Immunological Studies of Tumors

III. Effect of homologous antiserum upon¹ Yoshida sarcoma cells *in vivo*

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Received July 24, 1959

Studies on humoral cytotoxins have long been undertaken by many authors and repeatedly discussed in relation to tumor immunity and therapy. Many investigators have reported the cytotoxic action of homologous and heterologous immune sera, or of antisera prepared against various cell components *in vitro* and *in vivo* (Millar and Hsu 1956, Schrek and Preston 1956, Horn 1956, Colter *et al.* 1956, Malmgren 1958). Flax (1956) showed that the cytotoxic action of complement-dependent antibody in the purified gamma globulin on Ehrlich ascites tumor cells was related to the loss of a cellular capacity to metabolize glucose with retention of capacity to utilize succinate. Previously, the present author reported the presence in a homologous immune serum of a cytotoxin harmful to cells of the Yoshida sarcoma subline C. He showed that the immune serum was specific to any tumor type in experiments with four kinds of rat diploid ascites tumors (Matsumoto 1958), and further that the tumor cells treated with the immune serum *in vivo* showed a low stainability with basic dyes.

The present study was undertaken in order to obtain some detailed information on the metabolic effect of antiserum on tumor cells *in vivo*.

The author wishes to offer his hearty thanks to Professor Sajiyo Makino for his kind direction and improvement of the manuscript for publication. Further thanks are extended to Dr. S. H. Hori for his valuable advice.

Material and method

The here-employed anti-Yoshida-sarcoma-subline-C antiserum (AS) was prepared by repeated injections of Yoshida sarcoma subline C cells into Fischer rats which are non-susceptible to the tumor, according to the method previously adopted by the present author (Matsumoto 1958). Normal serum (NS) was obtained from Wistar rats which are susceptible to the tumor, by the same

¹ Contribution No. 445 from Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan. An article "Differential reactions of homologous antiserum to tumor cells of different rat ascites tumors" published in Jour. Fac. Sci. Hokkaido Univ., Ser. VI (Zoology), Vol. 14, No. 1: 31-38, should be referred to as No. 1 of "Immunological Studies of Tumors." Supported by a grant-in-aid to Dr. S. Makino from the Scientific Research Fund of the Ministry of Education.

procedure as described before (Matsumoto 1958).

The rats used in the present study were of pure inbred Wistar strain (W/Ma), weighing about 100 grams each. On the 1st day of the tumor transfer, sera (AS and NS) were injected into the peritoneal cavity of tumor-bearing rats.

For cytological studies, smear preparations of the tumor ascites were made at appropriate intervals following the injection of the sera. Each smear was fixed with absolute methanol and stained with May-Grünwald and Giemsa (MGG) according to the method of Jacobson and Webb (1952).

For demonstration of dehydrogenase activity, the neotetrazolium method devised by Hirono (1957) was used with a slight modification as follows: tumor ascites aspirated from rats bearing tumor, before and after the injection of sera was mixed with the incubation mixture which consists of 1 ml of 1 % neotetrazolium chloride in 0.2 M sodium phosphate buffer (pH 7.4) and 1 ml of either 0.2 M sodium succinate or 0.2 M sodium citrate or 0.2 M glucose. The mixture was incubated in a centrifuge tube for 2 hours at 37°C, and centrifuged for 5 minutes at 500 r.p.m. After repeated centrifugations of the precipitate in 0.85 % saline solution, the resultant sediment was lightly squashed on the slide and mounted with glycerin. Dehydrogenase activity of tumor cells was demonstrated in the cytoplasm as a deep purple formazan deposition of reduced neotetrazolium.

Results

An attempt was made at first to observe the effect of antiserum (AS) upon actively growing tumor cells. During from the 1st day to the 7th day of the tumor transfer, the antiserum was injected into peritoneal cavity of rats bearing tumor at various doses at one-day-interval. For comparison, injection of normal rat serum (NS) into tumor-bearing rats was also made at the same interval as in the antiserum.

1. Effect of antiserum on the survival time of host rats

Repeated injections of NS into tumor-bearing rats, amounting to 6 ml, showed no effect on the tumor cells nor on the survival time of the tumor-bearing rats. As shown in Table 1, all of eight animals treated with NS died of tumor, showing large metastatic tumors. On the contrary, the rats which had received antiserum injections showed a parallel relation existing between the life-span of tumor-bearing animals and the dose of AS. As shown in Table 1, tumor animals which received injections less than 3.5 ml of AS on the 1st day of tumor transfer did not survive longer than did the control animals, while a remarkable elongation of survival time was observed in rats which received 4 ml or more of AS. There occurred however two exceptional animals (asterisk in Table 1) of which one died of diarrhoea on the 14th day after tumor transfer, and the other showed the growth of tumor. Autopsy revealed no tumor cells either in the ascitic fluid or in the

organs of the former, while in the latter, tumor cells, though they had disappeared almost completely in the ascities on the 7th day of tumor transfer, appeared again and proliferated to lead to the subsequent death of animal. Such a renewed malignant growth of the tumor may have been due to the existence of tumor stem-cells which had remained unaffected by the antiserum.

Table 1. Life spans of tumor-bearing Wistar rats following transplantation of Yoshida sarcoma subline C, after treatment with antiserum (AS) and normal serum (NS)

	Sex	Body weight	Total dose	Life span
		(grams)	(cc/100 gr)	(days)
NS-treated	♂	70	2	9
	♂	70	2	7
	♂	80	3	8
	♂	80	3	7
	♂	90	3.5	7
	♂	100	4	8
	♂	100	5	9
	♂	150	6	8
	♂	150	6	—
AS-treated	♂	100	2	8
	♂	100	3	8
	♂	100	3	8
	♂	100	3	10
	♂	130	3.5	12
	♂	140	3.5	14
	♂	70	3.5	14
	♂	90	4	—
	♂	90	4	—
	♂	90	4	—
	♂	100	6	—
	♂	100	6	—
	♂	130	6	—
	♂*	100	6	9
	♂*	100	7	12

2. Cytological evidence

Acetic dahlia preparations: Tumor samples were taken from rats at various intervals after the application of NS or AS, and colored with acetic dahlia. One of the most obvious effects of AS was the agglutination of tumor cells. In AS-treated animals, the tumor cells disappeared rapidly from the ascites, showing a gradual breaking down of their cytoplasm during one to six hours after serum injection. Cells undergoing damage were usually characterized by enlargement of the cell body together with a pycnotic disintegration of naked nuclei. These are typical features of cell damage during 6 to 12 hours after injection (Figs. 3-4). The chromosomes in mitotic cells remained unaffected by the antiserum. It was found that there were some tumor cells which remained viable being enclosed by a viscous ascitic fluid. The reacting cells, such as leucocytes, lymphocytes and plasma cells, increased in number. Until after the 4th injection of AS (48 to 72 hours after the 1st), the complete damage of tumor cells continued without the death of the tumor-bearing animals nor the formation of metastatic tumors. However, tumor cells reappeared after 2 to 3 days unless booster injections of AS were made.

Mitotic frequencies of tumor cells in rats which had received AS and NS injections were studied in order to gain information about some quanti-

Fig. 1. Curves showing the change in occurrence of mitotic cells after injection of the sera. A, in AS-treated tumor cells. B, in NS-treated tumor cells.

Figs. 2 to 5. Yoshida sarcoma subline C cells stained with acetic dahlia. 400 $\times$. 2, the untreated tumor cells just before injection of sera. 3, showing degenerative change in tumor cells; taken from 3-hour sample after AS injection. Note the necrotic feature and pycnotic naked nuclei. 4, tumor cells from 12-hour-sample after AS injection. Residual tumor cells undergo degeneration. 5, seventy-two hours after AS injection. No tumor cells was found in the sample.

tative aspects of the effect of sera upon tumor growth. The results are graphed in Fig. 1. Generally, the mitotic index of tumor cells in NS-treated rats showed no remarkable shift from that in control rats, while in AS-treated rats the mitotic index showed a sudden decrease 1 hour after the serum injection, and then a striking increase occurred 3 hours after the 1st injection of the serum. Subsequently, a gradual decrease in number of mitotic cells was observed until the tumor cells disappeared completely from the ascites, up to the 3rd day after the 1st injection.

Figs. 6 to 9. MGG stained tumor samples after injection of the sera. 1000 $\times$. 6, NS-treated tumor cells 6 hours after injection. Note the density of the normal cytoplasm and dark nucleoli. 7, NS-treated cells 12 hours after injection. Some cells decrease the stainability to dye. 8, AS-treated tumor cells 6 hours after injection. Note the loss of basophilia and breaking down of the cytoplasm, all of which are enclosed by viscous ascitic fluid. 9, AS-treated cells from the same sample as Fig. 8. Note the extrusion of cytoplasmic buds and the irregularity of cell border.

From the above results, it may be concluded that the antiserum when injected into the peritoneal cavity of tumor-bearing rats inhibited tumor growth by injuring the cytoplasm of the tumor cells.

3. MGG preparations

In order to observe the process of damage in tumor cells, MGG and some other cytochemical stainings were applied to tumor ascites taken from NS- and AS-treated rats. Samples of tumor ascites were aspirated from rats at various intervals and stained with MGG, methyl-green and pyronin, and

Figs. 10 to 24. Yoshida sarcoma subline C cells, showing dehydrogenase activity demonstrated by granular deposit of pigment intracellularly. 100 $\times$. 10-14, succinate-added tumor cells. 10, untreated tumor cells showing strong dehydrogenase activity. 11, six hours after AS injection. Note the complete loss of the activity. 12, twenty-two hours after AS injection. The activity begins gradually to recover. 13, six hours after NS-injection, showing decrease of activity. 14, twenty-two hours after injection, showing the recovery of the activity.

azure B for nucleic acids. Among the methods used here, the May-Grünwald and Giemsa (MGG) preparations yielded the most satisfactory results in demonstration of nucleic acids, so that the following descriptions are based on use of MGG preparations.

As a result of the staining of normal tumor cells with MGG, the cytoplasm and nucleolus showed a blue coloration, while the chromatin in the resting cells and the early prophase chromosomes stained purple-red. Metaphase and telophase chromosomes stained black, i.e., purple-red superimposed upon blue. The stainability of NS-treated cells did not notably differ from the untreated ones even after the application of a large amount of serum. But decrease of stainability was observed in some cells (Fig. 7).

In AS-treated rats, the stainability of tumor cells with the dye was considerably altered, showing pycnotic granules stained black in degenerating cells. Generally the change appeared during from 12 to 24 hours after serum injection (Fig. 8). Twenty-four hours after injection, affected cells extruding many cytoplasmic buds underwent gradual disintegration showing the loss of cellular basophilia (Fig. 9).

4. Enzyme study: dehydrogenase activity as revealed by the neotetrazolium method

The capacity of affected cells to utilize glucose and succinate as the sole exogenous energy source was already reported by Flax (1957) in Ehrlich ascites tumor cells treated with anti-Ehrlich gamma globulin plus complement. In the present study, the cellular capacity to metabolize succinate, citrate and glucose as the exogenous energy source in untreated, NS- and AS-treated tumor cells was investigated by the use of Hirono's method (1957). Samples here employed were obtained from rats which had received a single injection of sera.

1) *Untreated tumor cells.* When succinate and citrate were used as substrate respectively, the normal tumor cells showed abundant granular formazan depositions of deep purple pigments indicating a striking dehydrogenase activity (Figs. 10 and 15). However, the activity was much weaker in the citrate-treated cells than in the succinate-treated ones. Little dehydrogenase activity was observed in the tumor cells when glucose was used as substrate, though there were a few cells which showed small granular depositions in the cytoplasm (Fig. 20). It is then evident that the enzymatic

15-19, citrate-added tumor cells. 14, untreated tumor cells, showing moderate dehydrogenase activity. Six hours (16) and twenty-seven hours (17) after AS injection. Six hours (18) and 72 hours (19) after NS-injection. 20-24, glucose-added tumor cells. 20, untreated tumor cells. Note the complete loss of dehydrogenase activity, except a few showing faintly stained (purple-red) small granular deposition. 21, tumor cells 6 hours after AS injection. Note the increase of dehydrogenase activity in tumor cells. 22, gradual decrease of the activity in a sample at 48 hours after AS injection. 23, slight increase of the activity, 6 hours after NS injection. 24, tumor cells showing the loss of the activity, 72 hours after NS injection.

activity of tumor cells occurs in decreasing order as follows: succinate-treated cells (+++), citrate-treated cells (+) and glucose-treated cells (almost zero) (Table 2).

2) *NS-treated tumor cells.* Six hours after NS-injection, tumor cells showed a higher enzymatic activity by using glucose as substrate than untreated ones. Conversely, with succinate or citrate as substrate, the enzymatic activity of treated tumor cells was greatly decreased below that of controls. This decrease continues temporarily for a few hours and then the activity recovered with time (Table 2, Figs. 13-14, 18-19 and 23-24).

Table 2. The effect of antiserum (AS) and normal serum (NS) on neotetrazolium reduction by the affected tumor cells
The amount of reduced neotetrazolium as formazan deposition was estimated, from 0 to ‡

	Substrates					
	Glucose		Citrate		Succinate	
	0		+		+++	
Control						
Tested serum	AS	NS	AS	NS	AS	NS
6 hours after injection	0	±	+	0	0	0
12 hours after	±	0	±	±	0	±
24 hours after	+	0	0	±	±	0
48 hours after	+	0	±	+	+	±
72 hours after	±	0	+	±	++	+
4 days after	0	0	+	+	+++	++

3) *AS-treated tumor cells.* The enzymatic activity of AS-treated tumor cells was variable. The reducing ability of neotetrazolium salt was completely lost in cells to which succinate was added six hours after the AS-injection, where many cells showed no formazan granules in the cytoplasm, though the cells seemed to be normal in morphology. Such a feature of enzymatic activity began to recover 24 hours after injection of the serum, and had returned to the normal level by 4 days after serum injection.

Discussion

In a therapeutic attempt of tumors with homologous antiserum, Aizawa *et al.* (1952) reported that repeated injections of anti-Yoshida sarcoma serum brought about a complete cure in a number of tumor-bearing rats, when 50 to 70 cc of serum was injected intraperitoneally over a 4- to 7-day period, and that smaller doses produced vacuolar degeneration and some necrosis of tumors. Similar toxic effects of antisera were also observed in the present study when tumor cells were treated with smaller doses of antiserum than that employed by the above investigators. Again, there occurred a temporary

retardation of tumor growth in AS-treated tumor-bearing animals, as shown by a marked change in mitotic frequency of tumor cells. From the data at hand, it seems likely that a possible correlation exists between the dose of the sera administered and the life-span of tumor-bearing animals which received the serum injection. Therefore, a complete cure of tumor-bearing animals may be expected from the injection of relatively large amount of sera into rats at suitable intervals after tumor transplantation.

The cytological study of the serum-treated tumor cells *in situ* revealed a remarkable difference in cellular basophilia from the untreated tumor cells. Further the agglutination of tumor cells by serum-injection was observed, irrespective of the nature of injected sera, whether normal or antiserum: in antiserum-treated tumor cells, the cell agglutination occurred 1 hour after the serum injection, and degeneration and necrosis of tumor cells followed during subsequent 12 to 24 hours. Therefore, the agglutination of tumor cells seemed to be a first step in the cellular degeneration as pointed out by Wissler and Flax (1957). On the contrary, normal serum generally lacks any ability to damage tumor cells.

MGG staining of tumor cells treated with antiserum plus complement made it clear that the affected cells had undergone nuclear breakage along with the diminution of cellular basophilia. Some cells showed a blebbing of cytoplasm (Fig. 9), though not frequent in many tumor cells. There were to be found tumor cells of small size which showed no reduction in basophilia: they seem to be active and residual tumor stem-cells which have remained alive being unaffected by antiserum and form through proliferation the primary source of the renewed growth of tumors. Wissler and Flax (1957) found that there occurred a few viable cells characterized by unusually large size containing multiple prominent nucleoli in the samples taken 36 hours after the treatment with anti-Ehrlich gamma globulin plus complement; further, they found that the surviving cells were significant in being polyploid in reference to the view of Hauschka (1953) involving the immunoselection of polyploid forms. In the present experiments, the surviving tumor cells are generally small-sized having compact and comparatively large nuclei. A similar feature has been reported in the therapeutic experiments with chemicals in several rat ascites tumors, showing that there are always present small-sized tumor stem-cells which have remained unaffected by the action of the drug, and that the proliferation of such cells induces the renewed malignant growth (Makino 1957).

The dehydrogenase activity of serum-treated tumor cells as determined by the neotetrazolium method showed an interesting effect of serum upon tumor cells. Some workers have investigated the mechanism whereby an antibody or some therapeutic agents injure the metabolism of tumor cells (Black and Speer 1953, Flax 1956). Using anti-Ehrlich gamma globulin plus complement and the neotetrazolium method, Flax (1956) ascertained that the

affected tumor cells could utilize succinate but not glucose as an exogeneous energy source. He stated that anti-Ehrlich gamma globulin plus complement might inhibit the glycolysis of tumor cells, while the treated cells retained the ability of utilizing succinate as substrate.

The tetrazolium method for dehydrogenase activity has yielded variable results in cells and tissues. Barka and Dallner (1958) observed a remarkable formazan-production by ascites tumor cells in the presence of glucose, whereas Rutenberg *et al.* (1950) reported that the purified succinic dehydrogenase was not able to reduce the tetrazolium salts. On the otherhand, Williams-Ashman (1953) found no glucose metabolizing activity of ascites tumor cells. Using the neotetrazolium method, Hirono (1957) reported on the succinic dehydrogenase in ascites tumors of rats and mice. The present study has shown that in untreated Yoshida sarcoma subline C cells, little dehydrogenase activity was observed when glucose was used as substrate, while an intense neotetrazolium reduction occurred in the presence of succinate. The diverse results noted above may probably be due to different conditions of the tumor used for experiments. The present author (unpublished) has found that the dehydrogenase activity of tumor cells as determined by the staining method and electrophotometric analysis differs in different tumors and in different samples taken from the same tumors at various intervals after tumor transplantation. The variation in dehydrogenase activity as observed in the present experiments seems to indicate the inhibitory action of serum on the metabolic function of tumor cells.

Summary

Effects of the homologous anti-Yoshida-sarcoma-subline-cell-serum upon antigen-cells was investigated in *in vivo*.

Intraperitoneal injection of the antiserum, resulted in no elongation of survival time in tumor-bearing rats in a maximum dose. Rats which received 4 to 6 ml of the antiserum on the 1st day of tumor transplantation survived over a relatively longer period than any others. Further, the tumor cells after treatment showed a marked fall in frequency of occurrence of mitosis and in cytoplasmic basophilia with a subsequent breakage of the cytoplasm.

Use of the neotetrazolium method yielded information that the tumor cells treated with antiserum were unable to utilize the exogeneous succinate, though there occurred an increased ability to utilize exogeneous glucose by tumor cells. Such a neotetrazolium reduction occurred 6 to 12 hours after the injection of antiserum. The gradual return to normal level was attained 3 to 4 days after injection in the case when booster injection was not given.

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