Effect of Attenuated Cells on the Growth of Small Implant of Yoshida Ascites Hepatoma AH109A

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ABE, I., HORI, K., SAITO, S., SUZUKI, M. and SATO, H. Effect of Attenuated Cells on the Growth of Small Implant of Yoshida Ascites Hepatoma AH109A. Tohoku J. exp. Med., 1981, 134 (4), 385-392 — The transplantability of small implants such as 10 cells of Yoshida ascites hepatoma AH109A, which grew lethally only in 2 out of 19 rats when transplanted subcutaneously, was improved by admixed implantation of attenuated tumor cells prepared with mitomycin C, presenting lethal take in 23 out of 27 animals. Similar effect was also obtained by liver homogenate or sonicated tumor cell suspension. Although the rate of tumor "take" of less than 10³ cells of AH109A inoculated in the subcutis increased by admixing the attenuated cells, growth characteristics of such a small implant of the tumor, once initiated, were not significantly different whether inoculated with or without the attenuated cells. Growth support effect of attenuated cells was discussed in relation with either the size of inoculum or growth ability of tumor cells. —— Yoshida ascites hepatoma AH109A; growth ability; growth support effect; attenuated cells

In most Yoshida ascites hepatomas, transplantability of subcutaneous inoculum is much lower than that of intraperitoneal inoculum (Asahina 1967); e.g., the cell number for 50% take (TD₅₀) of AH109A was smaller than 10 cells when transplanted intraperitoneally, while TD₅₀ after subcutaneous inoculation was about 500 (Satoh et al. 1967). This suggests that growth ability of a small subcutaneous implant of tumor is impaired by some factors or by a deficit of some factors. In this connection, several reports have dealt with growth ability of subcutaneous tumor (Révéz 1956; Toda et al. 1967; Hewitt et al. 1973; Dykes et al. 1976; Evans 1978; Van den Brenk et al. 1978). The present study was performed to describe difference in growth ability between intraperitoneal and subcutaneous implants of the rat ascites hepatoma, AH109A, and to elucidate further the effect of chemotherapeutically attenuated tumor cells on the growth of small implants of the same tumor.

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MATERIALS AND METHODS

Yoshida ascites hepatoma, AH109A, transplanted intraperitoneally in male Donryu rats (Nihon Rat Co., Urawa) weighing 120–150 g was aspirated on day 5 and adjusted to the appropriate number with saline. The cell suspension (0.1 ml) was inoculated in the back subcutis with or without attenuated cells. The size of inoculum of intact AH109A cells was given in legends for Figures.

The attenuated cells were prepared as follows: AH109A bearing rats were treated with 10 mg/kg body weight of mitomycin C (Kyowa Hakko Co., Osaka) given intraperitoneally on days 4 and 5, and tumor cells were collected 2 hr after the second treatment. One million cells of the attenuated AH109A suspended in 0.1 ml saline were admixed with 0.1 ml of viable AH109A cells, and they were injected subcutaneously unless otherwise specified. Usually, every 10⁶ cells of the attenuated tumor contained less than 1 viable cell which survived chemotherapy.

Sonicated cells were prepared by sonic disruption of 2.5 × 10⁷/ml of cell suspension with a Branson sonifier (model W-185, Branson Sonic Power Co., USA) at 20 kHz, position 5 for 2 min and subsequent centrifugation at 1,000 × g for 10 min to remove the remnant intact cells. The procedure was repeated again, and 0.2 ml of suspension was injected with viable cells where appropriate. About 2 cells were still alive in 0.2 ml of the sonicated cell suspension.

Liver homogenate was prepared by homogenization of the minced liver in saline with a teflon homogenizer and centrifugation at 1,000 × g for 10 min. The pellet was homogenized again and combined with the supernatant. Half ml of the homogenate from 0.1 g liver was admixed with 0.1 ml of viable tumor cell suspension and inoculated as above.

Peritoneal mononuclear cells were obtained by the method of Snyderman et al. (1971). Twenty ml/kg body weight of 0.5% glycogen was injected into the peritoneal cavity of normal rats. On the 4th day, 5 ml of saline was injected intraperitoneally and the abdomen was massaged for 2 min, then ascites was collected. The ascites was centrifuged as above, and the pelleted mononuclear cells were resuspended in saline at density of 10⁸/ml.

RESULTS

Effect of the attenuated AH109A cells on the growth of 10 living cells of the same tumor is shown in Fig. 1. Ten living cells of AH109A by themselves could not grow in the back subcutis in any of 6 animals. Either 10⁷ or 10⁶ cells of attenuated AH109A supported the growth of such small implants of the same tumor. In accord with an earlier report (Donelli et al. 1969), liver homogenate also supported the growth of AH109A.

Since mononuclear cells appeared in the ascites of mitomycin C-treated rats, though one-log order lower in number than the attenuated cells, the effect might not owe to the attenuated tumor cells but to the mononuclear cells. In order to eliminate the influence of contaminating mononuclear cells, the tumor cells were prepared from untreated ascites in the pure culture state and sonicated to be deprived of growth ability. The tumor take being 5 out of 11 rats after inoculation of 100 cells or less rose up to 9 out of 10 animals by admixing the sonicated cell suspension (p<0.05). On the contrary, as many as 10⁷ of the mononuclear cells only slightly improved the take of small implants of AH109A (p<0.10). The effect of the attenuated cells on the take of AH109A inoculated in the subcutis was summarized in Fig. 2. The take of 10 cells of AH109A significantly increased from 2 out of 19 rats (11%) when inoculated singly to 23 out of 27 animals (85%)
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by admixing the attenuated cells ($p<0.01$). That two cells of viable AH109A grew in 3 out of 8 animals implies that most, if not all, of the viable cells participated in tumor growth when initiated. TD$_{50}$ of AH109A was about 100 cells in subcutaneous inoculation into 104 rats throughout the present experiments. When the attenuated same cells were admixed, TD$_{50}$ lowered to less than 10 cells. When 100 cells of viable AH109A were inoculated remote from the attenuated cells, the tumor did not grow at all. Tumor growth was only temporary when AH109A was admixed with 10$^6$ cells of AH66F which grew only temporarily in the subcutis.

Allowing an appropriate lag period before the tumors became measurable depending on the size of inoculum, growth curves after inoculation of 10$^2$, 10$^4$, and 10$^6$ cells lay on the same line (Fig. 3). Doubling time (DT) was 0.8 day, independent of the presence of the attenuated cells. Fraction with growth initiating ability may not significantly change either with variety of size of inoculum or with admixing the attenuated cells. Therefore, the attenuated cells could improve only transplantability without affecting the growth characteristics of the initiated tumor.

The change of life span as a function of size of subcutaneous inoculum was biphasic (Fig. 4). The change of life span corresponding to the 2-fold change of size of inoculum (SD$_2$ after the definition of Sato et al. 1966) was variable depending
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Median life span after intraperitoneal inoculation of AH109A was similar irrespective of admixing the attenuated cells when the number of the living cells were the same (Fig. 5). Change of life span as a function of size of inoculum was linear through wide range of size of inoculum and SD was constant. The fact enabled us to estimate the absolute number of the viable cells in the attenuated cell population by bioassay. TD of intraperitoneal inoculum of AH109A was less than 10 cells either the attenuated cells were admixed or not.

**Discussion**

It was shown that small population of Yoshida ascites hepatoma, AH109A, grew in the subcutis in the presence of the admixed attenuated same tumor or normal liver homogenate. Effect of liver homogenate may not be unexpected but one would suspect that the sonicated cell suspension had not been effective since cytoplasm prepared from the irradiated cells could exert the growth support effect but the sonicated cells were not effective (Toda et al. 1967). Our observation that the fraction with the growth ability would not considerably decrease with decrease of size of inoculum seems different from that obtained with CBA “NT”
tumor by Hewitt et al. (1973) that clonogenic cell fraction decreased as the size of inoculum was decreased and that the impaired clonogenicity was recovered by the radiation inactivated cells. This discrepancy might, at least in part, originate from considerable difference in growth ability between the tumors used by these authors and us; TD\textsubscript{50} of CBA "NT" tumor used by them was 6900 and that of AH109A used in the present experiments about 100. It implies that clonogenic fraction was much higher in AH109A than in CBA "NT" tumor. It is probable that decrease in clonogenic fraction is not marked with decrease of size of inoculum in a tumor with relatively high transplantability. AH109A may be such an extreme example.

The mechanism by which the attenuated cells, sonicated cell suspension, and liver homogenate supported the growth of small implant of AH109A is unknown. Growth support effect of irradiated cells (Révész 1956; Hewitt et al. 1973), chemotherapeutically inactivated cells (Dykes et al. 1976), and normal tissues (Donelli et al. 1969) was reported. Evans (1978) reported that whole body

![Fig. 3. Growth curve of AH109A cells inoculated subcutaneously with or without the attenuated same tumor. Size of inoculum of AH109A was $10^2$ ($\triangle$, $\blacktriangle$), $10^4$ ($\circ$, $\bullet$), or $10^6$ ($\circ$, $\bullet$). The lefthand set is growth curve after inoculation of AH109A alone and the righthand set after inoculation of intact AH109A admixed with the attenuated same tumor ($10^6$ cells). The abscissa is days after inoculation of AH109A; the upper set of numbers for the experiment with $10^6$ living cells, the middle set with $10^4$ cells, and the lower set with $10^2$ cells. Solid symbols, size of inoculum of the intact cells; open symbols, tumor volume ($V$) calculated from the long ($L$) and short ($S$) axes of the tumor. Assumption was made that the tumor is ellipsoid, where $V$ equals $\pi LS^2/6$ and that $5 \times 10^6$ cells are 1 ml in volume. As seen, if allowance is made for the greater lag period in the smaller size of inoculum, the growth curves after 3 different sizes of inoculation lie on the same line. Doubling times (DT) were $0.8 \pm 0.1$ and $0.8 \pm 0.2$ days (3 experiments each) in AH109A inoculated singly and with the attenuated cells respectively.]}
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Irradiation resulted in the reduction of tumor take possibly because of depletion of monocytes. On the contrary, Peters et al. (1978) reported that local thoracic irradiation induced the increase in lung colonies not due to increased vascular trapping of the tumor cells, one of the major steps in inducing metastatic growth (Sato and Suzuki 1972), nor to immunological impairment but to improved physiological microenvironment for growth.

Although $SD_2$ defined by Sato et al. (1966) was assumed to be the same as DT in L1210 by Skipper et al. (1964, 1965) and $SD_2$ value was similar to DT in Yoshida sarcoma (Sato et al. 1966; Abe and Sato 1976) and rat ascites hepatoma AH66 (Sato et al. 1966), $SD_2$ and DT may reflect the different biological properties of the tumor.

Fig. 4. Life span of rats inoculated subcutaneously with AH109A singly or with the attenuated same tumor as a function of size of viable cell implant. Solid symbols show the median life spans after inoculation of the graded numbers of viable AH109A alone, and open symbols after inoculation of the intact cells with the attenuated same tumor ($10^6$). Different symbols show different experiments. The change of life span corresponding to the two-fold change of size of inoculum ($SD_2$ after the definition by Sato et al. 1966) was $0.4 \pm 0.4$ or $1.6 \pm 0.2$ days at size of inoculum over or under $10^4$ cells respectively.

Fig. 5. Life span of rats inoculated intraperitoneally with AH109A singly or with the attenuated same tumor as a function of size of viable cell implant. Solid circles show the median life spans after inoculation of the graded numbers of viable AH109A alone, and open circles after inoculation of the intact cells with the attenuated same tumor ($10^6$). One hundred million cells of the attenuated tumor (○) were presumed to contain about 25 viable cells, subsequently less than 1 viable cells was contained in every $10^6$ attenuated cells. Difference of size of inoculum by 1,000-fold from 10 to $10^4$ cells resulted in the difference of the median life span by 5.0 days. $SD_2$ was calculated to be 0.5 day, which was similar to the cell cycle time of the intraperitoneal growth of AH109A.
Effect of Attenuated Cells on Tumor Growth (Sato et al. 1966). Moreover, SD\textsubscript{2} may not be fixed or specific to each tumor but may change depending on the growing conditions of the tumor. The present result on SD\textsubscript{2} values of AH109A growing in the subcutis and intraperitoneal cavity supports the above point of view, and suggests that the attenuated cells had no influence on SD\textsubscript{2}. The observation further supports that the attenuated cells did not change the growth characteristics of the small implant of AH109A other than the rate of tumor take. In this connection, it was observed that 2 cells of AH109A inoculated with the attenuated cells, which grew exponentially in 3 out of 8 animals with DT of 0.8 day in the early phase, delayed to reach to say 1 ml in volume as compared with those which originated from more than 10 cells. The delay had a tendency to occur rather in the declining growth phase than in the initial phase. Elongation of SD\textsubscript{2} may be related, at least in part, to the growth delay after inoculation of very small size of tumor. Steel (1977) reported the growth delay which occurred in the initial stage when the size of inoculum of intact B16 melanoma cells admixed with the attenuated cells were below 10 cells. So far, what factor(s) would be involved in SD\textsubscript{2} elongation and growth delay in the small subcutaneous implant is not known. It is important to know the growth characteristics of the small population of tumor cells considering the remnant viable cells surviving various treatments. Dykes et al. (1976) pointed out that total cell kill should be necessary for complete cure of cancer because the small surviving population of tumor mass which had escaped from anticancer effect would begin to grow again supported by the attenuated cells. Even if tumor mass is eliminated in a mode of fractional cell kill (Skipper et al. 1964, 1965), the larger the tumor before treatment, the more is the number of the attenuated cells produced. Therefore, it should be more difficult to cure the tumor of larger size even when anticancer drugs are effective, since it produces more attenuated cells which might eventually lead to relapse, unless the host defence mechanism is well operative.

References

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