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Effects of Single Cyclophosphamide Doses on the Kinetics of Thoracic Duct Lymph and Blood Leukocytes in Calves[1]

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SUMMARY

After 5 or 20 mg cyclophosphamide per kg body weight, given once i.v., the output in thoracic duct lymph of small and large lymphoid cells, cells incorporating [^{3}H]thymidine *in vitro*, mitotic cells, pyknotic cells, and/or the number of lymphocytes and neutrophils in peripheral blood were measured in six calves. The median grain count of labeled cells and the DNA content of pyknotic nuclei were determined. After both doses there was an exponential decrease and subsequent recovery of the median grain count. The larger dose caused a temporary cessation of lymphoid cell division, reduced the output of nondividing small lymphoid cells, and probably impaired proliferation of neutrophil precursors. The results suggest that increased cell production during recovery was due to changes in the growth fraction and that feedback mechanisms acting on G_0-G_1 cells controlled the proliferation of lymphoid cells.

INTRODUCTION

CYT,[3] a compound yielding a number of alkylating metabolites after activation by a microsomal enzyme system in the liver (1, 2), has considerable antineoplastic activity against a variety of experimental tumors and human cancers (7) and is one of the most potent immunosuppressive agents (6). Although its effects on humoral and cellular immunity and related inflammatory processes have been investigated extensively (8), little is known of its influence *in vivo* on the kinetics of normal cells in lymphatic tissues. An assessment of these CYT effects appears to be desirable for several reasons: *(a)* cytokinetic events involved in immune responses and related regulatory mechanisms need further elucidation; and *(b)* based on a better knowledge of these events, the therapeutic value of cytotoxic agents may be enhanced, *e.g.*, by dose fractionation, and better antineoplastic or immunosuppressive activity might thus be obtained (4).

For evaluation of the time course and the dose response of cytokinetic events in lymphoid and nonlymphoid cell renewal systems following administration of cytotoxic drugs, repeated sampling at short time intervals is required. Calves with thoracic duct-jugular vein shunts allow for such sampling of blood and lymph (3). Well-documented kinetic studies of normal cells in lymphatic tissues of this species are available (12). The influence of a single small or larger dose of CYT given i.v. on the kinetics of TDL cells and WBC of calves is presented. Preliminary results have been published (14).

MATERIALS AND METHODS

Six healthy calves were used (Table 1). A thoracic duct jugular vein shunt was established in all but 1 animal, and TDL flow was continuously monitored. [^{3}H]TdR (Schwarz/Mann, Orangeburg, N. Y.; specific activity, 1.9 Ci/mmole; 31 mCi/liter in 0.9% NaCl solution; dose, 0.15 mCi/kg body weight; infusion rate, approximately 0.9 mCi/hr) was infused for approximately 16 hr. One hr after the termination of the [^{3}H]TdR infusion, CYT (Mead Johnson Laboratories, Evansville, Ind.; dose, 20 or 5 mg/kg body weight; concentration, 20 g/liter in 0.9% NaCl solution) was infused over a 15-min period (~8 ml/min). Lymph and peripheral blood samples were obtained at various intervals. Absolute numbers of neutrophils and mononuclear cells in the blood were determined. The output of large and small TDL cells was assessed as described previously (10). Aliquots of each lymph sample were incubated for 30 min at 38° with [^{3}H]TdR (Schwarz/Mann; specific activity, 6.7 Ci/mmole; concentration, 1 mCi/ml) at a concentration of 1 μCi/ml. After incubation the lymph was centrifuged at 180 × *g* for 5 min at 4°. Following decantation of the supernatant, cells were processed and evaluated as described previously (14). Radioautographs were exposed to show *in vitro* but not *in vivo* incorporation of [^{3}H]TdR. Results of cytophotometric DNA determinations on TDL cells with pyknotic nuclei were used to calculate the data summarized in Table 3 (pyknotic nuclei with a DNA content higher than the mean + 2 S.D. nuclear DNA content of diploid unlabeled TDL cells were considered to have a DNA content greater than 2n). All results were normalized and expressed as a percentage of the mean pre-CYT values (Table 2).

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[3] The abbreviations used are: CYT, cyclophosphamide; TDL, thoracic duct lymph; TdR, thymidine; 2n, diploid nuclear DNA content.

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RESULTS

The effect of the single i.v. CYT dose on TDL and blood cells can be divided into 4 phases (Charts 1 and 2; Table 3).

Phase 1 is an initial phase of damage, lasting 8 to 10 hr, characterized by: *(a)* a dose-dependent decline of the output of large TDL cells (Chart 1*B*); *(b)* a dose-dependent, exponential decline of the median grain count of TDL cells labeled *in vitro* (Chart 2*C*); *(c)* a dose-dependent decline of the output of TDL cells incorporating [^{3}H]TdR *in vitro* (Chart 2*A*); *(d)* a decline inversely related to the dose of the output of TDL cells in mitosis (Chart 2*B*); and *(e)* an increased output of TDL cells with pyknotic nuclei (Chart 2*D*; Table 3).

Phase 2 is an intermediate, postdamage-prerecovery phase, lasting at least 10 hr, characterized by: *(a)* a dose-dependent recovery of the median grain count (Chart 2*C*). By use of a normal time scale, it became apparent that this recovery most probably was exponential; *(b)* a dose-dependent variation, after 5 and 20 mg CYT per kg body weight, of the output of TDL cells incorporating [^{3}H]TdR *in vitro* (Chart 2*A*) and TDL cells in mitosis (Chart 2*B*); *(c)* an increased output of TDL cells with pyknotic nuclei after 20 mg CYT per kg body weight (Chart 2*D*; Table 3); and *(d)* a beginning decline of the neutrophil number in the blood (Chart 1*D*).

Phase 3 is the phase of recovery, starting on Day 2 and lasting for at least 4 days, characterized by: *(a)* an increasing output of large TDL cells with a dose-dependent overshoot (Chart 1*B*); *(b)* a decreased output of small TDL cells after 20 mg CYT per kg body weight (Chart 1*A*); *(c)* a return of the median grain count to predrug levels after 5 mg CYT per kg body weight and a rise of the median grain count to a peak slightly above predrug levels after 20 mg CYT per kg body weight (Chart 2*C*); *(d)* an increasing output of TDL cells incorporating [^{3}H]TdR *in vitro* and TDL cells in mitosis with a dose-dependent overshoot (Chart 2, *A* and *B*); *(e)* a reduced output of TDL cells with pyknotic nuclei (Chart 2*D*; Table 3); and *(f)* an increasing number of blood neutrophils after 5 mg CYT per kg body weight and a decreasing neutrophil number after 20 mg CYT per kg body weight (Chart 1*D*).

Phase 4 is a phase of stabilization, characterized by: *(a)* a normalization of the output of small and large TDL cells (Chart 1, *A* and *B*); *(b)* a median grain count in the predrug range (Chart 2*C*); *(c)* after the overshoot, a declining output of TDL cells incorporating [^{3}H]TdR *in vitro* and TDL cells in mitosis (Chart 2, *A* and *B*); *(d)* a high output of TDL cells with pyknotic nuclei after 5 mg CYT per kg body weight but a normal output of TDL cells with pyknotic nuclei after 20 mg CYT per kg body weight (Chart 2*D*; Table 3); and *(e)* a normalization of the blood lymphocyte and neutrophil number after the smaller dose of CYT and a rising number of blood lymphocytes and neutrophils after the larger dose of CYT (Chart 1, *C* and *D*).

Table 1
Details of CYT studies

No.	Strain	Calf Wt (kg)	Calf Age (mos.)	CYT (mg/kg)	Samples Lymph	Samples Blood
1	Holstein	75	3.5	5	Yes	Yes
2	Holstein	111	6.3	5	Yes	Yes
3	Holstein	78	4.75	20	Yes	Yes
4	Holstein	80	4.75	20	Yes	Yes
5	Holstein	134	5.5	20	Yes	Yes
6	Holstein	165	7	20	No	Yes

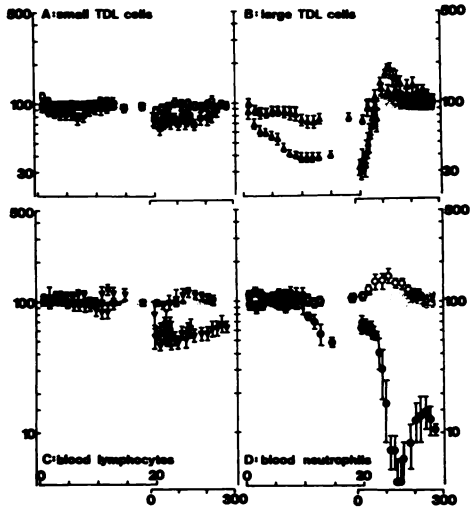

Chart 1. *Abscissa*, time in hr after a single i.v. injection of CYT; *ordinate*, percentage of initial value; *open symbols*, running mean ± S.E. of calves treated with 5 mg CYT per kg body weight; *closed symbols*, running mean ± S.E. of calves treated with 20 mg CYT per kg body weight; *hatched area*, mean ± S.E. of initial values. *A*, output of small TDL cells; *B*, output of large TDL cells; *C*, number of lymphocytes in peripheral blood; *D*, number of neutrophils in peripheral blood.

Table 2
Mean pre-CYT values

Calf	Output (× 10^6/kg body wt/hr) Small TDL cells	Large TDL cells	TDL cells incorporating [^{3}H]TdR *in vitro*	TDL cells in mitosis	TDL cells with pyknotic nuclei	Median grain count/labeled TDL cell	No. μl Blood lymphocytes	Blood neutrophils
1	132 ± 9[a]	15.2 ± 2.7	6.6 ± 0.7	0.14 ± 0.05	0.14 ± 0.06	51 ± 5	5940 ± 320	4201 ± 294
2	176 ± 12	17 ± 2.4	6.9 ± 1.2	0.13 ± 0.04	0.29 ± 0.14	50 ± 3	5468 ± 489	8325 ± 814
3	65 ± 6	5.2 ± 0.5	1.4 ± 0.2	0.04 ± 0.01	0.07 ± 0.01	35 ± 1	4613 ± 308	5407 ± 702
4	124 ± 13	24 ± 6.9	4 ± 0.4	0.04 ± 0.03	0.3 ± 0.02	32 ± 2	6876 ± 560	9164 ± 1254
5	85 ± 9	7.3 ± 0.9	3.2[b]	0.11[b]	0.35[b]	59[b]	3491 ± 208	4819 ± 1393
6							4793 ± 210	3100 ± 395

[a] Mean ± S.E.
[b] Only 1 pretreatment value available.

DISCUSSION

The interpretation of the present results is largely based on the assumptions that *(a)* CYT did not selectively alter the rate of influx or efflux of proliferating *versus* nonproliferating cells in TDL and peripheral blood; *(b)* CYT effects on TDL and blood cells reflected alterations in lymphopoietic and hemopoietic tissues; and *(c)* [^{3}H]TdR toxicity due to *in vivo* labeling was negligible.

Effects of CYT on TDL Cells

Median Grain Count of TDL Cells Incorporating [^{3}H]TdR. The results shown in Chart 2*C* indicate that both doses of CYT induced a progressive inhibition of the DNA synthesis rate, assuming that the median grain count reflects DNA synthesis. The extent but not the duration of this initial inhibition was dose dependent. These observations fit well with pharmacological investigations. Brock (1) reported that the maximum alkylating activity in serum of mice, rats, and dogs was reached within 15 to 30 min after administration of large doses of CYT (125 to 1000 mg/kg body weight). The alkylating activity remained at maximum values for 1.5 to 2 hr, was about 25 to 50% of the maximum value at 4 hr, and was no longer detectable at 8 hr, at least after 125 mg/kg body weight. In man a slower activation rate in the liver and a longer persistence of the alkylating activity in the blood were found. After 30 mg CYT per kg body weight, maximum alkylating activities were reached within 1 to 2 hr and persisted up to 8 hr after drug administration (2).

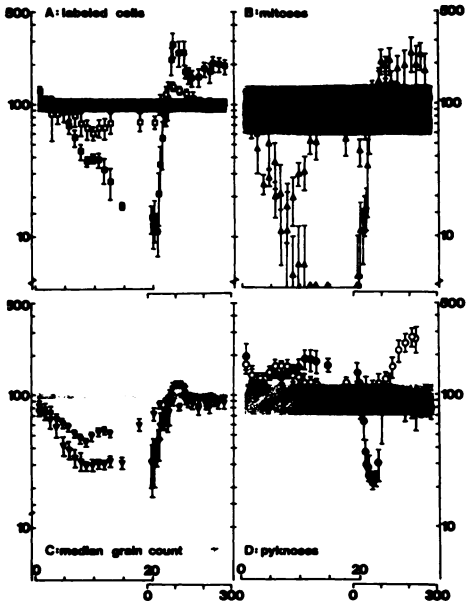

Chart 2. *Abscissa*, time in hr after a single i.v. injection of CYT; *ordinate*, percentage of initial value; *open symbols*, running mean ± S.E. of calves treated with 5 mg CYT per kg body weight; *closed symbols*, running mean ± S.E. of calves treated with 20 mg CYT per kg body weight; *hatched area*, mean ± S.E. of initial values. *A*, output of TDL cells labeled *in vitro*; *B*, output of TDL cells in mitosis; *C*, median grain count per TDL cell labeled *in vitro*; *D*, output of TDL cells with pyknotic nuclei.

The rate at which the median grain count reached control levels was also dose dependent. Evidence for the existence of repair mechanisms after CYT-induced alkylation has been published (5), but it is not known whether CYT-damaged cells can repair DNA during all parts of the cell cycle or whether this is restricted to the S phase (9). Our results suggest that repair mechanisms might have been active for about 1 day after the smaller dose of CYT and for more than 1 day after the larger dose of CYT.

The observations summarized in Chart 2*C* (little or no overshoot of the median grain count) and Chart 2*A* (significant rise in the output of labeled TDL cells above predrug values) demonstrate that recovery processes were associated with changes in the growth fraction in addition to changes in the DNA-synthetic rate. Earlier findings indicated that the cell cycle phase transit times did not change during recovery following extracorporeal irradiation of circulating blood (12).

Output of TDL Cells Incorporating [^{3}H]TdR and Output of TDL Cells in Mitosis. Only a small fraction of large TDL cells incorporated [^{3}H]TdR *in vitro* (Table 2). Since in normal calves G_2 transit time is approximately 30 to 40 min and DNA synthesis time is 3.5 hr (12), the large TDL cells not labeled *in vitro* could not all be G_2 cells but probably represented cells in G_0-G_1 or in late G_1. During the 1st 20 hr after 20 mg CYT per kg body weight, the output of TDL cells with pyknotic nuclei in the diploid range increased steadily (Table 3). Our results therefore suggest that the larger dose of CYT affected DNA-synthesizing cells as well as non-S cells, most probably cells in G_0-G_1.

After 20 mg CYT per kg body weight, the output of pyknotic nuclei with a DNA content greater than 2n increased but was not important in absolute numbers (Table 3). If only

Table 3

Mean output of TDL cells with pyknotic nuclei of 2n or more than 2n nuclear DNA content

Time after CYT (hr)	Calves 1 and 2 (5 mg CYT/kg body wt)			Calves 3, 4, and 5 (20 mg CYT/kg body wt)		
	Output (× 10^6/kg hr) of pyknotic nuclei with DNA of		No. of pyknotic nuclei measured	Output (× 10^6/kg/hr) of pyknotic nuclei with DNA of		No. of pyknotic nuclei measured
	2n	>2n		2n	>2n	
0	0.196 ± 0.065	0.006 ± 0.002	62	0.205 ± 0.060	0.003 ± 0.001	124
0.25-5	0.275 ± 0.030	0.019 ± 0.002	264	0.271 ± 0.060	0.026 ± 0.006	488
6-10	0.273 ± 0.044	0.041 ± 0.007	153	0.327 ± 0.059	0.038 ± 0.0007	370
11-20	0.241 ± 0.043	0.014 ± 0.003	113	0.603 ± 0.066	0.014 ± 0.002	363
21-100	0.187 ± 0.019	0.008 ± 0.001	95	0.119 ± 0.028	0.001 ± 0.0002	296
101-200	0.336 ± 0.047	0.017 ± 0.002	167	0.316 ± 0.047	0.010 ± 0.002	355
>200	0.426 ± 0.017	0.022 ± 0.001	102	0.267 ± 0.037	0.007 ± 0.001	159

" Mean ± S.E.

a small fraction of S cells was lysed after the larger dose of CYT, it is difficult to explain the declining output of cells incorporating [^{3}H]TdR (Chart 2*A*) without postulating that the influx of cells into S decreased. It therefore appears that, after 5 mg CYT per kg body weight, damaged cells continued to divide despite the fact that their progression through S and possibly other cell cycle phases was delayed, while there was an almost complete depletion of the proliferating compartment after the larger dose. One might speculate, furthermore, that the increased output of TDL cells with pyknotic nuclei observed during the phase of stabilization after 5 mg CYT per kg body weight was due to a late lysis of CYT-damaged cells.

The results presented in Chart 2, *A* and *B*, finally demonstrate that the patterns of recovery and overshoot after the 2 doses of CYT used were similar in time but different in magnitude. This could indicate that recovery was initiated at the same time but was amplified in a different way.

Output of TDL Cells with Pyknotic Nuclei. Prior to CYT injection, the output of TDL cells with pyknotic nuclei (Table 3) amounted to approximately 3 to 7% of the output of [^{3}H]TdR-incorporating cells (Table 2). Only 1.5 to 3% of the pyknotic nuclei had a DNA content greater than 2n (Table 3).

Following CYT at least 4 factors might have influenced the output of TDL cells with pyknotic nuclei (Table 3): *(a)* lysis of cells in S and/or G_2 during the phase of damage after 5 and 20 mg CYT per kg body weight; *(b)* lysis of cells in G_0-G_1 during the phase of damage and the intermediate phase after the larger dose of CYT; *(c)* late lysis of damaged cells during the phase of stabilization after the smaller dose of CYT; and *(d)* recovery. The inverse relation between the output of [^{3}H]TdR incorporating TDL cells or of TDL cells in mitosis and the output of TDL cells with pyknotic nuclei during the recovery phase (Chart 2, *A*, *B*, and *D*) indicated that the number of proliferating TDL cells was proportional to the rate of cell lysis in the G_0-G_1 compartment. These results suggest that feedback mechanisms control the proliferation of lymphoid cells. These mechanisms may act on G_0-G_1 cells and destroy the excess of newly formed cells, thus preventing an overgrowth of a stimulated clone of antigen-responsive cells.

Output of Small and Large TDL Cells. The time sequence of the recovery of TDL cell output, *i.e.*, first of the large and then of the small TDL cells (mainly long-lived T-cells), suggests that the loss of the latter was compensated for by an influx of cells resulting from the division of large TDL cells, *i.e.*, mainly immunologically committed "lymphoblasts."

Effects of CYT on Blood Cells

The marked effect of 20 mg CYT per kg body weight on the number of blood neutrophils is reminiscent of changes seen after whole-body irradiation. The overshoot in the number of neutrophils following 5 mg CYT per kg body weight may be tentatively related to regeneration after a slight initial depletion and/or shifts within compartments of distribution. The more profound depression and the slow recovery of mononuclear cells in the blood, *i.e.*, mainly lymphocytes, compared to the early recovery of the output of small TDL cells indicate that CYT also may affect mechanisms controlling the production of B-cells.

Empirically, continuous immunosuppressive therapy (*i.e.*, daily drug doses) is given to recipients of organ transplants, while intermittent chemotherapy is often used for treatment of patients with malignant disease. One might therefore speculate that a continuous but moderate interference with cell proliferation in lymphatic tissues is essential for immunosuppression. Conversely, massive side effects of an antineoplastic therapy such as an almost complete temporary depletion of the proliferative compartment and even a lysis of cells in G_0-G_1 are acceptable, provided that sufficient time for recovery is available between drug pulses. In humans and calves the majority of dividing TDL cells were found to have an extremely short DNA synthesis time of 7 and 3.5 hr, respectively, and a cell cycle time of 9 and 5.5 to 6 hr, respectively (12, 13). Since cell division in lymphatic tissue is more rapid than is division of bone marrow cells or of neoplastic cells (11), it is conceivable that an interference with DNA synthesis in lymphoid cells exerts more pronounced effects than in other cell types. This might explain why a continuous administration of cytostatic drugs in small daily doses is useful for immunosuppression. If the time required for recovery is related to generation time, intermittent drug pulses might allow for recovery of normal but not of neoplastic cell systems.

An assessment of cytokinetic drug effects as used in the present study is time consuming. Our results indicate that TDL cell output data and blood leukocyte counts might suffice for a rapid analysis of the effect of single or repeated administration of 1 drug or drug combinations.

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