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Effect of *in Vitro* Glucocorticoid Treatment on Acid Ribonuclease Activity in P1798 Lymphosarcoma Cells (33815)

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Lymphosarcoma P1798 has been shown to regress rapidly following subcutaneous injection of glucocorticoids (1, 2) with a concomitant rise in tumor acid ribonuclease activity (3, 4). Burton *et al.* (5) showed that newly suspended P1798 cells are sensitive to corticoids *in vitro*. The present experiments show that the effect of corticoid treatment on the acid ribonuclease of the tumor is caused by a direct action of the drug on the tumor cell rather than by an indirect action mediated through some other part of the host body. Cells from the corticoid sensitive strain of lymphosarcoma P1798 were grown in tissue culture and shown to be sensitive to *in vitro* treatment with 9α-fluoroprednisolone (9FP) and dexamethasone phosphate. Gabourel and Aronow (6) demonstrated similar sensitivity of cultured ML-388 cells *in vitro*. The P1798 cells from our established cultures have now been shown to exhibit an increase in acid ribonuclease activity following *in vitro* treatment with 9FP.

Methods. Cultures of lymphosarcoma P1798 have been quite difficult to establish. Repeated culturing similar to the method of Sato and Buonassisi (7) was used. Solid tumors were minced in medium A (MEM which had BME vitamins substituted for MEM vitamins and supplemented with 100 μmoles/liter each pyruvate and asparagine as well as 10% fetal calf serum), filtered through cheese cloth, and incubated at 37° for 24 hr. The cell suspension was then injected subcutaneously into female BALB/c mice and after the resultant tumor grew to approximately 10-mm diameter (14–18 days) it was again put into culture. The second culture was incubated for 2–4 days before it was injected into another host mouse while the third culture generation was kept for 5–7 days and the fourth was kept in culture 1–4 weeks. Corticoid sensitive, strain 1 (CS-1) reported here was established in the fifth culture generation and has now been maintained for 2 years while strain 2 (CS-2) was established in the fourth culture generation and has been maintained for 1 year. These cells grow in suspension so that trypsin is not necessary for passage and they have not been subcultured more than once every 4–6 weeks. The stock cultures were maintained in a volume of approximately 50 ml and twice a week 40 ml of the culture was replaced with fresh medium B (1:1 mixture of Fischer's media and media A described above).

Established cultures from the corticoid resistant strain (CR-1) of the P1798 lym-

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phosarcoma (2) were obtained in the same manner as the sensitive except that Fischer's medium (purchased from Grand Island Biological Co.) was used in the fourth and fifth culture generations. All of the CR-1 experiments reported here were performed on cells maintained in our medium B for 18 months.

Corticoid sensitive cells (CS-1 from culture generation 4) were incubated for 3 months in the presence of 1 μg/ml of dexamethasone phosphate (DMP) to develop a resistant strain *in vitro*. This culture was fed with medium A containing dexamethasone phosphate (1 μg/ml) approximately every other week. Development of a corticoid resistant strain *in vitro* was similarly accomplished by Aronow and Gabourel (8). The cells were injected into a host mouse after 3 months and the resultant tumor was put into culture as described above. This fifth culture generation was established as a resistant strain, designated CS-R-1, and has been maintained in medium B for 18 months. Twice weekly 15 ml of culture was replaced with fresh media. If a volume of more than 20 ml was used in the stock culture, the cells did not survive. The reason for this peculiar trait is unknown.

Cells from these established lines were grown subcutaneously in BALB/c female mice (ca. 20 g) and the tumor sensitivity to 9FP *in vivo* was determined as described previously for asparaginase sensitivity (9). The mice were injected (sc) with 0.5 μg of 9FP suspended in 0.2 ml of steroid suspending media (formulated by Armour Pharm. Co., Kanakee, Ill., and distributed through CCNSC) while control animals received injections of suspending media alone.

The effect of *in vitro* treatment of steroids on the acid ribonuclease activity of cultured cells was determined by addition of the steroid dissolved in 95% alcohol to the cell culture so that the steroid was in a concentration of 0.1 μg/ml and the alcohol concentration was less than 0.01%. Alcohol alone was added to the control cultures. After the cultures were incubated at 37° for the designated time period, the cells were removed by centrifugation, washed with 2 × 10 ml of culture media which did not contain serum (the serum was found to contain RNase activity) and once with 10 ml of 0.25 *M* sucrose. The cells were resuspended in 1 ml of 0.25 *M* sucrose and broken by sonic oscillation for 3 min on a Branson Sonifier. Microscopic examination of the preparations showed more than 95% broken cells. The samples were centrifuged to remove cell debris and the supernatants were frozen. Ribonuclease activity was measured within 1–2 days of sonication according to the method of Ambellan and Hollander (10) using citrate phosphate buffer, pH 5.8. The substrate, a highly purified yeast RNA, was purchased from Mann Biochemical Corp. Protein was measured by the method of Lowry *et al.* (11) using serum albumin as the standard. Ribonuclease activity was calculated as units per/milligram of protein and expressed as percentage of control in the Tables I and II.

Results and Discussion. The *in vivo* sensitivity of tumors derived from the established tissue culture strains is similar although not identical to that of the original tumor strain in regular transplant. The cells which were maintained in the presence of DMP (CS-R) gave rise to a tumor which is almost completely resistant to 9FP treatment. The DMP did not cause regression of the tumor but did retard its growth while 9FP mediated a definite tumor involution. This difference is believed to be related to the solubility of the steroid. Dexamethasone was given to some mice to relate its effectiveness to DMP and it was as effective as 9FP in causing tumor regression. More experiments are planned to elucidate this discrepancy.

Ribonuclease activity of the cells was measured after incubation with 9FP for various time periods to establish the mode of the increased activity reported in the tumor following *in vivo* treatment (3, 4). Table I shows that 9FP does, indeed, act on the cells in culture to produce an increase in acid ribonuclease activity in all of our established strains except the CS-R which had been transformed to a resistant culture *in vitro*. The increase in the cellular RNase was measura-

TABLE I. The Effect of Steroids *in Vitro* on Acid Ribonuclease Activity of Cultured Cells of P1798 Lymphosarcoma.

		Ribonuclease activity (% of control)			
Cell line	Steroid[a] (hr):	6	18	24	48
CS-1	9FP	115 ± 21 (2)[b]	126 ± 31 (3)	195 ± 50 (3)	177 ± 38 (4)
	DMP	—	—	—	106 ± 18 (2)
	Testosterone	—	—	—	98 (1)
	Progesterone	—	—	—	86 (1)
CS-2		—	—	134 (1)	137 ± 1 (2)
CR-1	9FP	—	—	152 (1)	176 ± 25 (2)
CS-R-1	9FP	—	—	104 ± 11 (2)	97 ± 17 (4)
	DMP	—	—	—	101 (1)

[a] Steroid concentration was 0.1 μg/ml in treated cultures.

[b] Mean ± SE (no. of experiments).

ble in two of the three experiments done 18 hr after the addition of 9FP to cultures of CS-1. A small increase in activity was seen in one experiment after 6-hr exposure to 9FP so that we feel that the onset of the definite increase is later than 6 hr but earlier than 24 hr—probably near the 18-hr period.

Neither progesterone nor testosterone is capable of increasing the acid ribonuclease activity of the cultured cells and, surprisingly, dexamethasone phosphate did not elicit an increased RNase either (Table I). More experiments are planned to ascertain the cause of the lack of effectiveness of DMP. It is particularly puzzling in view of the fact that cells which were maintained in the presence of DMP became resistant to the action of 9FP.

TABLE II. Effect of 18 hr *in Vivo* Treatment with 9FP on Ribonuclease Activity of Tumors Derived from Tissue Culture and Regular Transplants.[a]

	Ribonuclease activity (% of control)	
Cell line	Tissue culture strain	Regular transplant
CS-1	95 ± 12 (3)	138 ± 10 (2)
CS-2	133 ± 7 (3)	186 (1)
CR-1	114 ± 27 (3)	109 ± 12 (2)
CS-R-1	104 ± 3 (2)	

[a] Animals were injected subcutaneously with 0.5 μg of 9FP suspended in 0.2 ml of suspending medium. Control animals were injected with suspending medium alone (0.2 ml). Mean ± SE (no. of experiments).

When the established cell cultures were injected subcutaneously into BALB/c mice (Simonsen Laboratories, Gilroy, Cal.) a tumor always was palpable within 14 days. These tumors were transplanted into a group of mice and the effect on tumor RNase of 9FP given *in vivo* was measured. The results of these experiments are shown in Table II, along with the results of similar experiments done simultaneously on the original tumors in regular transplant. It is interesting that CS-1 did not show an increase in RNase activity while CS-2 had only a modest elevation. The parent strains in regular transplant showed RNase activity significantly higher than control values. On the other hand, Table I shows that following *in vitro* treatment, CS-1 has increased RNase activity greater than CS-2. These observations seem to indicate that the host animal does play a role in part related to the elevation in acid RNase following corticoid treatment.

Strain 1 was established approximately 1 year before strain 2. The CS-2 was stable at the fourth generation in the development of the culture while CS-1 was not established until the fifth generation. We, therefore, assume that although the exposure to 9FP *in vitro* is able to cause an increase in the acid RNase, the activation is lost *in vivo* after 2–3 years in culture. Perhaps CS-2 will also lose

its sensitivity *in vivo* after longer culturing. Long-term culturing may change the permability of the cell. The CS-R strain which was derived from CS-1 and the resistant culture CR-1 which was established within a couple of months of CS-1 showed no significant increase in RNase activity following *in vivo* treatment with 9FP for 18 hr. The increased RNase activity observed in the regular transplant of the sensitive strain has been noted as early as 6 hr and is always present after 18 hr (4). Here, some experiments were done after 24 hr to see if the time period was critical but the same results were obtained.

The increased RNase activity has been seen in all regressing tumors of P1798 *in vivo* regardless of the lympholytic agent used (4). Here we have shown that 9FP also mediates the increased activity *in vitro*. In the 24- and 48-hr experiments with 9FP there is no decrease in cell number, but merely a decrease in the growth of the cells. In other words, the cells are now multiplying more slowly than the control but they are still growing. In view of this finding the increase in RNase activity must not be related to the regression of the tumors but to the cessation of growth of the tumors which undoubtedly preceeds the regression. This hypothesis is supported by the finding of Ambellan and Hollander (4) that the resistant strain of P1798 did not regress following 9FP treatment but did stop growing and the acid ribonuclease was increased 50%. Likewise, when 5-fluorouracil was given to animals bearing the corticoid sensitive strain of P1798, the tumor growth was arrested and the ribonuclease level was increased. Further, when the animals were treated with agents which had no effect on the growth of the tumor, there was no increase in nuclease activity. However, in contrast to that, all of the tumors derived from the cell cultures except CS-R-1 also ceased to grow when the host was treated with 9FP but the RNase activity was increased in only the CS-2 tumors. The reason for this discrepancy remains obscure and will be the subject of future work.

Summary. Cell lines of P1798 lymphosarcoma which have been established in culture for more than 1 year exhibit corticoid sensitivity and growth characteristics similar to the parent, regular transplant when injected into host BALB/c mice. These cells also develop increased acid ribonuclease activity after incubation with 9α-fluoroprednisolone *in vitro*. The experiments described show that the increased ribonuclease previously reported is related to a direct action of the steroid on the cells.

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