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Immunology

Kinetics of cell death and disintegration in human lymphocyte cultures

(proliferation kinetics/DNA release/phytohemagglutinin/trypan blue)

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ABSTRACT **In order to quantitate lymphocyte proliferative responses, we explored the role of cell death in the kinetics of phytohemagglutinin-stimulated cultures. Unless the disintegration time (t_{DIS}) of nonviable lymphocytes in culture is known, the *rate* of cell death cannot be calculated. To obtain t_{DIS}, we determined the time interval between total and viable cell population decay after various killing events. Two subpopulations of lymphocytes were observed, the major (80%) with a mean (± SEM) t_{DIS} of 16 ± 2 hr and the minor (20%) with a t_{DIS} of 45 ± 7 hr. Kinetic balance sheets were constructed predicting total culture DNA content (cells plus medium), as calculated both from proliferation rates and from observed death and disintegration rates. In an experiment characterized by extensive cell death, the two tallies were well-matched when the above data were utilized. The large discrepancy between predicted and observed DNA contents of the medium indicates that the DNA of disintegrated lymphocytes is extensively degraded. We conclude that cell death explains proliferation deficits in stimulated lymphocyte cultures. Our approach to quantitation of cell death may have general applicability to kinetic studies of cultured cells.**

In kinetic studies of cultured cell proliferation, the rate of cell death is particularly difficult to quantify and yet is quite important, because it may affect results to a great extent. Cell viability is usually assayed by simple tests such as trypan blue exclusion (1), giving only the instantaneous number of dead cells. Nonviable cell *counts* can be utilized to calculate the death *rate* only if the disintegration time (t_{DIS}) of a nonviable cell is known.

Our interest in quantitation of cell death was stimulated by the observations made by ourselves and others (2–8) that in phytohemagglutinin (PHA)-stimulated lymphocyte cultures, at widely used cell concentrations (2×10^6 lymphocytes per ml), an increase in cell number or DNA content is most often not demonstrable, in spite of considerable thymidine (Td) incorporation and mitotic activity, raising the question of the fate of newly synthesized DNA. It has been demonstrated that after mitogen stimulation of lymphocytes a major portion of the newly synthesized DNA is eventually found in the culture medium (5, 9, 10). This DNA contains specific, apparently amplified sequences that may be present in 3- to 4-fold greater amounts in phytohemagglutinin (PHA)-stimulated than in noncycling human lymphocytes (11, 12). Because cell death was not accurately quantified in these studies (2–12), it was of interest to evaluate its overall importance in lymphocyte cultures and to determine the fate of DNA in dead cells.

Abbreviations: t_{DIS}, disintegration time; PHA, phytohemagglutinin; AraC, cytosine arabinonucleoside; β_S, lymphocyte population with a short disintegration time; β_L, lymphocyte population with a long disintegration time.

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MATERIALS AND METHODS

Human lymphocytes from normal volunteer donors were purified (>95%) and cultured according to our published methods (13, 14). Weakly agglutinating, purified PHA (Wellcome Reagents Ltd., Beckenham, England) was added to bulk cultures containing 2 to 33×10^5 cells per ml to a final concentration of 3 μg/ml, which gave optimal stimulation. Cultures were pulsed with [^{3}H]Td, 2 μCi/ml (6 Ci/mmol) to assess incorporation into acid-precipitable material and autoradiographic labeling indices (15). The DNA contents of cell pellets and culture medium were assayed fluorometrically with diaminobenzoic acid after acid precipitation and extractions with alcohol and ether (16).

Enumeration of Living and Dead Cells. The total cell number was determined in quadruplicate in a hemacytometer. The percentage of cells with a blue nucleus after incubation in 0.1% trypan blue for 5 min at room temperature was determined as the mean of quadruplicate counts of 100 cells each. Only those stained lymphocytes that had a morphology sufficiently unaltered for them to be included in the total cell count were scored as dead, i.e., blue-stained debris was ignored. Alternatively, viable (Pronase resistant) and dead cell nuclei were counted with a Coulter model D electronic particle counter according to Stewart *et al.* (7), which gave values differing by no more than 10% from results obtained by hemacytometer counts.

Determination of t_{DIS} of Dead Cells by Population Decay after a Lethal Intervention. The principle of the method is that the time interval between the decay curves of live and dead cells defines the t_{DIS}. Experimental observations were begun 3 or 6 days after initiating cultures, at which time the cultures were exposed to a killing intervention consisting of 3000 rads in 10 min from a ^{60}Co source or incubation for 1 hr in 0.05 M KCN/0.1 M potassium phosphate, pH 7.3 at 37°. Colcemid (0.2 μg/ml) and 50 μM cytosine arabinonucleoside (AraC) were added to prevent surviving cells from dividing and increasing cell counts. These two blocking agents caused extensive death in proliferating cultures and a lower mortality in unstimulated cultures. After the various treatments, the lymphocytes were distributed to replicate tubes and reincubated at 37°. Paired cultures were then collected at regular intervals, agitated on a vortex mixer to break up clumps, and counted.

RESULTS

Measurement of sensitivity to killing events and t_{DIS} by population decay

In the population decay analysis (Fig. 1), irradiated cells that are dead at time t_1 will have disintegrated at some later time t_2, when the total cell number (alive plus dead) will represent only the cells that were viable at time t_1. The interval $t_2 - t_1$

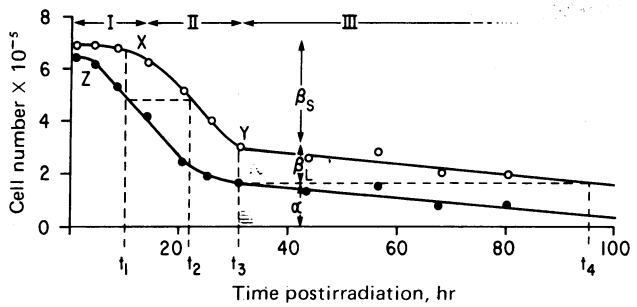

FIG. 1. Population decay analysis. A PHA-stimulated culture initiated at 2×10^5 lymphocytes per ml was irradiated on day 6 and cell counts were obtained by electronic particle counting. O, Total (viable plus dead) cell count; ●, viable cell count. $\alpha = 0.24$, $\beta_L = 0.20$, $\beta_S = 0.56$, $[t_{DIS}]_L = 63$ hr, $[t_{DIS}]_S = 12$ hr.

then represents the time between cell death and disappearance from the culture, i.e., t_{DIS}. Insofar as t_{DIS} has any dispersion and if there is more than one population of cells characterized by different values of t_{DIS}, the curves describing total and live cell numbers will diverge with increasing time after the killing event. In practice, only a slight divergence of the curves with time was seen.

As shown in Fig. 1, the plots of viable and total cell numbers could be divided into three areas corresponding to three temporal phases of survival after a killing intervention. Phase I precedes point X, when lymphocytes killed by the lethal challenge begin to disintegrate. Point Z defines the time on the viable cell curve at which the earliest effect of killing is seen after a variable lag phase. Phase II is the time interval between points X and Y, during which most cells sensitive to the lethal challenge disintegrate. Their t_{DIS} was estimated at the median point of Phase II. Assuming a Gaussian distribution of t_{DIS}, this value represents the mean disintegration time. Phase III begins at point Y, when the death rate decreases sharply to a low value in some experiments and to nearly zero in others.

The patterns of the two curves in Fig. 1 reveal a subpopulation of killed lymphocytes with a long t_{DIS}. Beyond point Y, the total cell number curve does not converge to the viable cell number curve as would occur if the same t_{DIS} prevailed. Rather, the curves closely parallel each other for a prolonged period, defining a t_{DIS} of $t_4 - t_3$. The data are most readily interpreted by considering that the situation at point Y defines three subpopulations of cells according to their fate after killing intervention. The first subpopulation, α, consists of lymphocytes that are relatively resistant to the killing event, as has also been observed in other systems (17), and that die at a slow rate insofar as culture conditions are adverse. Cells that die as a result of the killing event can be divided into two subpopulations. One, β_S, consists of lymphocytes with a short disintegration time, $[t_{DIS}]_S$, which are the majority of cells disintegrating in Phase II; the other, β_L, contains cells with a long disintegration time, $[t_{DIS}]_L$, which constitute the major disintegrating cell population in Phase III. Because of cross contamination, $[t_{DIS}]_S$ is slightly overestimated whereas $[t_{DIS}]_L$ is slightly underestimated. It is assumed, in the absence of any evidence to the contrary, that both populations, β_L and β_S, have the same killing event-induced death rate and differ only in t_{DIS}. Table 1 summarizes 31 experiments that gave data defining the size of these three populations. The variability between individual experiments was such that the differences between β_S/β_L ratios are not significant ($P \geq 0.65$, Student's t-test).

We performed 20 population decay experiments, in which at least seven pairs of measurements were available in Phases I and II. The $[t_{DIS}]_S$ values, grouped by experimental situations, are summarized in Table 2. In all cases but one, the observed differences in mean $[t_{DIS}]_S$ were not statistically significant. In the one significantly different situation, the mean $[t_{DIS}]_S$ was increased by one experiment that yielded an extremely long time value. Cell concentrations in the culture at the time of killing did not exercise an apparent influence ($P = 0.16$, rank correlation test). The overall mean $[t_{DIS}]_S \pm$ SEM was 16 ± 2 hr.

Nine experiments included five or more pairs of measurements spanning point Y and Phase III, thereby permitting es-

Table 1. Lymphocyte subpopulations after a killing intervention, by culture age

Treatment and population	PHA-stimulated 3 days	PHA-stimulated 6 days	Unstimulated 3 days	Unstimulated 6 days
Irradiation				
	(10)	(8)	(2)	(3)
α	0.20 ± 0.02	0.20 ± 0.02	0.19 ± 0.06	0.23 ± 0.07
β_L	0.16 ± 0.03	0.25 ± 0.06	0.16 ± 0.04	0.23 ± 0.05
β_S	0.64 ± 0.03	0.55 ± 0.06	0.65 ± 0.04	0.54 ± 0.05
KCN				
	(2)			
α	0.50 ± 0.02			
β_L	0.13 ± 0.03			
β_S	0.37 ± 0.03			
None (besides AraC and Colcemid)				
	(2)	(2)		(2)
α	0.23 ± 0.02	0.23 ± 0.02		0.58 ± 0.01
β_L	0.19 ± 0.05	0.20 ± 0.05		0.12 ± 0.01
β_S	0.58 ± 0.05	0.57 ± 0.05		0.30 ± 0.01

Lymphocytes were cultured in the presence or absence of PHA for 3 or 6 days. The cultures were then irradiated with 3000 rads or treated with KCN, and/or resuspended in medium containing AraC and Colcemid (see *Materials and Methods*). α, β_L, and β_S subpopulations are defined in the *text* and Fig. 1. The data are expressed as the mean fraction ± SEM of the initial lymphocyte population. The number of experiments is indicated in parentheses.

Table 2. Mean disintegration times of β_S subpopulation

Experimental conditions	$[t_{DIS}]_S \pm$ SEM, hr		
Proliferation condition			
Stimulated (10)	19.3 ± 3.0	0.08	
Unstimulated (10)	13.1 ± 1.1		
Killing event (in addition to AraC and Colcemid)			
KCN (5)	15.9 ± 1.4	0.12	0.25
Irradiation (10)	12.9 ± 1.1	0.03	
None (5)	22.9 ± 5.6		
Day of killing event			
Day 3 (9)	15.0 ± 1.2	0.57	
Day 6 (11)	17.0 ± 3.0		
Method of cell counting			
Hemacytometer (11)	14.9 ± 1.0	0.43	
Electronic particle counter (9)	14.7 ± 2.2		

The number of experiments is given in parentheses. Significance (Student's t test) of differences between t_{DIS} values is shown at right.

timation of $[t_{DIS}]_L$. In six of these, the mean value was 45 ± 7 hr, whereas in three others $[t_{DIS}]_L$ was more than 100 hr. There was no apparent difference in $[t_{DIS}]_L$ between stimulated and nonstimulated lymphocytes. For spontaneous cell death in nonstimulated cultures (without killing intervention and in the absence of AraC and Colcemid), t_{DIS} was in the 15–30 hr range, but the number of dead cells was too small to assess more precise estimates.

As a check of the population decay method, we analyzed time-lapse cinematomicrographic films of human lymphocytes proliferating in response to various stimulants. The films were kindly provided by William Marshall and have been extensively described (18). t_{DIS} was the time interval between the moment a cell became immobile and rigid and the time it fragmented or assumed the shape of debris. A total of 24 mobile and dividing lymphocytes were clearly observed to die, and 4 disintegrated visibly with a t_{DIS} ranging from 8 to 18 hr, in good agreement with $[t_{DIS}]_S$ by population decay. The remainder of the dying cells coalesced into aggregates of cell debris and platelets and was lost into this background. A portion of the cells already dead at the beginning of the film failed to disintegrate for periods up to 100 hr, and evidently belonged to what we term subpopulation β_L.

Cell death rate

Utilizing the above values for t_{DIS}, we can now calculate the rate of cell death. Contrary to the population-decay experiments, lymphocyte cultures are normally exposed not to a massive killing event, but to a permanently and increasingly adverse culture situation. Because killing with a variety of agents chosen for their relative lack of potential to cause structural damage (irradiation, KCN, AraC, and Colcemid) gave similar results, "spontaneous" cell disintegration can be expected to follow the same kinetic patterns. This point is supported by the cinematomicrographic observations. Since there was wide variability in $[t_{DIS}]_L$, $[t_{DIS}]_S$, and the β_S/β_L ratio in different population decay experiments, mean values for these parameters must be used in death rate calculations unless one undertakes elaborate population-decay experiments in parallel with each culture study. To simplify an actuarial determination of the death rate in a particular culture, we can round off the mean $[t_{DIS}]_S$ and $[t_{DIS}]_L$ values utilized in calculations to 15 hr and its integer multiple, 45 hr, respectively. The β_S/β_L ratio can be put at 4, so that 80% of all cells dying in any time interval are assigned a t_{DIS} of 15 hr and 20% are assigned a t_{DIS} of 45 hr. The rate of cell death can then be assessed by actuarial tabulation at time intervals of 15 hr, making corrections for the persistence of dead β_L cells through three such intervals. The death rate per hour during each 15-hr interval is calculated from the trypan blue-positive cell counts by the formula:

$$\frac{(n_D)_{t-15\text{ to }t}}{15} = \frac{(n_D)_t - (n_D)_{L(t-45\text{ to }t-15)}}{15} \quad [1]$$

in which $(n_D)_{t-15\text{ to }t}$ = the number of cells that died during the 15-hr interval preceding time t, $(n_D)_t$ = the number of dead cells observed at time t, and $(n_D)_{L(t-45\text{ to }t-15)}$ = the number of residual dead β_L cells tallied in $(n_D)_t$ that died prior to t − 15. Similarly, the disintegration rate between time t and t + 15 of cells dead at time t is equal to:

$$\frac{(n_D)_{S(t-15\text{ to }t)} + (n_D)_{L(t-45\text{ to }t-30)}}{15} \quad [2]$$

in which $(n_D)_{S(t-15\text{ to }t)}$ = the number of β_S cells that died during the 15-hr interval preceding t, and $(n_D)_{L(t-45\text{ to }t-30)}$ = the number of β_L cells that died during the interval 45–30 hr preceding t.

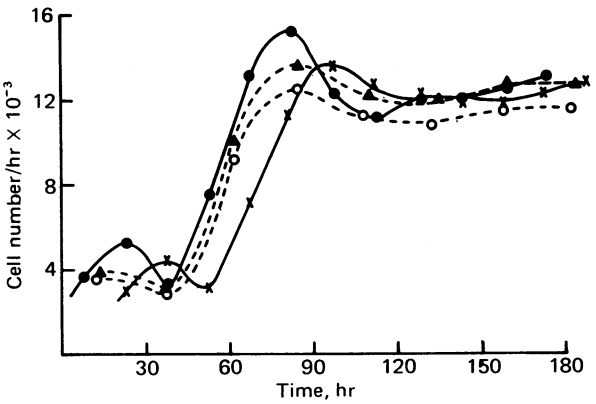

FIG. 2. Death and disintegration rates. Replicate 0.5-ml cultures containing 1.4 × 10^6 lymphocytes were stimulated with PHA and harvested at daily intervals as described in *Materials and Methods*. The number of trypan blue-positive cells present at 15-hr intervals was obtained by interpolation. ●, Death rate from Eq. 1; x, disintegration rate from Eq. 2; ▲, death rate utilizing daily trypan blue-positive cell counts and a mean t_{DIS} of 22 hr or ○, of 24 hr.

The results of such an actuarial tabulation for an experiment carried out under conditions of cell concentration that caused extensive cell death are shown in Fig. 2. The death rate was biphasic. An initial wave, possibly reflecting nonadaptation of some cells to culture conditions, was followed by a gradual increase. Later, the curve for the death rate reached a plateau as the percent trypan blue-positive cells increased and the total cell number decreased. The disintegration rate followed after a lag = $[t_{DIS}]_S$. As a further simplification in the death rate calculation, the mean t_{DIS} could be assigned an approximate value of 22 hr, the weighted mean of $[t_{DIS}]_L$ and $[t_{DIS}]_S$. Then, the dead cells in any cell count represented the number dying in the preceding 22 hr, and dividing this number by 22 hr gave the average death rate per hour over that interval (Fig. 2). For all practical purposes, an instantaneous dead cell count gave a close approximation of the death rate, assuming a t_{DIS} of 24 hr. Thus, the absolute number of cells dying in the culture was only slightly underestimated by summing the dead cell counts at 24-hr intervals.

Release and turnover of DNA in the culture medium

Using the experiment described in Fig. 2, we generated a balance sheet, attempting to account for all DNA originally present and newly synthesized in a PHA-stimulated lymphocyte culture during an 8-day period. Fig. 3*B* presents serial measurements of the DNA assayed by the diaminobenzoic acid fluorescence method in cell pellets and in the culture medium. To explain the decrease in DNA content in the face of extensive blastogenesis and considerable [^{3}H]Td uptake (27,000 cpm compared with 1800 cpm in unstimulated controls on day 3), we tested the hypothesis that cell death accounts for the deficit in cell proliferation. Two methods were used to calculate a projected "maximal DNA content" of the culture, assuming an absence of DNA catabolism: in Fig. 3*A* (left bars), the amount of newly synthesized DNA was estimated from the rate of cell division and was added to the amount of DNA originally present; in Fig. 3*A* (right bars), the amount of DNA in the cell pellet was estimated from the observed cell counts, and the value for the DNA of disintegrated dead cells (no longer present in the pellet) was added to it.

The DNA values plotted in Fig. 3*A* were calculated as fol-

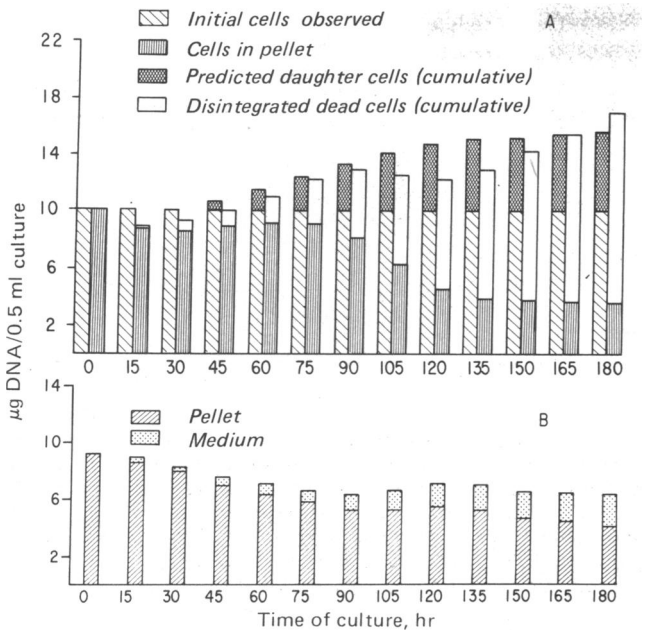

FIG. 3. Balance sheet of (*A*) DNA content estimated from initial cell count and proliferation rates (left bar) and from observed cell counts and disintegration rates (right bar), and (*B*) DNA content of the cell pellet and medium assayed by diaminobenzoic acid fluorescence.

lows: (*i*) To estimate cell DNA contents, we multiplied the cell counts by 7 pg for G_0 lymphocytes (average of seven determinations) and by the average value of 10.5 pg for cycling cells. The number of cycling cells is given by cell number × growth fraction. The growth fraction (GF) is: $LI \times t_C/(t_S + t_P)$, in which LI = labeling index, t_C = cell cycle time = 17.5 hr, t_S = duration of S phase = 9.5 hr, and t_P = duration of [^{3}H]Td pulse = 2 hr. The values of t_C and t_S were obtained from pulse-labeled mitoses curves and agree well with published data (19).

(*ii*) The left bars of Fig. 3*A* present one estimate of the "maximal DNA content" of the culture. The lower left bars represent the DNA contributed by the G_0 lymphocytes originally present (a constant). The upper left bars are a cumulative estimate of the DNA contributed by cells that divide and were obtained as follows: The instantaneous rate of entry into S phase was calculated (20) as $LI/(t_S + t_P)$. It was presumed that all cells entering S go on to mitosis and contribute an additional G_0 DNA complement, with the exception of those cells that die in S, G_2, and M. For lack of any published evidence to the contrary, it was assumed that cells may die at any point of the cell cycle. The number of cells dying in S, G_2, and M during each interval was obtained as follows:

$$n_{D(S+G_2+M)t-15\text{ to }t} = \text{death rate} \times 15\text{ hr} \times \frac{t_S + t_{G_2} + t_M}{t_C} \times GF \quad [3]$$

in which the death rate is calculated by Eq. 1 and $t_{G_2} + t_M$ = 5.5 hr (from the pulse-labeled mitoses curves).

(*iii*) The right bars of Fig. 3*A* estimate the "maximal DNA content" of the culture (cells plus medium) if no degradation of DNA from disintegrated cells occurs. The lower right bars represent the calculated DNA content of living and dead cells obtained by multiplying the observed cell number by the cell DNA content and weighting for the growth fraction as described above. The upper right bars represent a cumulation of all DNA released from disintegrated lymphocytes (Eq. 2).

Comparison of the left and right bars of Fig. 3*A* shows a fair match between the two largely independent methods of estimating the "maximal DNA content." Equally well-matched balance sheets were obtained from other similar experiments and from cultures initiated at the more optimal cell concentration of 2×10^5/ml.

A number of points can be made from the data and calculations presented in Fig. 3. (*i*) The theoretical "maximal DNA content" of the culture after 180 hr is predicted to be 16 μg (Fig. 3*A*, left bar), whereas the DNA content actually observed in the cell pellet was 4 μg (Fig. 3*B*, lower bar), demonstrating a massive deficit. (*ii*) A calculated estimate of DNA released into the medium by disintegrating cells (Fig. 3*A*, upper right bars) closely approximates the deficit between calculated "maximal DNA content" (Fig. 3*A*, left bars) and the calculated (or observed) amount of DNA in the cell pellet (Fig. 3*A*, lower right bars). The proliferation deficit is therefore attributable to cell death. (*iii*) The great difference between the predicted (13 μg) and observed (2.4 μg) DNA contents of the medium suggests that degradation of DNA, released into the medium by dead lymphocytes, to nonacid-precipitable metabolites is extensive. (*iv*) During the first 2 days, a number of cells that cannot be accounted for by the observed number of trypan blue-positive cells disappear from the culture. These cells could have very short disintegration times, or they may adhere to culture vessel walls.

DISCUSSION

The amount of cell death in a culture system *in vitro* is clearly an important parameter that must be taken into account when the effects of experimental perturbations are analyzed. For proliferating lymphocyte cultures, operational concepts such as stimulation, suppression, and allogeneic effects would be better defined if cell death could be quantified. To accurately quantify cell death *rates*, we require the t_{DIS} of nonviable lymphocytes. We have demonstrated that there are at least two populations of lymphocytes with markedly different t_{DIS} values. Two distinct morphological expressions of cell death have been described: (*i*) swelling with subsequent autolysis, and (*ii*) denaturation with "mummification" of the cell (21, 22). It is possible that these phenomena account for our observations.

In the example experiment, the cells were grown under concentrated culture conditions (2.8×10^6 cells per ml) that we and others have shown are far from optimal (6, 7). Cell death provides a plausible explanation for the observation that cell number and culture DNA content do not increase in crowded culture conditions, despite considerable [^{3}H]Td incorporation and significant proliferation indices. In cultures initiated at a concentration of 2×10^5 cells per ml, several division cycles can be achieved, and the small difference between potential and actual cell proliferation is also explained by cell death, by using similar calculations. The data suggest that a majority of the DNA released into the culture medium by dying and disintegrating cells is degraded to acid-soluble material. An interpretation of studies providing biochemical characterizations of released DNA (11, 12) must take these observations into account.

The good matches observed in several balance-sheet experiments validate our methods for quantifying cell death, as well as the assumptions underlying these methods: cell death may occur in any phase of the cell cycle, and all cells that initiate DNA synthesis go on to mitosis unless they die. Of great practical significance is the fact that by utilizing a mean t_{DIS} value of 22 hr we get a satisfactory approximation of lymphocyte death rate (Fig. 2); this means that instantaneous, nonviable cell

counts only underestimate the actual death rate per day by a small amount. This is true up to day 6 after stimulation, when β_L is only insignificantly increased over its value on day 3 (Table 1). Our preliminary evidence indicates that at later times β_L may increase further and $[t_{DIS}]_L$ may be considerably longer than 45 hr, resulting in increased accumulation of dead cells.

We do not know to what extent our particular values for t_{DIS} and our characterization of two dead cell populations are restricted to the lymphocyte system and to our particular culture conditions. Quantitation of cell death generally has been neglected in kinetic studies of dividing cell cultures, yet it is a major factor during the decay phase of cell proliferation. The methods used in our studies with lymphocytes have important potential applications in the general area of cytokinetics.

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