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Patterns of Cell Survival Following Treatment with Antitumor Agents *In Vitro**

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Abstract—*Cell cultures provide a rapid, efficient and economic research system to investigate cell killing effects of antitumor agents. Only the colony-formation technique appears appropriate to determine post-drug treatment cell survival. Utilizing this technique, the lethal effect of 22 antitumor agents on a human lymphoma cell line were determined after brief exposure (1 hr) to the agent. The resulting survival curves corresponded to one of five patterns: simple exponential, biphasic exponential, threshold exponential, exponential plateau and ineffectual. These patterns may be useful in the classification of cell-antitumor drug interactions.*

INTRODUCTION

CELL cultures provide a rapid, efficient and economic system for cytotoxicity screenings of antitumor agents. The main assumption for *in vitro* studies with chemotherapeutic agents is that the survival response of the cultured cells will reflect that of *in vivo* cells once the drug has reached the neoplastic elements [1–4]. Drug-induced cell killing is the result of an interplay between the type, extent and duration of the damaging effect caused by a drug to critical biosynthetic pathways or subcellular structures, and the capacity of living elements to bypass or repair such damage. A lethally damaged cell may not only complete DNA synthesis, but may even divide several times before the entire progeny perishes from the lethal damage inherited from their single ancestor [5–7]. Therefore, dye exclusion tests which measure cell membrane integrity, and tests which determine inhibition of DNA synthesis, under the assumption that a cell which completes DNA synthesis is viable, may grossly under- or over-estimate the killing effectiveness of injurious agents [8, 9]. Hence, for proliferating populations, the lethal effects of an antitumor agent must be defined by its impairment of the reproductive integrity of the individual cells. *In vitro*, this impairment can be assessed by the inability of cells to proliferate indefinitely forming colonies under the appropriate experimental conditions [10]. Applying this method to cell culture systems, dose–response effects can be analyzed quantitatively since the exact concentration of the drug bathing the cells and the duration of exposure is known. The quantitative responses can be used to compare the efficacy of different agents on a given cell type or the activity of a specific drug on different cell classes.

We have conducted extensive cellular pharmacological investigations utilizing inhibition of colony-formation techniques exclusively. This report summarizes our findings on the lethal effects of 22 antitumor agents used to treat cultured human lymphoma cells. Most of the survival data presented in this report has already been published in papers describing the activity of the individual agents [1, 11–23]. The purpose of this article is to provide a general perspective of the sorts of cell-drug interactions that can be observed in *in vitro* cytotoxicity screenings and to demonstrate that the survival of cells treated *in vitro* follows one of 5 patterns which may be used to compare and analyze these interactions.

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MATERIALS AND METHODS

Cell line

For our studies, we used a human immunoglobulin-producing cell line (T_1 cells) derived from the tumorous lymph node of a patient with lymphocytic lymphoma [24]. Monolayer cultures were maintained in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine and antibiotics. Under these conditions, the doubling time of exponentially growing cells was 44 hr and the cell cycle time determined by the pulse-labeled mitosis (PLM) method was 31 hr, the pre-DNA synthesis period (G_1) was 15 hr, the DNA synthesis period (S) was 10 hr, and the post-DNA synthesis period (G_1) was 6 hr (25). The growth fraction, estimated from continuous ^{3}H-TdR-treatment experiments averaged 82%. The proportions of cells in each stage of the cell cycle, as defined by flow cytometry were: $G_{1/0}$ 61%; S, 26%; and G_2 + M, 13% [25].

Drugs

Drug solutions in growth medium were always prepared immediately before an experiment and the pH adjusted, if necessary, to 7.2. Water soluble drugs were first diluted in saline solution. Liquid soluble drugs (i.e., nitrosourea derivatives) were first diluted in pure ethanol or in 20% ethanol-80% propylene glycol. At the concentrations used, solvent alone did not affect the viability of the treated cells.

Cell survival

Stock cultures were harvested, aliquots of 5×10^5 cells were seeded in 60 mm plastic Petri dishes, and incubated in fresh medium at 37°C in a 5% CO_2, in air, atmosphere. After cells reached exponential growth (usually about 48 hr), the medium was decanted and freshly prepared drug solutions in medium were added to the dishes. Following treatment for 1 hr at 37°C, the drug was decanted, cells were washed, harvested as a single cell suspension by previously reported standard procedures [26], and counted with an electronic particle counter (Coulter Counter Model ZBI, Coulter Electronics, Hialeah, Florida). Known aliquots were dispensed into 60 mm Petri dishes so that 50–100 colonies would appear after 21-day incubation in a 5% CO_2-humidified atmosphere at 37°C. The colonies were stained with 2% crystal violet in 95% ethanol and counted with a stereomicroscope. Viability was defined as the ability of single cells to give rise to a colony of ≥ 50 cells. In every experiment, three replicate dishes for each dose point were inoculated and the plating efficiency (PE) of at least six controls was assessed simultaneously. PE was defined as the ratio of the number of colonies to the number of inoculated cells. Control cells were exposed to all mechanical manipulations undergone by the treated cells but without receiving drugs. Surviving fractions for each concentration point were calculated in reference to the control cultures by (PE treated cells)/(PE control). The variance of the surviving fraction was calculated using Finney's formula 2.3 [27], and the standard error was given by the square root of the variance.

RESULTS AND DISCUSSION

To characterize the lethal effects of antitumor drugs we employed the technique of survival analysis which is based on the examination of the individual members of the cell population to whom the measured event, cell death, has not occurred. Results were expressed in the form of survival curves representing the decreasing proportions of surviving clones as the drug concentration increased. The shape of the survival curve is a function of the biological properties of the treated cells and the physicochemical characteristics of the antitumor drug. Therefore, analysis of the shape of the elicited survival curve may provide vital information on the mechanisms leading to cell death and contribute important knowledge for the design of improved therapeutic schemes. Unfortunately, the profusion of factors underlying the form of the survival curve and the limited knowledge about their interrelationship prevents a clear definition of the components involved in determining its shape.

One method, commonly used in radiobiology to analyze the shape of survival curves, is based on target theory, developed by Lea [28] to interpret results of cell survival following treatment with ionizing radiations. Target theory assumes the intracellular existence of a number of distinct, discrete structures or entities engaged in some essential activity necessary to retain reproductive integrity. A fixed number of targets must be inactivated biologically before cell survival is abolished. A fraction of inactivated targets less than this critical total will not lead to reproductive death (sublethal damage) while the sum above this level results in "overkill".

We believe that utilization of some concepts and the nomenclature of target theory can be a useful first approximation to the classification of cell-antitumor drug interactions. This approach has two distinct advantages: (a) It does not require *a priori* knowledge of drug effects at the molecular level or interpretation of the biochemical mechanisms leading to enhancement or decline of the cytotoxic effect; and (b) It provides a frame of reference to derive useful quantitative parameters for efficacy comparisons. For the analysis of drug-induced cytotoxicity, targets can be operationally defined as essential macromolecules or critical biosynthetic pathways, and the levels of damage (lethal and sublethal) envisioned as a reflection of the net intracellular concentration of drug molecules over fixed periods of time.

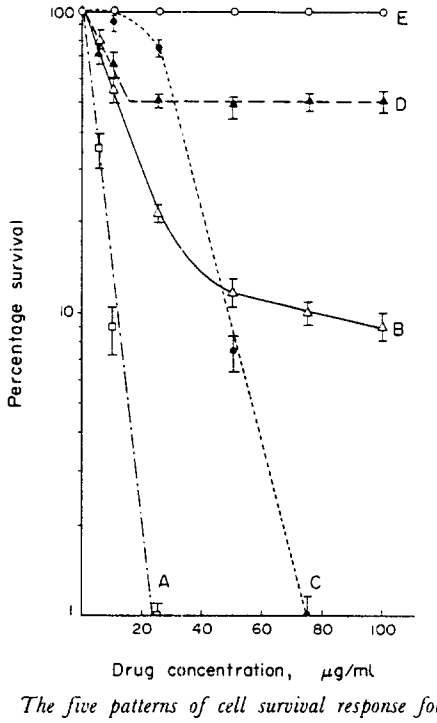

Fig. 1. The five patterns of cell survival response following treatment with antitumor drugs in vitro. *A, cis-diamminedichloroplatinum* (II); *B, bleomycin; C,* 1-(2-*chlorethyl*)-3-*cyclohexyl*-1-*nitrosourea, CCNU; D, prednisolone; E, Arabinofuranosylcytosine, ara-C. Points represent the average of at least two experiments with three replicates per dose point. Bars indicate the standard error.*

All of the survival curves of asynchronous exponentially growing T_1 cells treated for 1 hr with increasing concentrations of a variety of antitumor agents corresponded to one of five patterns (Fig. 1).

1. *Type A: simple exponential*

This pattern was observed for T_1 cells treated with adriamycin, adriamycin–DNA complex, rubidazone, *cis*-diammine-dichloroplatinum (II), 4'demethylepipodophyllotoxin ethylidine glucopyranoside (VP-16), and 3,3 dimethyl triazeno-*o*-benzoic acid (OA) (Table 1). The survival curve was characterized by a logarithmic decrease in the number of surviving cells as a function of increasing drug concentrations. On a semi-logarithmic plot the surviving fraction $= e^{-kd}$, where D is the concentration and k is the constant numerical value of the slope. k can be depicted as $1/D_0$, where D_0 is numerically equal to the concentration that will reduce the surviving cells by 63%. In the context of target theory nomenclature, the D_0 represents the sensitivity of the critical cellular target (macromolecules, biosynthetic pathways, etc.) and can be used to compare the magnitude of the cytotoxic effect of different drugs on a given cell population or the sensitivity of different cell types to a single drug. It must be emphasized that the survival response to antitumor drugs depends not only on the concentration of the agent but also on the time interval of exposure [1, 13, 15, 20, 29–31]. Hence, survival must be expressed not only as a function of dose (C) but also of time (T) or rather the product $C \times T$ (or some mathematical species thereof). This product has been termed the integral dose [19, 32] or when defined more precisely, the exposure dose [31]. The equation can be approximated when the length of *in vitro* treatment is only 1 hr and, hence, the function is reduced to the numerical value of the concentration—i.e. $D_0 = ?$ μg/ml, 1 hr). For varying exposure times, i.e., 30 min, 2 hr, 4 hr, etc. the D_0's cannot be compared unless the appropriate formulation for the time component is introduced [31].

2. *Type B: biphasic exponential*

This survival pattern was noted after treatment with bleomycin, camptothecin sodium, and *m*-[di-(2-chloroethyl)-amino]-L-phenylalanine (peptichemio) (Table 1). The curve was also characterized by a logarithmic decrease in survival as the drug concentration increased. However, it consisted of two parts: an initially steep slope followed by a much shallower slope. By analogy with the preceding pattern this curve could be adequately described by the D_0 corresponding to each segment of the curve (sometimes achieved only by extrapolation) and by the concentration after which the killing rate changes (inflexion point). The inflexion point (I.P. = ? μg/ml, 1 hr) indicates the concentration which

Table 1. Parameters of survival patterns of T_1 cells treated with various antitumor agents

Type A		Type B				Type C			Type D				Type E	
Drug	D_o*	Drug	D_{o1}*	D_{o2}*	I.P.*	Drug	D_o*	D_q*	Drug	D_o*	I.P.*	P_s(%)	Drug	C_{max}
Adriamycin	0.15	Bleomycin	17	140	37	L-Phenylalanine mustard	1.5	0.5	Methotrexate	52	10	82	Arabinofuranosylcytosine	5000
Adriamycin–DNA complex	0.5	Camptothecin sodium	20	226	10	1-Propanol 3,3′ iminodimethanesulfonate (ester) HCl (Yoshi 864)	12	48	Hydroxyurea	1400	400	75	5-Fluorouracil	5000
Rubidazone	0.5													
Cis-diammine-dichloroplatinum (II)	5.5	*m*-[di-(2-chloroethyl)-amino]-L-phenylalanine	1.0	2.5	3.2	1,3-Bis (2-chloroethyl)-1-nitrosourea (BCNU)	17	17	0^2, 2′-Cyclocytidine	95	32	70	Pyrimidine deoxiribose	5000
									Prednisolone	21	10	50	N_1-2′-furanidyl-5-fluorouracil	
4′ Demethyl-pipodophyllotoxin ethylidine glucoside (VP-16-213)	3.0					1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)	11.5	22						
3,3 Dimethyl triazeno-*o*-benzoic acid	18.5					1-(2-Chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU)	19.5	25						
						Cyclohexanecarboxylic acid, 4-(3-[2-chloroethyl]-3-nitrosoureido), *cis*	20	20						

*All units are in μg/ml, 1 hr.

eradicates all of the more sensitive cells from the population. The shape of such curves can be explained by two separate mechanisms: (1) The drug may kill cells in a given stage of the cell cycle with an efficacy several fold greater (first slope) than that exerted on cells in other stages (second slope); or (2) Distinct cell populations, in terms of drug sensitivity, coexist within the seemingly homogenous cell line and the biphasic curve results from differential killing of these two populations regardless of their position in the cycle. These two different possibilities can be distinguished in experiments that use synchronized cells; the biphasic curve should disappear if mode 1 is in operation. It is notable that for both bleomycin and camptothecin sodium, our results supported the second mode [14, 16].

3. *Type C: threshold exponential*

For certain agents, notably radiomimetic drugs (Table 1), the survival response was characterized by the so-called threshold or type C curve [33] where at low concentrations no significant decrease of survival is observed, but higher concentrations produce an exponential killing effect. The linear exponential curve defined by the survival at high concentrations does not extrapolate to 1 (or 100% when the ordinate is expressed as per cent survival) when the concentration is 0 and thus the curve is composed by a shoulder region and a linear part. In the context of target theory nomenclature, the shoulder reflects the capacity of the targets to absorb damage without expressing a cellular lethal effect or inactivation of an insufficient number of targets. This results from the capacity of cells to accumulate sublethal damage but does not imply cellular capacity to repair such damage [19, 23]. The extent of the shoulder can be quantified by extrapolating the linear part of the curve to the abscissa and defining the "quasi-threshold" concentration (D_o) [34] applying the same time-exposure factor considerations previously discussed for the D_o [31]. Thus, Type C curves can be compared on the basis of their D_q (1 hr) and their D_o (1 hr).

4. *Type D: exponential plateau*

This response is typical of cell cycle stage sensitive antitumor agents, usually antimetabolites (Table 1). These drugs produce an initially exponential decrease in survival. Survival reaches a plateau when all sensitive cells are sterilized and subsequent increments in drug concentration fail to augment the killing effect. These curves can be characterized by the D_o (1 hr) of the initial part of the curve, (obtained by extrapolation); the I.P. (1 hr) between the exponential and plateau segments of the curve; and the percentage survivors (P_s, 1 hr) attained at plateau. For certain drugs, P_s may be reached at levels above those calculated from proportions of cells in the sensitive stage. For instance, a $P_s = 82\%$, 1 hr, was noted for methotrexate, an S phase sensitive agent. Yet the proportion of T_1 cells in S phase measured by both autoradiography and flow cytometry averaged 26%. Thus, 30% of cells in S phase were unaffected even by high concentrations of methotrexate. This result suggests that not all of the cells in a given stage, purportedly sensitive to a given agent, are necessarily candidates for sterilization by this drug.

5. *Type E: ineffectual*

Type E survival, where concentrations as high as 5 mg/ml failed to elicit any killing effect, was noted after treatment for 1 hr with arabinofuranosylcytosine (ara-C); 5-fluorouracil; and pyrimidine-deoxyribose N_1-2′-furanidyl-5-fluorouracil (Ftorafur). No quantitative parameter can be used to describe type E survival curves. However, the magnitude of the resistance can be indicated by presenting the largest concentration that failed to elicit *in vitro* cell killing ($C_{max} = ?$ μl, 1 hr). Drugs that inhibit DNA synthesis kill cells by the mechanism of "unbalanced growth" [35, 36]. To achieve lethality by this mechanism, inhibition of DNA synthesis must be maintained for a period longer than the generation time [37], lest the cells resume their growth, synchronized but capable of unlimited proliferation. For drugs which bind irreversibly to their target enzymes, degrade slowly, and are exposed to cells which have a limited capacity to synthesize new enzymes, continuous DNA inhibition may be achieved even after brief exposure to the drug. If one or more of these conditions are not satisfied, lethality can only be obtained if the cells are continuously incubated with the antitumor agents. Thus, T_1 cells incubated for extended periods with arabinofuranosyl–cytosine readily demonstrated considerable decrements in cell survival [18].

Although the above described survival patterns characterize the lethal effects of most antitumor drugs and provide an operational framework to derive quantifiable parameters for comparing their efficacy, it would be

premature to predict that they may have an immediate clinical application. Whether the proposed classification has relevance in the design of chemotherapeutic protocols must await appropriate clinical investigation, and be used in conjunction with other pertinent factors such as pharmacokinetics, cell population growth kinetics, etc. Yet, on a first approximation, the following guidelines may apply: (a) For type A survival curves, the drugs in the group appear potentially effective in reducing tumor burden singly and independent of scheduling; (b) Drugs in the type B survival curve group mimic, in part, the behavior of type A drugs. Yet, a considerable fraction of cells may be less sensitive to the agent and best results may be expected by utilizing these drugs in combination; (c) For drugs with type C survival responses, low concentrations may yield clinically irrelevant killing effects; superior results may be observed by rapid escalation of the employed dosages; (d) All drugs of the type D survival curve group appear cell cycle stage sensitive. Hence, judicious scheduling or combination with other agents should provide the best results for these agents; (e) Drugs in group E are marginally effective in a "push" modality and best results can be anticipated by administration via continuous intravenous infusion.

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