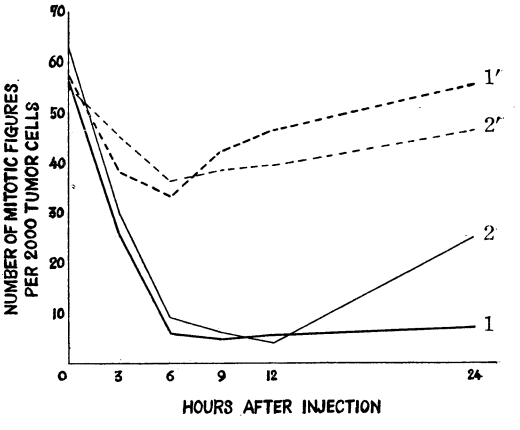

FIG. 3. Frequency of mitotic cells following the intraperitoneal or subcutaneous injection of 1 mg of MBAO on the 5th day after inoculation.

Solid graphs=original Yoshida sarcoma, 1; intraperitoneal injection. 2; sub. cutan. injection.

Dotted graphs=resistant subline, 1'; MBAO-i.p. resistant sub. (intraperit. inj.), 2'; MBAO-s.c. resistant sub. (sub. cutan. inj.).

it may be assumed that these sublines possess clearly marked resistance. On the behavior of dividing cells 12 hours after the injection of 2 mg of MBAO, there was no difference between MBAO-2 mg × 1 resistant subline and the original control strain, but 24 hours after injection the former showed a more notable recovery in number than the latter. This behavior continued to about the 10th transfer generation, and after the 12th generation, dividing cells showed also a fairly marked resistance, but these dividing cells in MBAO-2 mg × 1 resistant subline were more resistant to 1 mg than 2 mg as shown in fig. 4.

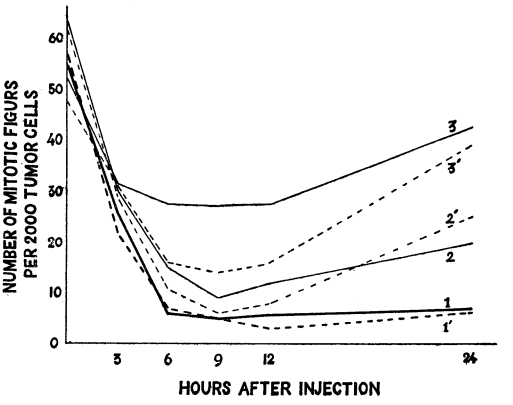

FIG. 4. Behavior of mitotic cells in MBAO-2 mg × 1 resistant subline to the intraperitoneal injection of 1 or 2 mg on the 5th day after inoculation.

Solid graphs = Injection of 1 mg. 1; original strain. 2; the 5-10th transfer generations of MBAO-2 mg × 1 resistant subline. 3; the 11-16th transfer generations.

Dotted graphs = Injection of 2 mg. 1'; original strain. 2'; the 5-10th transfer generations of MBAO-2 mg × 1 resistant subline. 3'; the 11-16th transfer generations.

The change in total number of tumor cells

The changes in total number of tumor cells in the original strain and MBAO-s.c. resistant subline are shown in fig. 5. In the original strain and MBAO-s.c. subline treated with consecutive injections of daily doses of 1 mg from just after inoculation with 2×10^6 tumor cells until death of the host, the total numbers of tumor cells in 1 cmm of ascites were counted. The tumor cells in the original strain from which the resistant subline was derived, even if 10×10^6 of tumor cells were inoculated, proceeded to decrease in number without multiplication and they disappeard from the ascites fluid within 4 or 5 days after inoculation and all cases were completely healed. On the contrary, those of the resistant subline continued to multiply in spite of consecutive treatment, and 4 days after inoculation showed a state of pure culture of tumor cells, and continued to multiply resulting in the death of the host, like as in the untreated control animals. MBAO-i.p. resistant subline showed resistance to continued injections with daily doses of 1 mg, given intraperitoneally from the 5th day after inoculation, and the decrease of tumor cells in number was scarcely seen.

Average survival time in untreated control strain was 9 days. On the other hand that in resistant sublines, after they acquired a resistance to 1 mg, was 9, 5 days in MBAO-s.c. subline and 11 days in i.p. subline, differing from the complete recovery or notable prolongation of survival time in treated control strain. In MBAO-2 mg × 1 subline after the 12th generation, it was 11 days. The infiltrative growth of tumor in resistant subline was of the same degree

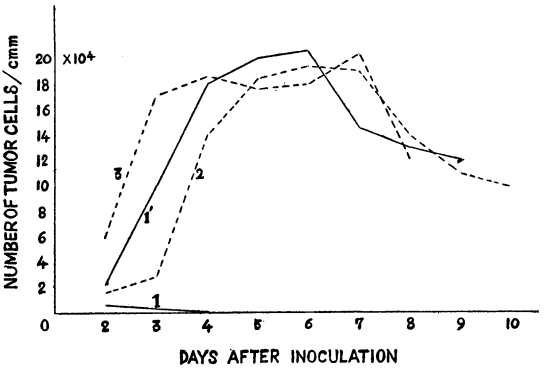

FIG. 5. Change of total number of tumor cells through a transplant generation in original strain and resistant subline.

Solid graphs = original strain. 1; Rats received the inoculatton of 2×10^6 or 10×10^6 tumor cells and were treated with consecutive injections with daily doses of 1 mg of MBAO started from right after the inoculation. Both results were quite similar. 1'; untreated animals inoculated with 2×10^6 tumor cells.

Dotted graphs = MBAO-s.c. resistant subline. 2: Rats were inoculated with 2×10^6 tumor cells. Treatment was done as in 1. 3; Inoculation with 10×10^6 tumor cells. Treatment as above.

as in untreated control animals, in spite of treatment with MBAO (see figs. 18–21).

DISCUSSION

From to these results it is evident that the development of resistance to MBAO could not be avoided by any mode of administration of the agents. In case of injection, consecutive or intermittent, intraperitoneal or subcutaneous, the development of resistance was always observed. In the resistant subline, not only did the resting cells escape injury, but the dividing cells continued their multiplication without receiving any considerable influence. Burchenal *et al.* used average survival time as criterion in their studies on the development of resistance of leukemia AK-4 to repeated doses of 4-amino-N^{10}-methyl PGA (A-methopterin). Law and Boyle used the mean weight in milligrams of the localized lymphoma tissue at 9 days after subcutaneous inoculation as a criterion of development of resistance to folic acid antagonists in a transplantable lymphoid leukemia. On the other hand I have employed principally the number of tumor cells and mitotic figures and their morphological findings. At the same time the survival time and the degree of infiltrative growth were used as accessories. Since MBAO is relatively lacking in toxic effects when compared with Nitrogen Mustard, death by intoxication of host animals could not be considered in this series of doses. Nevertheless, if death by intoxication has to be considered, the survival time is of course, unsuitable as an indicator of tumor resistance. As a criterion of tumor-resistance, exact measurement of the growth-rate of tumor is most desirable. For this porpose the criterion used in the

present study, which was to judge by the behavior of the tumor cell itself, may be a reasonable one.

On the biological and immunological findings of these resistant sublines and mechanism of development of tumor resistance it will be published later.

SUMMARY

Successive transplantations of the Yoshida sarcoma have been carried out with consecutive injections of Nitrogen Mustard N-oxide, and three resistant sublines to this agent were obtained. Resistance always developed sooner or later by any mode of administration of this agent and it was found to increase in a stepwise fashion. These sublines have continued to be resistant in subsequent transfer generations, following the emergence of resistance.

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EXPLANATION OF FIGURES

FIG. 6. A large degenerative tumor cell. Several basophilic cells surrounding it belong to the so-called 1st period type. Giemsa staining. 800×.

FIGS. 7–14. Abnormal mitosis of original Yoshida sarcoma cells at 24 hours after intraperitoneal injection of 1 mg of MBAO. Giemsa staining. 1 000×.

FIG. 7. Breaking down of spiral chromonemata within chromosomes.

FIG. 8-9. Scattering of chromosomes at anaphase.

FIG. 10-11. Coagulation and stickiness of chromosomes.

FIG. 12. Chromosome bridge at telophase.

FIG. 13. Chromosome bridge and lagging chromosomes at telophase.

FIG. 14. Dissociation of cytoplasmic and nuclear division.

FIG. 15. 24 hours after intraperitoneal injection of 1 mg in the 20th transfer generation of MBAO-i.p. resistant subline. Three regular mitotic figures at metaphase are observed. Giemsa staining. 700×.

FIGS. 16-17. 5 days after inoculation in the 29th transfer generation of MBAO-s.c. resistant subline. There are seen many regular mitotic figures despite continued injection with daily doses of 1 mg. Giemsa staining. 700×.

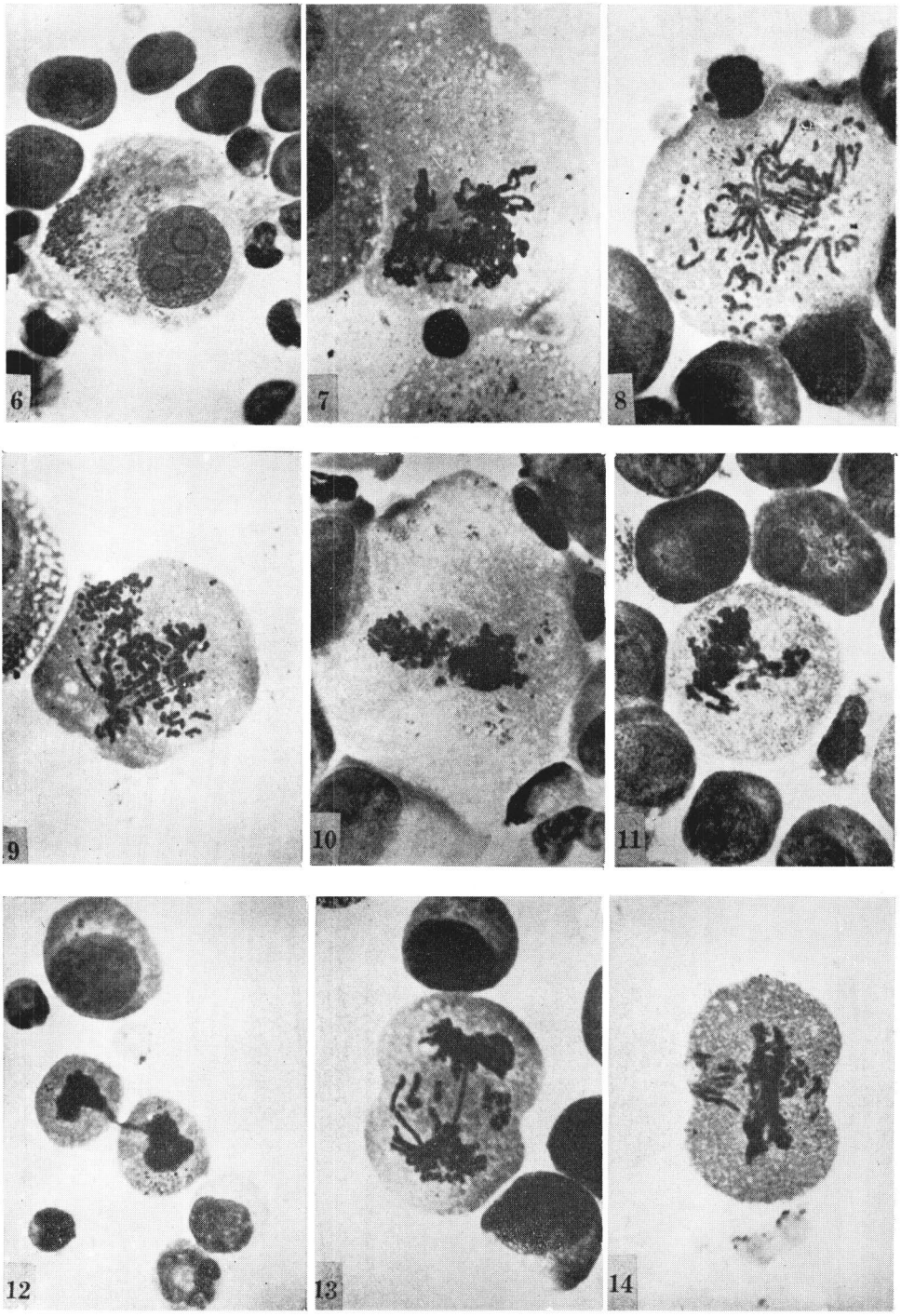
FIGS. 18-21. The infiltrative growth of tumor in original strain and resistant sublines are demonstrated (Tumor tissues are indicated by arrows.).

FIG. 18. Original strain with no treatment (survival time 9 days).

FIG. 19. 23th transfer generation of MBAO-i.p. resistant subline (surv. t. 10 days).

FIG. 20. 10th transfer generation of MBAO-s.c. resistant subline (surv. t. 9 days).

FIG. 21. 7th transfer generation of MBAO-2 mg×1 resistant subline (surv. t. 8 days).



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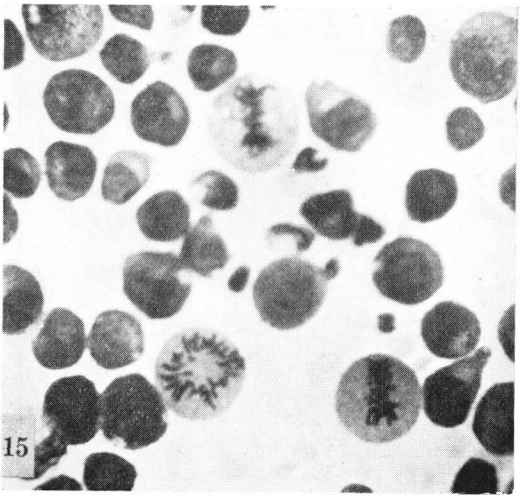
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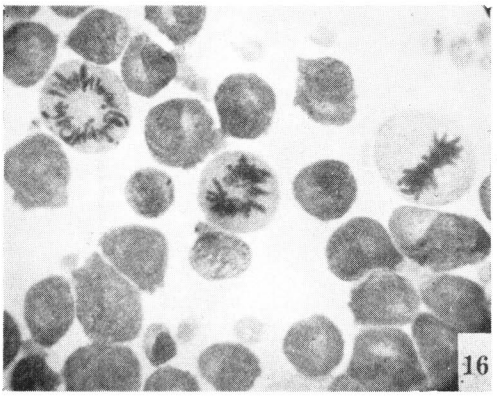
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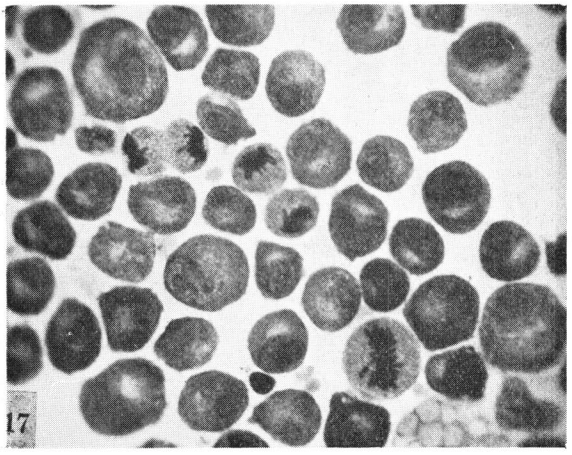
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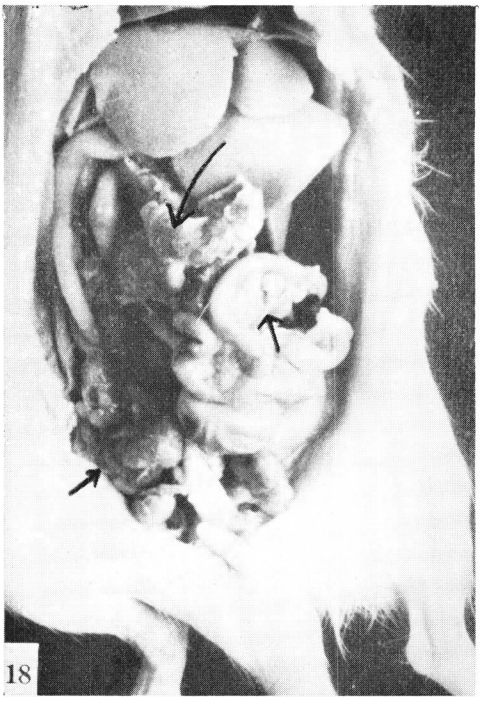
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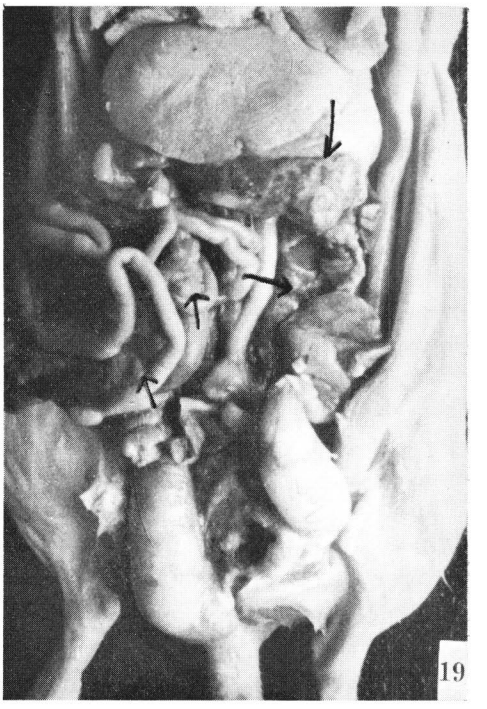
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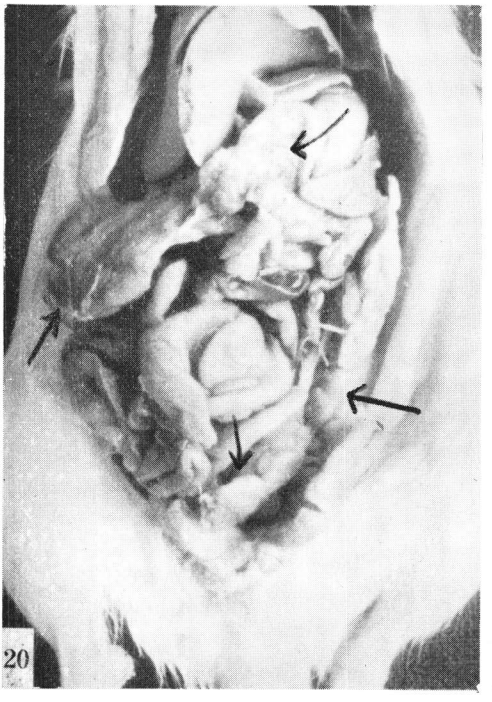
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