[CANCER RESEARCH 44, 2464–2466, June 1984]

Treatment of Murine Testicular Leukemia[1]

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ABSTRACT

The murine leukemia P388 is being evaluated as a potential model for testicular leukemia since this tumor is responsive to most of the drugs used to treat childhood acute lymphocytic leukemia. Infiltration of P388 cells into testes occurs, but the tumor cells plateau at about 10^5 to 10^6 cells/testes, a number inadequate to produce macroscopic disease. Therefore, disease could be evaluated only by bioassay, *i.e.*, suspending and implanting the testes into recipient mice. In advanced disease (at plateau), the tumor cells are responsive to Adriamycin; when the tumor cells are proliferating in testes, they respond to treatment with Adriamycin, methotrexate, and vincristine. Therefore, in this model, the testicular leukemia cells do not appear to reside in a pharmacological sanctuary.

INTRODUCTION

Leukemia of the testes is a significant clinical problem among boys with acute lymphocytic leukemia (7). Of all such male children who have successfully completed induction, consolidation, and maintenance therapy for this disease, 10 to 33% of them are subsequently found to have active disease in their testicles within 1 year after cessation of therapy (8–10, 16). An overwhelming majority of these boys progress to bone marrow involvement and finally succumb to the leukemic disease process. Recent data suggest that the incidence of testicular leukemia is significantly influenced by prior drug therapy, such that certain investigational multidrug chemotherapy regimens appear to have a lower incidence of late testicular relapse than do some of the established treatment regimens.[3]

When therapeutic interventions have been required for testicular disease, they have consisted chiefly of sterilization of the gonads with ionizing radiation in conjunction with further chemotherapy. Neither approach has been completely acceptable or successful (10). Therefore, the need for an animal model to develop therapeutic strategies and to understand the pathological mechanisms of this disease process is obvious. This report describes our efforts to use P388 murine leukemia for this purpose. Further, little is known concerning the possible role of the blood-testis barrier (1, 2, 14, 16) in excluding certain drugs selectively from the testicular milieu. The hypothesis that such an anatomic barrier may play a role in the propensity for testicular leukemia is also examined in this study. Preliminary results of this approach have been presented in abstract form (4, 5).

MATERIALS AND METHODS

P388 leukemia cells were maintained in male DBA/2 mice by weekly transfer of 10^6 cells i.p. Experiments were performed using male C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice. The tumor cells were bioassayed in various organs as follows. The brain, spleen, and testes were suspended into 1 ml of 0.9% NaCl solution (saline) by passage through stainless steel mesh screens (Tissue Sieve; E. C. Apparatus Corp., St. Petersburg, FL). The suspensions or a 100-μl aliquot of whole blood (obtained by cardiac puncture) were then injected i.p. into recipient mice. The number of viable tumor cells implanted was estimated from the median day of death observed in groups of 8 or more animals. The plot of log number of tumor cells implanted *versus* day of death is approximately linear (Table 1, control). The least-squares regression of the data yields the relationship

$$y = 10.05 - 0.37x$$

where y is the $\log_{10}$ number of cells and x is the median day of death. The presence of suspensions of brain clearly altered this relationship such that, for a given tumor implant size, animals died earlier than did controls. For implants of 10^5 or 10^4 cells, the presence of testicular or splenic suspensions produced a similar effect. In most experiments, animals were anesthetized with ketamine (45 mg/kg i.m.), and a total body "wash-out" procedure was performed after removal of a 100-μl aliquot of whole blood. At least 10 ml of saline were perfused into the left ventricle with a right ventricular outflow tract. This procedure removed more than 95% of the RBC from the testes. Also, testes were rinsed with 5 ml of saline, and the tunica albuginea testis was removed prior to suspending the organ. Comparison of the quantal data obtained via the bioassay utilized the 2-sample rank test (3) or, in some cases, the χ^2 test.

Materials. Mice were obtained from The Jackson Laboratory, Bar Harbor, ME. The initial inoculum of P388 cells was supplied by Dr. Joseph Mayo, National Cancer Institute. Drugs were obtained as parenteral forms from The University of Texas M. D. Anderson Hospital Pharmacy.

RESULTS

Following i.p. or i.v. inoculation, P388 cells infiltrated the testes of $BD2F_1$ mice (Chart 1). Infiltration occurred more rapidly following i.p. administration, suggestive of intrainguinal spread of disease in this model. The number of cells in the testes observed in this and numerous other similar experiments plateaued at about 10^5 and 10^6 cells/testes. Infiltration was further confirmed by comparing the results of bioassay in a group of animals receiving the whole-body wash-out procedure described in "Materials and Methods" (Table 2). The estimated capillary volume of testes is inadequate to provide the 10^5 to 10^6 cells observed in advanced P388 leukemia, and it is not surprising that the wash-out did not remove a detectable number of tumor cells.

Since infiltration of P388 tumor cells into the testes occurred, we wished to evaluate the chemotherapeutic response of cells in the organ to drugs. Response to 3 agents used to treat childhood leukemia is shown in Table 3. The reduction in viable tumor cells due to drug treatment was compared for brain (a

[1] Supported by Grant CA-28034 from the National Cancer Institute, and by development funds from the Department of Pediatrics, The University of Texas Medical School at Houston.

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Received November 10, 1983; accepted March 12, 1984.

Table 1

Bioassay of P388 leukemia cells

P388 cells were removed from the ascites of $BD2F_1$ mice 7 days after implantation of 10^6 cells i.p. from DBA/2 stock animals. The cells were injected into groups of 8 animals in the absence (control) or presence of tissue suspensions of the spleen, brain, blood, or testes of untreated mice. An equivalent of 100 μl of whole blood or the entire organ was administered i.p. in the bioassay. Data reflect the median day of death of recipient animals.

	Median day of death after following no. of cells were implanted						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
Control	9	10	13.5	17	19	21.5	24.5
Spleen		10	13	15[a]			
Brain		8.5[a]	9.5[a]	12[a]			
Blood		11	13.5	15			
Testes		10	12[a]	13[a]			

[a] Significantly different from control, $p < 0.05$ (2-sample rank test).

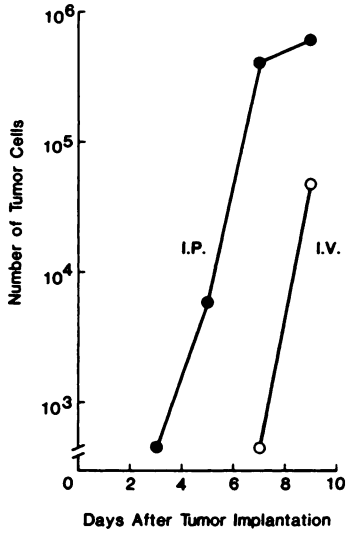

Chart 1. Infiltration of the testes of $BD2F_1$ mice implanted with 10^5 P388 cells i.p. or i.v. on Day 0. Animals were sacrificed on the days shown after tumor implantation, and the infiltration of tumor cells was assessed by bioassay, as described in "Materials and Methods." The median days of death for simultaneously implanted groups of mice were 11 and 12.5 days for i.v. and i.p. implants, respectively.

Table 2

Infiltration of P388 leukemia cells into the testes of $BD2F_1$ mice

Mice were anesthetized 6 days after i.p. implantation of 10^6 P388 cells. The vasculature of 8 mice was extensively washed with saline prior to removal of testes; another 8 mice were anesthetized only (controls). The testes were suspended and injected into recipient mice for bioassay of P388 cells, as described in "Materials and Methods."

Treatment	Days of death	Median
Vascular washed	10,11,11,13,13,14,21,22	13[a]
Controls	9,10,11,11,13,13,14,15	12

[a] Not significantly different from control, $p > 0.05$ (2-sample rank test).

known pharmacological sanctuary), blood (a readily accessible pharmacological space), and testes (pharmacological status essentially unknown). Excellent 1- to 2-log cell kill was achieved in blood with the approximate 10% lethal doses used confirming the ready access of tumor in blood to the agents used. Cells in brain and, to some apparent degree, in testes were responsive to a 10% lethal dose of VCR,[4] an agent known to be active against brain-localized tumors (12). On the other hand, Adriamycin failed to reduce the brain tumor burden but was significantly effective against tumor cells in testes. Therefore, ability to cross classic membrane barriers (such as the brain) does not appear to adequately explain refractoriness of testes to MTX or VCR since Adriamycin (ineffective in brain) was very effective in testes. Thus, 2 cell cycle-specific drugs, MTX and VCR, were relatively ineffective against tumor cells in testes whereas the cell cycle-nonspecific drug Adriamycin was effective. These results suggest that the refractoriness relates to the relatively nonproliferating status of the tumor cells in testes of animals bearing advanced P388 leukemia (see Chart 1). Therefore, we examined the chemotherapeutic response of P388 cells in the testes of mice having a lower tumor burden, *i.e.*, early disease (Table 4). In this model, tumor cells in testes were responsive to both MTX and VCR; 1- to 2-log cell kill was achieved on Days 3 and 5. Testes are not unique in this regard as can be observed in the data given in Table 5. As the extent of disease advanced, the response of tumor cells in spleen also diminished when MTX was administered (Table 5).

Attempts to select for a P388 cell line that would "home" to testes by serial passage of the testes of animals bearing P388 leukemia for several months [analogous to the method used by Nicolson for other tumors (11)] were only marginally successful (5). That is, after 8 weeks, there was evidence of enhanced infiltration by the selected cell line; however, after 5 months of

Table 3

Comparative chemotherapeutic effects in brain, blood, and testes of mice bearing advanced P388 leukemia

P388 cells (10^6) were implanted i.p. into $BD2F_1$ mice. Seven days later, the drugs shown were administered i.p., and the brain, blood, and testes were removed for bioassay 4 hr later. The organs were then suspended and implanted into recipient animals. The results shown are the median days of death for recipient animals.

Treatment	Median day of death		
	Brain	Blood	Testes
Control	19	13	14
MTX, 100 mg/kg		18.5[a]	14.5
VCR, 3 mg/kg	23.5[a]	19[a]	16.5
Adriamycin, 12 mg/kg	18	19[a]	19[a]

[a] Statistically different from control median day of death, $p < 0.05$ (2-sample rank test).

Table 4

Chemotherapeutic response of early P388 testicular leukemia to MTX or VCR

P388 cells (10^5) were implanted i.p. into $BD2F_1$ mice. On Days 3, 5, and 7 thereafter, the animals (8/group) were treated i.p. with MTX (100 mg/kg) or VCR (3 mg/kg). Four hr later, the testes were removed, suspended, and implanted into recipient mice to estimate the P388 cell number by bioassay, as described in "Materials and Methods."

Treatment	Median day of death	
	Control	Treated
Day 3		
VCR	17	24[a,b]
MTX	19	21[c]
Day 5		
VCR	13.5	17.5[a]
MTX	14.5	17[a]
Day 7		
VCR	12	16[a]
MTX	13	12

[a] Significantly different from control median day of death, $p < 0.05$ (2-sample rank test).
[b] Three of 8 drug "cures" (survival of the animal for >35 days after inoculation) median calculated for dying animals only.
[c] Five of 8 drug "cures," significantly different from control (0 of 8), $p < 0.05$ (χ^2 test). Median calculated for dying animals only.

[4] The abbreviations used are: MTX, methotrexate; VCR, vincristine.

Table 5
Chemotherapeutic response of early P388 splenic leukemia to MTX
The spleens of animals treated with MTX (see Table 4) were bioassayed for P388 cells, as described in Table 4 and "Materials and Methods."

Day of Treatment	Median day of death	
	Control	Treated
3	16	19[a,b]
5	13.5	14
7	12	12

[a] Significantly different from control, $p < 0.05$ (2-sample rank test).
[b] Three of 8 drug "cures." Dying animals only used for calculation of median.

further serial passage, the selected and wild-type cells infiltrated testes in a very similar manner (data not shown).

DISCUSSION

The biological mechanism for testicular relapse in childhood leukemia is unknown. Although the existence of a Sertoli cell blood-testis barrier to drugs is clearly established (1, 2, 14, 16), biopsy of children with macroscopic testicular leukemia indicates presence of disease in the interstitial space rather than behind the Sertoli cell barrier (9). We have attempted to use P388 murine leukemia as a model for this disease process. The P388 leukemia would appear logical for this purpose since it is responsive to a number of the chemotherapeutic agents used to treat the human disease (13). In mice bearing P388 leukemia, infiltration of the tumor cells into testes occurs (Chart 1; Table 2). Cells in this organ are unresponsive to the cell cycle specific agents MTX and VCR in advanced disease (Table 3). This refractoriness may be due to the nonproliferating state of the cells rather than indicating a pharmacological sanctuary, however, since tumor cells in testes are responsive to these agents during early disease (Table 4). Further cell cycle kinetic data are required to prove this hypothesis definitively. This refractoriness probably does not relate to unique environmental aspects of the testicular disease since a similar refractoriness to MTX was observed in the spleens of mice bearing advanced P388 leukemia (Table 5).

Although the blood-testis barrier did not exclude drugs in the doses utilized here, this may not be true of lower, more frequent dosing schedules of these same drugs. A recent report by Jackson *et al.* (6) describes use of L1210 cells in $BD2F_1$ mice to examine the pharmacological barrier to cyclophosphamide in murine testes. Using 100 mg of that chemotherapeutic drug per kg on Day 3 following inoculation of 5×10^3 L1210 cells intratesticularly resulted in a shortened survival when compared to inoculation of an equal number of cells i.m. The authors interpreted this finding as indication that the testis can provide a pharmacological sanctuary for leukemic cells with reference to cyclophosphamide. These results, when coupled with our data, suggest that the question of whether testes are a pharmacological sanctuary for mammalian leukemia may not be simply answered. In addition to the presence of an anatomic or Sertoli cell barrier that appears to be variably exclusive to drugs, factors such as tumor growth properties and tumor-drug interaction probably influence the occurrence of isolated testicular relapse.

In summary, the data establish that infiltration of P388 leukemia into testes occurs and that tumor cells in the testes are responsive to at least 3 agents used to treat childhood acute lymphocytic leukemia. Macroscopic evidence of the disease does not occur following i.p. inoculation of the tumor since proliferation of the cells plateaus at only 10^5 to 10^6 cells/testes. Selective cure of the P388 tumor in other tissues of the mouse may allow for more extensive proliferation of the tumor in testes, thereby creating macroscopic disease.

ACKNOWLEDGMENTS

The authors wish to acknowledge the efforts of Rhonda Shannon and Mark Delbello, students at The University of Texas Medical School at Houston who participated in this project during tenure in the summer program for medical students. Also, Barbara Herbert provided expert technical assistance in the performance of many of the bioassays.

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