Short Communication

Resistance of human T-CFU<sub>e</sub>s to activated cyclophosphamamide: A feature common with critical marrow stem cells?

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It has been known for over a decade that the alkylating agent Cyclophosphamide (CY) has a differential effect on the immune system, exerting greater toxicity against B than T lymphocytes (Poulet & Turk, 1972; Stockman et al., 1983; Turk et al., 1972). CY selectively depletes the B-cell rich peripheral splenic pulp and exerts a greater inhibition of stimulation of B-cells by pokeweed mitogen than T cells by phytohaemagglutinin (PHA; Stockman et al., 1973). This differential effect is found even at the earliest stage of life; CY given to newly hatched chicks leads to a severe depression of antibody synthesis in adult chickens while T cell functions are virtually unimpaired (Lerman & Weidanz, 1970). Recent experiments have indicated that part of these effects are due to inhibition of T suppressor cell activity (Turk & Poulter, 1982). The exact mechanism by which this selectivity is exerted could not previously be studied in vitro since CY requires activation in the liver (Hill, 1975). We show here that the newly available active form of CY, phosphoramid mustard (PM, Fenselau et al., 1977) is virtually inert against colony-forming T cells in tissue culture at levels highly cytotoxic to other cells. Cultured human T lymphoma cells retain a significant amount of this resistance; recent work in other laboratories suggests that the same is true for important human marrow-repopulating stem cells (see below).

To determine the effect of PM on T lymphocytes we used the quantitative colony assay technique we developed for examining the proliferation-dependency of cytotoxicity in vitro. In this assay (Byfield & Calabro-Jones 1981) purified preparations of mononuclear cells from human blood are treated with PHA and the number of colonies (T-CFU<sub>e</sub>, T cell colony-forming units in culture) are subsequently counted. The addition of active cytotoxic drugs inhibits this colony formation and yields survival curves similar to those found with permanently explanted lines (Byfield & Calabro-Jones, 1981). In the experiments reported here we studied the effect of PM exposure both prior to and after the induction of proliferation by PHA, using an identical protocol employed by us with other alkylating agents (Byfield & Calabro-Jones, 1981).

We have previously shown that the sequence of exposure to PHA and drug influences the shape of the resultant survival curves, the effect being a function of solubility characteristics of each agent (Byfield & Calabro-Jones, 1981). The survival curves for log phase WI-L2 cells (a malignant human B cell line) and CEM cells (a human T lymphoma line) exposed to PM were also obtained. In vitro activity of the PM was confirmed using HeLa cells cultured by our standard colony-counting method (Byfield et al., 1981). (The PM was kindly synthesized by Dr. R. Struck.)

We found (Figure 1) that the population of human T cells that form colonies in semi-solid medium after PHA stimulation were virtually unaffected by PM at concentrations that reduced Hela cell survival by almost 3 logs. All other malignant lines we have studied thus far, both rodent and human (Byfield et al., 1981; Murnane et al., 1980), were also quite sensitive to PM. Unlike other alkylating agents (cf. Byfield & Calabro-Jones, 1981), the time of exposure to PHA made no difference (Figure 1). The colony-forming human T cells were almost totally resistant to PM whether or not they were actively proliferating at the time of PM exposure.

The resistance to PM of some human T cells carried over into a human malignant T cell line (CEM cells) when compared to a B cell line (WI-L2 line, Figure 2). In this case resistance was relative rather than almost absolute as in the case of the normal human T CFU<sub>e</sub>s. However, at PM concentrations likely to be encountered in vivo during human lymphoma therapy with CY (about a 1.0 μM peak, cf. Wagner et al., 1977, equal to the lowest PM concentration studied here), this difference is likely to be significant.

The level of resistance of the colony-forming T cells is impressive and seems capable of explaining...
Figure 1 Effect of activated cyclophosphamide (Phosphoramide mustard, PM) on the survival human T cell in vitro colony forming units (T-CFU_{E}). The cells were exposed to PM either before (resting, ●) or after (cycling, ○) PHA exposure. Survival of HeLa cells measured with same batch of PM, ▲.

Figure 2 Survival of malignant human B cells (WIL2) (○) or T cells (CEM) (●) after a 60 min exposure to various concentrations of Phosphoramide mustard.

the results of much of the earlier in vivo data. Since activated CY has been shown to substantially inhibit normal B cell colony formation in vitro (Winklestein, 1982), the degree of resistance seen here for colony-forming T cells appears sufficient to explain the histological manifestations of CY treatment in vivo (Stockman et al., 1973; Turk & Poulter, 1982). Presumably part of the functional manifestations of CY treatment stem from the greatly enhanced ratio of surviving T to B cells.

However, it has been shown by others that some human T cell functions, including mitogenic stimulation of DNA replication can be inhibited by activated CY (Korbling et al., 1982). This suggests that the T cells that form colonies in semi-solid medium after PHA exposure are probably a unique population of the overall mass of T cells (only a small fraction of T cells form such in vitro colonies). Thus the resistance of T cells to CY killing seems not to be universal but rather limited to a select T cell fraction. This differential toxicity may well contribute to the various functional effects seen in vivo.

Finally, we would note that recent experiments on both rodent and human marrow stem cells have also suggested a select and differential toxicity of activated CY. Thus it was initially shown that in vitro treatment of rat bone marrow with 4-hydroperoxycyclophosphamide (a synthetic metabolic precursor of PM) led to elimination of leukaemic cells without seriously impairing the capacity of the treated marrow to repopulate marrow-depleted animals (Sarkis et al., 1980). Similar effects were then shown in humans where CFU_{E} could be totally depleted in culture by activated CY. Nevertheless, patients receiving donor marrow pre-treated with activated CY in vitro showed essentially normal marrow recovery kinetics (Kaizer et al., 1982). These data strongly suggest that true pluripotential marrow stem cells share with the PHA-dependent colony-forming lymphocyte subset a great resistance to killing by activated CY. Clinical data on granulocyte and platelet recovery patterns in patients (with lung cancer) treated with massive doses of CY have also indicated normal marrow recovery kinetics (Smith et al., 1983). Overall, these results by other investigators show clearly that CY exerts a selective toxicity against various marrow clonogenic cells, some pluripotent stem cells apparently being spared while the semi-committed or committed cells that form most of the colonies in vitro are killed. The widespread use of CY against many malignant conditions may therefore be based on this selective sparing of the most important renewal cells in the marrow, a form of selective toxicity not hitherto recognized.

The mechanism of cellular resistance shown by marrow stem cells and the subset of T cells studied
here is not as yet known but physical exclusion of the drug from these cells seems likely. Alternatively, the drug could be inactivated within the cells. We have shown that activated CY induces molecular (excision) repair of CY-induced damage and that these repair processes can be aborted by methylated xanthines (Byfield et al., 1981; Murnane et al., 1980). However, repair-related resistance of the magnitude found here has not previously been reported and seems unlikely to us. Our previous experiments showed that a significant relative resistance to alkylating agents stems from the degree to which cell penetration can be expected (Byfield & Calabro-Jones, 1981). This is especially true for drugs that are taken up by cellular transport processes. In such cases the toxicity exerted by each alkylating agent was a function of the activity of those transport processes. (Byfield & Calabro-Jones, 1981). We believe the most plausible explanation of our results is that PM does not enter the colony-forming T cells to a significant degree; if correct it would presumably hold true for pluripotent marrow stem cells as well. This suggests that either the two cell types are close in the cellular hierarchy of haematolymphogenous differentiation and, as a consequence, share this drug exclusion property or that exclusion (or inactivation) of compounds like PM is a conferred property of some resting stem cells. If this explanation is correct then the synthesis of other agents with useful differential effects based on pre-defined cell-entry characteristics may be possible. Our initial experiments had already suggested the potential existence of anti-cancer agents with this kind of selective toxicity (Byfield & Calabro-Jones).

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