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A comparative kinetic analysis of proliferation *in vitro* of Con-A-treated splenocytes and syngeneic leukaemia cells

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Abstract. The growth kinetics of Con-A-treated mouse splenocytes and syngeneic leukaemia cells cultured *in vitro* were compared with respect to (i) the total cell number, (ii) the rate of [^{14}C]thymidine incorporation (measured by pulse-labelling the cells at various times of incubation), and (iii) the labelling index of the cell populations. By correlating the thymidine incorporation, labelling index and cell number data, it has been established that, for both types of cells, the rate of [^{14}C]thymidine incorporation is directly proportional to the number of cells synthesizing DNA. A new approach to cytokinetic analysis has been developed, showing that important information can be obtained by determining the cumulative kinetics of [^{14}C]thymidine incorporation. The latter has been calculated by integrating the area underneath the time course of the rate of thymidine incorporation, and was directly proportional to the overall growth of both leukaemia cells and Con-A-stimulated splenocytes. Based on this proportionality, an estimate of the average duration of the S phase for both types of cells was calculated, suggesting that normal and neoplastic blasts maintain this parameter at a constant value (7·6 and 5·9 hr, respectively) throughout different stages of growth. The percentage of Con-A-responsive cells within the initial splenocyte population and their overall proliferation *in vitro* have been determined by a procedure which measures the cumulative kinetics of thymidine incorporation and the kinetics of cell total number in the presence or in the absence of the lectin, as well as in the presence of Con-A plus colcemid. A minor fraction (11%) of the initial splenocytes is recruited into cycle by Con-A, proliferating with similar kinetics to that of leukaemia cells in the same conditions. The great majority of the initial splenocyte population is unaffected by Con-A, decaying exponentially throughout the incubation with the same half-life (28 hr), both in the presence or in the absence of the lectin.

The *in vitro* culture of lectin stimulated lymphocytes and syngeneic leukaemia cells has provided a realistic model for comparative studies of normal and neoplastic cells, particularly of the transition from the non-cycling to the cycling state, the regulation of which may be important for cell transformation (Baserga, 1976; Gelfant, 1977). However, these studies are restricted by the fact that mitogenic triggering of non-cycling lymphocytes is a complex and

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still largely obscure process, which requires the co-operation of different types of cells normally present in unfractionated lymphoid populations (Habu & Raff, 1977; Andersson *et al.*, 1979; Coutinho *et al.*, 1979; Larsson & Coutinho, 1979). Thus, *in vitro* lymphocyte activation employs heterogeneous populations, whose cytokinetics are difficult to define (Tice, Thorne & Schneider, 1979), especially when the size of the responsive fraction and the fate of the bystander cells are unknown.

In this paper we present evidence that the above restriction to the comparative kinetic analysis of unfractionated lymphoid populations and leukaemia cells may be overcome in a simple experimental model, where mouse splenocytes stimulated by Concanavalin A (Con-A) are cultured in parallel with syngeneic leukaemia cells. The cytokinetics of this model have been analysed by means of a new methodological approach based on conventional experimental techniques (cell counts, pulse-labelling with radioactive thymidine, autoradiography). This approach, first worked out for leukaemia cells, has been applied to the complex cytokinetics of the splenocytes *in vitro*, leading to the development of a simple mathematical procedure for determining the growth parameters of the mitogen-stimulated fraction within a heterogeneous population.

MATERIALS AND METHODS

Leukaemia cells

RI leukaemia cells, derived from a radiation induced leukaemia of the CBA mouse (Hewitt, 1962) were obtained from the Chester Beatty Research Institute, London and passaged in the ascitic form in CBA mice. A tissue culture line of RI cells, grown in MEMx4 medium with 5% foetal calf serum, was used. Experimental cells were collected from cultures which had reached the growth plateau, washed once and resuspended in the culture medium.

Splenocytes

Splenocytes were obtained from CBA T6T6 mice aged 8–12 weeks. Mice were killed by decapitation and spleen cells were collected by squeezing spleens in MEMx4. After centrifugation these cells were treated with 0·87% NH_4Cl to lyse erythrocytes, washed once in MEMx4 and resuspended in the culture medium.

Culture conditions

RI cells ($2{\cdot}5 \times 10^4$/well) or splenocytes (about 6×10^5/well) were seeded at time zero in flat bottom microwells (Microtest II, Falcon 3040) containing 0·2 ml (final volume) of freshly prepared MEMx4 with 5% foetal calf serum (Gibco) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml). Incubation was carried out at 38°C in a 95% air, 5% CO_2 atmosphere in a humidified incubator.

Splenocyte stimulation

Splenocyte stimulation was produced by adding to the cultures, shortly after seeding, 0·8 μg/well of Con-A (Pharmacia, Uppsala, Sweden) purified on G50 Sephadex (Agrawal & Goldstein, 1967).

Cell counting

At fixed times of incubation the cells were carefully recovered from the microwells and each sample was counted twice in a Bürker counting chamber. The number of dead cells was estimated by the trypan blue exclusion test, diluting the cell suspension at a 1:1 ratio with a 1% trypan blue solution.

Pulse-labelling with [^{14}C]thymidine and radioactivity measurements

One hour pulse-labelling of individual cultures was performed at various times after the beginning of incubation by adding, to each of the selected wells, 10 μl of MEMx4 containing 0·2 μCi of 2-[^{14}C]thymidine (Radiochemical Centre, Amersham, specific activity 60 mCi/mmol). At the end of the labelling period the contents of the cells were collected with a multiple cell culture harvester (Skatron, Lierbyen, Norway) and counted in a scintillation mixture (4·0 g of 2,5-diphenyloxazolone and 0·05 g of 2,2′-*p*-phenyloxazolone in 1 l of toluene), using a Tri-Carb 460 CD scintillation spectrometer.

Autoradiography

Individual cultures were pulse labelled for 1 hr at various times after the beginning of incubation by adding 4 μCi/well of methyl-[^{3}H]thymidine (Radiochemical Centre, Amersham, specific activity 21 Ci/mmol). At the end of the labelling period the contents of the wells were recovered, diluted 1:5 with a 10^{-4} M solution of cold thymidine in MEMx4 and immediately spun at 1000 ***g*** for 10 min. The sedimented cells were washed four times in the above cold thymidine solution and then spread onto glass slides pretreated with a solution containing 0·05% $KCr(SO_4)_2$ and 5% gelatine. After drying, the slides were fixed in methanol for 10 min and then dipped into Ilford SCK 5 emulsion and kept in the dark at 4°C for 20–30 days. After this period the slides were developed for 5 min at room temperature in Kodak D-19 Developer, fixed for 15 min in Kodak 'Unifix' fixer and stained with Giemsa.

RESULTS AND DISCUSSION

Kinetic indices of RI cells

Figure 1 shows the time course of cytokinetic indices relative to RI cells cultured *in vitro* under the conditions indicated above. Such indices are: (i) the total number of cells per well at time t, $C(t)$; (ii) the percentage of cells in S at a given time, as measured by autoradiography [labelling index, $LI(t)$]; (iii) the amount of [^{14}C]thymidine incorporated by the cell population during the pulse labelling and expressed as DPM × 60 min per well, $R(t)$ (rate of [^{14}C]thymidine incorporation).

Starting with $2·5 \times 10^4$ cells, $C(t)$ increases up to 6×10^5 in 80 hr according to a sigmoid kinetics, with a minimum doubling time of 10 hr during the interval 20–40 hr. Judging from the trypan blue exclusion test the number of dead cells never exceeded 2–3% of the total

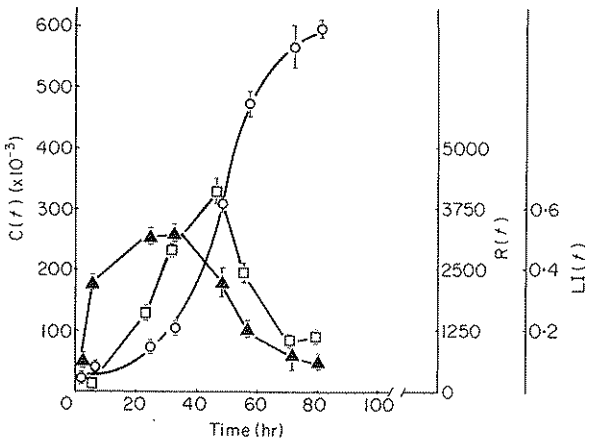

Fig. 1. Time course of the total number of cells [$C(t)$], of the labelling index [$LI(t)$] and of the rate of [^{14}C]thymidine incorporation [$R(t)$] of RI leukaemia cells. Each point reported in the graph is mean ± SEM of four separate samples. ○, $C(t)$; ▲, $LI(t)$; □, $R(t)$ = DPM × 60 min per well. The number of cells seeded in each well at time zero [$C(0)$] was $2·5 \times 10^4$.

population up to 80 hr (not shown). As can be seen from the LI(t) kinetics, the percentage of cells in the S phase within the RI population undergoes a sharp increase during the very first hours of incubation, attaining a maximum (about 50%) between 20 and 35 hr, and thereafter falling progressively. On the other hand, the time course of R(t) is characterized by a progressive increase which lasts up to 48 hr, followed by a rapid decline from this time onwards. As stated above, R(t) corresponds to the amount of [^{14}C]thymidine incorporated by DNA synthesizing cells in the whole population during the pulse labelling period. Although this amount is often assumed to be an index of the total number of cells synthesizing DNA within a cell population, it eventually depends on (1) the number of cells synthesizing DNA, (2) the rate of DNA synthesis during the labelling period, and (3) factors determining the metabolic pathway of precursor thymidine, such as the rate of thymidine transport into the cells, the size of the thymidine endogenous pool, etc. (Cleaver, 1967; Nicolini, 1975; Maurer, 1981). It is thus evident that data on R(t) may be used for determining the variations in the number of DNA-synthesizing cells only when all the factors mentioned under (2) and (3) are constant for cells in the S phase at the time of observation. Thus, although evidence has been provided for a linear relationship between the observed rate of thymidine uptake and the number of cells in S in several experimental systems, including lectin stimulated lymphocytes and tumour cells (Gunther, Wang & Edelman, 1974; Stewart & Hamill, 1977; Olivotto, Boddi & Dello Sbarba, 1979; Olivotto & Paoletti, 1980; O'Leary *et al.*, 1980), this relationship should be checked for any experimental model adopted. Olivotto & Paoletti (1980) have proposed a method for determining whether, for a growing cell population, there is a direct proportionality between R(t) and the number of cells in S at the corresponding time, based on parallel measurements of R(t), LI(t) and C(t). In fact, LI(t) represents the ratio of the labelled *v.* the scored cells within a population which is pulse-labelled and processed for autoradiography at time t. Thus, knowing the total cell number at time t [C(t)] it is possible to calculate the total number of cells in S at time t [$N^*(t)$] by multiplying LI(t) by C(t). Figure 2 shows that the relationship between R(t) and $N^*(t)$ for RI cells, in our conditions, is approximately linear and may be expressed by the equation:

$$R(t) = k_1 N^*(t) \qquad (1)$$

where k_1 is a constant (0·050), representing the amount of [^{14}C]thymidine (expressed as DPM) incorporated per hour into DNA by one cell in S in our conditions. In any experimental model where the above relationship can be shown to hold and k_1 can be determined, it is possible to estimate the total number of cells in S at a given time simply by measuring R(t) instead of using the cumbersome and less precise autoradiographic measurements of $N^*(t)$.

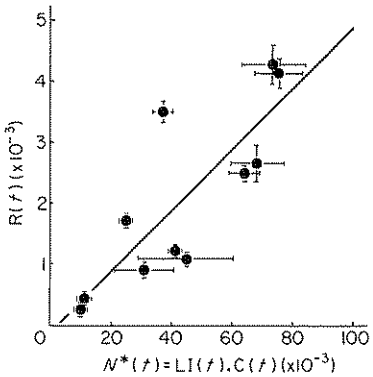

Fig. 2. Relationship between the rate of [^{14}C]thymidine incorporation [R(t)] and the total number of cells in S [$N^*(t)$] for RI cells. RI cells were pulse labelled with either [^{14}C]thymidine or [^{3}H]thymidine for 60 min, to obtain contemporaneous measurements of R(t) and LI(t), respectively. $N^*(t)$ values were calculated by multiplying LI(t) values by C(t) values measured at the corresponding times. Values reported in the graph are means ± SEM of four determinations, carried out on separate samples, of both R(t) and $N^*(t)$ obtained in two separate experiments. The straight line traced in the figure represents the least square regression line of R(t) on $N^*(t)$ according to the equation $R(t) = -125 + 0{\cdot}050N^*(t)$ where 0·050 is k_1 of eqn 1 and is expressed as DPM/cell in S. The standard error of k_1 has been calculated to be 0·0118 and the correlation coefficient r is 0·815 ($n = 11$).

As a further step towards the definition of the cellular cytokinetics, the cumulative kinetics of [^{14}C]thymidine incorporation of RI cells was calculated by integrating the area underneath the broken line joining the experimental values of $R(t)$ reported in Fig. 1 (shaded area in Fig. 3a). As shown in Fig. 3a, measurements of this area from zero up to time t [$\int_0^t R(\tau)\,d\tau$] display a general trend practically coincident with that of cell number increase, $C(t) - C(0)$. Such strict relationship between $\int_0^t R(\tau)\,d\tau$ and $C(t) - C(0)$ is confirmed by data shown in Fig. 3b, where values of $\int_0^t R(\tau)\,d\tau$, gathered throughout six separate experiments, are plotted against the corresponding values of $C(t) - C(0)$. This reveals a good correlation between these two parameters ($r = 0{\cdot}97$), and leads to the following equation:

$$\int_0^t R(\tau)\,d\tau = k_2[C(t) - C(0)] \qquad (2)$$

where k_2 is a constant (0·285) which represents the amount of [^{14}C]thymidine (in DPM) incorporated into DNA, required for the cell number to increase by a factor of 1 in our conditions. Thus, the overall increase in cell number from zero up to time t closely corresponds to a parameter which, according to eqn 1, registers the total number of cells found in S throughout the same interval and which, derived from pulse labelling measurements, is not affected by cell loss taking place between one measurement and the other. Obviously such a correspondence can only occur when, as in the case of RI cells, cell loss is practically negligible throughout the incubation.

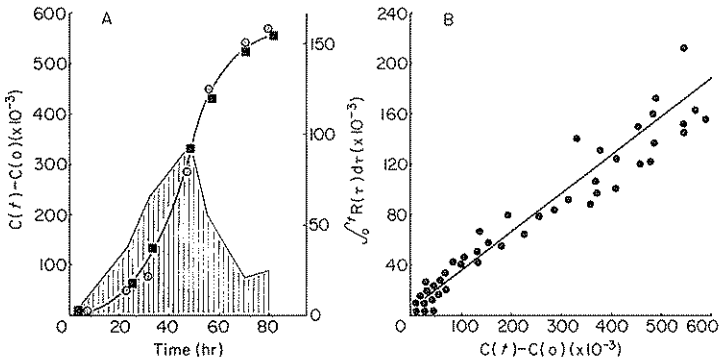

Fig. 3. (a) Time course of the cumulative [^{14}C]thymidine incorporation [$\int_0^t R(\tau)d\tau$] and of cell increment [$C(t) - C(0)$] during incubation of RI cells. Shaded area = area delimited by the broken line joining the experimental $R(t)$ points reported in Fig. 1; ■, integration of the shaded area from zero up to time t: [$\int_0^t R(\tau)d\tau$]; ○, cell increment from zero up to time t, [$C(t) - C(0)$]. Values were obtained by subtracting C(0) ($2{\cdot}5 \times 10^4$ cells) from $C(t)$ experimental values reported in Fig. 1. (b) Relationship between $\int_0^t R(\tau)d\tau$ and $C(t) - C(0)$ for RI cells. Values reported in the graph are means of four determinations, carried out on separate samples, of both $\int_0^t R(\tau)d\tau$ and $C(t) - C(0)$ obtained in six separate experiments. The straight line through the points represents the least squares regression line of $\int_0^t R(\tau)d\tau$ on $C(t) - C(0)$ according to the equation $\int_0^t R(\tau)d\tau = 7100 + 0{\cdot}285[C(t) - C(0)]$ where 0·285 is k_2 of eqn 2 and is expressed as DPM, incorporated into DNA, required for the cell number to increase by a factor of 1. The standard error of k_2 has been calculated to be 0·0115 and the correlation coefficient r is 0·967 ($n = 45$).

RI cytokinetics

The following cytokinetic analysis, elaborated for RI cells, will be subsequently used for Con-A-treated splenocytes. Integrating eqn 1 we obtain:

$$\int_0^t R(\tau)\,d\tau = k_1 \int_0^t \dot{N}^*(\tau)\,d\tau \qquad (3)$$

Assuming that the time spent by the cells in G_2 and in M is constant ($T_{G_2} + T_M = T_{G_2+M}$) and that cell loss is negligible, after eqn 3 it can be stated:

$$\int_0^t R(\tau)\,d\tau = k_1\,\bar{t}_s(t)[C(t + T_{G_2+M}) - C(T_{G_2+M}) + N^*(t)/2] \quad (4)$$

where $\bar{t}_s(t)$ is the average time spent in S by cells passing through S from the beginning up to time t, and $C(t + T_{G_2+M}) - C(T_{G_2+M})$ is the cell number increase attributable to these cells. The last term in eqn 4, $N^*(t)/2$, accounts for the contribution to $\int_0^t R(\tau)\,d\tau$ of the cells presently in S at time t, $N^*(t)$, which on the average have been in S for a time equal to $\frac{1}{2}\bar{t}_s$. For values of t much larger than T_{G_2+M} we have, from eqn 4:

$$\int_0^t R(\tau)\,d\tau = k_1\,\bar{t}_s(t)[C(t) - C(0) + N^*(t)/2] \quad (5)$$

By means of (1) and (5), if k_1 is known, measurements of $C(t) - C(0)$ and $\int_0^t R(\tau)\,d\tau$ are sufficient for estimating $\bar{t}_s(t)$ of cell populations which satisfy the above mentioned conditions, i.e.: (i) $R(t)$ is directly proportional to $N^*(t)$; (ii) cell loss is negligible throughout the incubation time; (iii) T_{G_2+M} is also negligible as compared to t. It is worth repeating that $\bar{t}_s(t)$ represents the mean of the times spent in S by all the cells passing through this phase from zero up to t. Therefore, essentially, $\bar{t}_s(t)$ is a mean value established from all data prior to time t. This implies that changes in t_s, occurring late in the experimental period, will not significantly influence the mean value $\bar{t}_s(t)$, because of their progressively smaller weight in the computation of the mean value.

Data reported in Table 1 refer to $\bar{t}_s(t)$ measurements obtained for RI cells according to eqns 1 and 5. From these data, $\bar{t}_s(t)$ for leukaemia cells appears practically constant (mean = 5·9 hr) throughout the incubation, suggesting that the average S duration is also constant for these cells through the different stages of growth *in vitro*. However, because of the sensitivity of the computation of $\bar{t}_s(t)$ to large changes in t_s (see above), variations of t_s during the late period of incubation cannot be excluded. Finally, from data reported in Table 1, it is possible to calculate that the weight of $N^*(t) = R(t)/k_1$, as compared to $C(t) - C(0)$, rapidly diminishes along with incubation, due to rapid increase of $C(t) - C(0)$. Thus, except for the early times of incubation, eqn 5 can be simplified as follows:

$$\int_0^t R(\tau)\,d\tau = k_1\,\bar{t}_s(t)[C(t) - C(0)] \quad (6)$$

and, recalling that $\bar{t}_s(t)$ is a constant,

$$\int_0^t R(\tau)\,d\tau = k_2[C(t) - C(0)] \quad (7)$$

The latter is in fact eqn (2), experimentally found for RI cells and discussed above. Thus it appears that plotting $\int_0^t R(\tau)\,d\tau$ against $C(t) - C(0)$, as in Fig. 3b, one is essentially plotting $k_2[C(t) - C(0)]$ against $C(t) - C(0)$. This accounts for the finding, illustrated in the same figure, that the experimental points are fitted by a straight line passing close to the origin (eqn 2). As the slope of this line (k_2) is equal to $k_1\,\bar{t}_s(t)$, one can immediately obtain a slightly less accurate estimate of $\bar{t}_s(t)$, having determined both k_1 and k_2. Such an estimate gives a $\bar{t}_s(t) =$ 5·7 hr for RI cells in our conditions, i.e. a value close to that previously determined by means of eqn 5. Such estimates of the duration of S are in keeping with those previously reported for murine lymphocytic leukaemia and lymphoma cells (6·5 hr) (Harris, Shon & Meneses, 1973; Janik & Steel, 1972).

Table 1. Estimate of the average time $[\bar{t}_s(t)]$ spent in the S phase by RI cells from the beginning up to different times of incubation. Values are calculated from eqns 1 and 5 as follows: $\bar{t}_s(t) = \int_0^t R(\tau)\,d\tau/\{0{\cdot}050.[C(t) - C(0)] + R(t)/2\}$. The number of cells seeded in each well at time zero [C(0)] was $2{\cdot}5 \times 10^4$

Time of incubation (hr)	R(*t*) (DPM × 60 min)	$\int_0^t R(\tau)\,d\tau$	$C(t) - C(0)$	$\bar{t}_s(t)$ (hr)
23·0	1104·0	18,275	50,500	5·94
31·5	1961·3	37,813	78,900	7·68
47·0	2731·3	92,363	285,200	5·91
55·5	1619·3	120,099	448,000	5·17
70·5	682·0	145,989	538,000	5·36
79·5	769·3	155,375	568,000	5·40
				$\bar{m} \pm$ SEM
				5·91 ± 0·38

Kinetic indices of Con-A-treated splenocytes

Figure 4 shows the time courses of C(*t*), R(*t*) and $\int_0^t R(\tau)\,d\tau$ for splenocytes incubated in the presence of Con-A under the standard conditions used for RI cells. To facilitate comparison with the corresponding parameters for RI cells, the same scales are used in this figure as in Figs 1 and 3. Starting with $6{\cdot}4 \times 10^5$ splenocytes at time zero, the C(*t*) time course is characterized by a progressive fall between zero and 40 hr, followed by a sharp rise between 50 and 100 hr. On the other hand, the R(*t*) kinetics shows a bell shape curve similar to that of RI cells (*cf.* Fig. 1), although delayed by about 30 hours relative to RI cells. Accordingly, values of $\int_0^t R(\tau)\,d\tau$, calculated as previously described by integrating the area under the R(*t*)

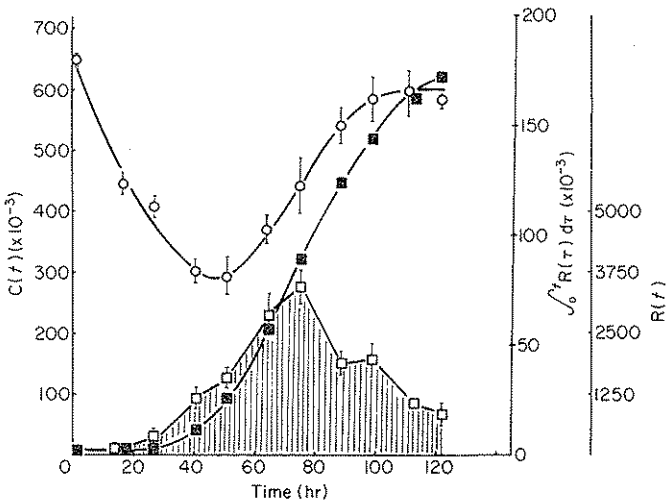

Fig. 4. Time course of the total number of cells [C(*t*)], of the rate of [^{14}C]thymidine incorporation [R(*t*)], and of $\int_0^t R(\tau)\,d\tau$ for Con-A-treated splenocytes. ○, C(*t*), means ± SEM of four separate samples; □, R(*t*), means ± SEM of four separate samples; ▩, $\int_0^t R(\tau)\,d\tau$, calculated by integrating the area under the broken line joining R(*t*) experimental points (shaded area).

The number of cells seeded in each well at time zero [C(0)] was $6{\cdot}4 \times 10^5$. R(*t*) values of the control cultures without Con-A were around 80 DPM/min throughout incubation.

time course, are fitted by a sigmoid curve similar to the kinetics of $\int_0^t R(\tau)\,d\tau$ for RI cells (*cf.* Fig. 3).

The relationship between $R(t)$ and the number of cells in S at the corresponding times, $N^*(t)$, were then investigated for Con-A-treated splenocytes applying the procedure previously described for RI cells, i.e. by plotting $R(t)$ against the product $LI(t).C(t)$. As shown in Fig. 5, here again the linear relationship between these two parameters may be expressed by the equation:

$$R(t) = k_1' N^*(t) \tag{8}$$

where k_1' is a constant, whose value (0·044 DPM/cell in S) is not significantly different from the value of eqn 1 determined above for RI cells (Student's test; significance level $P = 0{\cdot}05$).

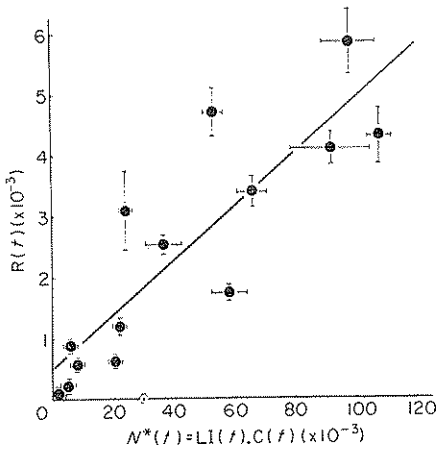

Fig. 5. Relationship between the rate of [^{14}C]thymidine incorporation [$R(t)$] and the total number of cells in S [$N^*(t)$] for Con-A-treated splenocytes. Splenocytes were pulse labelled with either [^{14}C]thymidine or [^{3}H]thymidine for 60 min, to obtain contemporaneous measurements of $R(t)$ and $LI(t)$, respectively. $N^*(t)$ values were calculated by multiplying $LI(t)$ values by $C(t)$ values measured at the corresponding times.

Values reported in the graph are means ± SEM of four determinations, carried out on separate samples, of both $R(t)$ and $N^*(t)$ obtained in three separate experiments. The straight line traced in the figure represents the least squares regression line of $R(t)$ on $N^*(t)$ according to the equation $R(t) = 558 + 0{\cdot}044N^*(t)$ where 0·044 is k_1' of eqn 8 and is expressed as DPM/cell in S. The standard error of k_1' has been calculated to be 0·0072 and the correlation coefficient r is 0·870 ($n = 14$).

To evaluate the cell growth induced by Con-A in our system, the time course of the total splenocyte number was also determined, both in the absence of Con-A and in the presence of Con-A plus colcemid, which blocks Con-A-induced lymphocyte proliferation (Steen & Lindmo, 1978). As shown in Fig. 6, the total number of cells in the absence of Con-A, [$U(t)$], decays throughout the time according to the following equation:

$$U(t) = U(0).e^{-qt} \tag{9}$$

where q is a constant, whose reciprocal (1/q) is the number of hours required for the cell number to decrease by a factor of 1/e. In our conditions q is 0·025 hr^{-1}, which corresponds to a half-life for $U(t)$ of about 28 hr. Thus, less than 10% of cells seeded at time zero survive after 100 hr of culture without Con-A. It is important to note that the $U(t)$ kinetics practically coincide with that of the total number of cells incubated with Con-A plus colcemid [$CB(t)$]. These findings are in keeping with those reported by Wilson, Blyth & Nowell (1968), who

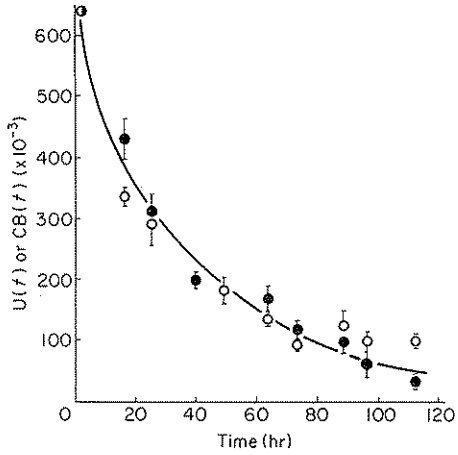

Fig. 6. Time course of the total number of splenocytes incubated either in the absence of Con-A [U(t)] or in the presence of Con-A plus colcemid [CB(t)]. Values reported in the graph are means ± SEM of four separate samples. ●, U(t); ○, CB(t). The number of cells seeded in each well at time zero was $6{\cdot}4 \times 10^5$. Colcemid (0·012 μg per well) was added at time zero.

The line passing through U(t) points represents the U(t) decay kinetics [$U(t) = U(0).e^{-qt}$, eqn 9] obtained calculating the least squares regression line of ln U(t) on the times of incubation. The equation of this line is ln U(t) = ln U(0) − 0·025t with a correlation coefficient $r = 0{\cdot}982$ and a standard error of the slope (s_q) = 0·0018 ($n = 9$). The above q and s_q estimates correspond to a U(t) half life of 27·7 hr with 95% confidence limits of 23·6 and 33·5 hr, respectively.

showed that, having blocked the lymphocyte proliferation in a mixed lymphocyte reaction by colchicine, the total number of cells in the cultures decayed exponentially with the same half life as in the unstimulated controls. It is clear that, once the mitogenic effect of Con-A is abolished by colcemid, this lectin does not substantially affect the spontaneous decay taking place in resting cultures. This point is relevant to the procedure adopted below to quantify the Con-A-dependent cell growth in our conditions.

Analysis of Con-A-treated splenocyte kinetics

Mouse spleen cells are a heterogeneous population, a minor part of which proliferates in response to Con-A. The responsive and unresponsive subpopulations may be denoted P(t) and Q(t), respectively. Recalling that, in the absence of Con-A, the whole population, U(t), decays exponentially according to eqn 9 and having no reason for assuming that, in these conditions, P(t) has different decay kinetics compared to Q(t), it is possible to write:

$$U(t) = [P(0) + Q(0)].e^{-qt} \tag{10}$$

where q is 0·025 hr^{-1} in our conditions. In the presence of Con-A, P(0), instead of decaying, will grow up to P(t), while, as indicated by colcemid experiments (p. 630), this lectin does not affect the overall cell decay of the unstimulated populations. Thus the total cell number in the presence of Con-A, C(t), will vary according to the equation:

$$C(t) = P(t) + Q(0).e^{-qt} \tag{11}$$

Thus,

$$C(t) - U(t) = P(t) - P(0).e^{-qt} \tag{12}$$

From eqn 8, let us now apply the same mathematical treatment previously derived from eqn 1 for RI leukaemia cells to the P(t) population. Integrating eqn 8,

$$\int_0^t R(\tau)\,d\tau = k_1' \int_0^t N^*(\tau)\,d\tau \tag{13}$$

Assuming that cell loss in the P(t) population is negligible throughout the interval 0–t, P(t)–P(0) represents the actual increase in this population during this interval. In these conditions, and so far as T_{G_2+M} may be considered a constant, one can write:

$$\int_0^t R(\tau)\,d\tau = k_1' \bar{t}_s(t)[P(t + T_{G_2+M}) - P(T_{G_2+M}) + N^*(t)/2] \tag{14}$$

(*cf.* eqn 4). For values of t much larger than T_{G_2+M} we have

$$\int_0^t R(\tau)\,d\tau = k_1' \bar{t}_s(t)[P(t) - P(0) + N^*(t)/2] \tag{15}$$

(*cf.* eqn 5) and for values of P(t) − P(0) much larger than $N^*(t)/2$

$$\int_0^t R(\tau)\,d\tau = k_1' \bar{t}_s(t)[P(t) - P(0)] \tag{16}$$

(*cf.* eqn 6). According to eqns 16 and 12 plotting $\int_0^t R(\tau)\,d\tau$ *v.* C(t) − U(t) is equivalent to plotting $k_1' \bar{t}_s(t)[P(t) - P(0)]$ against $P(t) - P(0).e^{-qt}$. In these conditions, so long as $\bar{t}_s(t)$ remains constant we have a function which starts at zero at time zero and, along with the decay of $P(0).e^{-qt}$, approaches asymptotically the straight line representing $k_2'[P(t) - P(0)]$ *v.* P(t), where $k_2' = k_1' \bar{t}_s(t)$ (see inset of Fig. 7). This straight line has a slope equal to k_2' and intercepts the abscissa at P(0). In fact, as shown in Fig. 7, plotting experimental values of $\int_0^t R(\tau)\,d\tau$ against C(t) − U(t), the points (open and closed circles) are fitted by a function which starts from zero and rapidly approaches a straight line. This indicates that the above conditions, including the constancy of T_{G_2+M} and $\bar{t}_s(t)$, and a negligible P(t) cell loss, are substantially satisfied in our experimental conditions. Thus, by extrapolating the $k_2'[P(t) - P(0)]$ *v.* P(t) straight line, both P(0) and $k_1' \bar{t}_s(t)$ may be estimated. Only very large values of t should be used to extrapolate this straight line. More precisely, any point is acceptable as far as $P(0).e^{-qt}$ becomes negligible as compared to P(t), so that $P(0).e^{-qt}/P(t)$ is less than or equal to the experimental error of cell counting. In our system q is known from eqn 9 (0·025 hr^{-1}), while both P(0) and P(t) are unknown. Therefore, it is only possible, letting P(0) = P(t), to have a cautious estimate of the time beyond which $P(0).e^{-qt}/P(t)$ becomes less than or equal to the experimental error. This time ($\hat{t}$) is the value of t such that

$$e^{-q\hat{t}} = SD/M \tag{17}$$

where SD/M is the standard deviation/mean ratio (coefficient of variation) of cell counting in our conditions. Thus,

$$\hat{t} = -(1/q).\ln (SD/M) \tag{18}$$

As in our experiments $\hat{t}$ has been calculated to be 74·8 hr, the straight line traced in Fig. 7 has been accordingly calculated by fitting the experimental points at $t \geqslant 74{\cdot}8$ hr (black points in Fig. 7). As stated above, dividing the slope of this line (0·33) by k_1' of eqn 8, it is possible to obtain an estimate of $\bar{t}_s(t)$ for Con-A-stimulated mouse splenocytes in our conditions. This estimate (7·6 hr) is close to the 8-hr duration of S calculated by Jones (1973) for the same

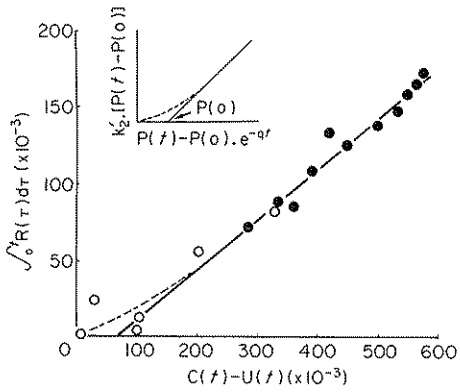

Fig. 7. Relationship between $\int_0^t R(\tau)\,d\tau$ and $C(t) - U(t)$ in splenocyte cultures. Values reported in the graph are means of four determinations, carried out on separate samples, of both $\int_0^t R(\tau)\,d\tau$ and $C(t) - U(t)$. The estimate of $\hat{t} = 74{\cdot}8$ hr has been calculated according to eqn 18, using the q value of eqn 9 (0·025) and the SD/M value of 0·154. The latter is the mean of 60 SD/M determinations, each carried out on four separate samples, obtained in seven experiments.

Open circles, experimental points at $t < \hat{t}$; closed circles, experimental points at $t \geqslant \hat{t}$. The straight line traced in the figure represents the least squares regression line of $\int_0^t R(\tau)\,d\tau$ on $C(t) - U(t)$ for times $\geqslant \hat{t}$, according to the following equation $\int_0^t R(\tau)\,d\tau = -22{,}500 + 0{\cdot}332[C(t) - U(t)]$ with a standard error of the slope = 0·0271 and a correlation coefficient $r = 0{\cdot}97$. The slope of this line corresponds to $k_2' = k_1' \bar{t}_s(t)$ of eqn 16. The intercept of this line with the abscissa gives an estimate of P(0) = 67,600. The least squares regression line of $C(t) - U(t)$ on $\int_0^t R(\tau)\,d\tau$ for times $\geqslant \hat{t}$ (not shown) leads to the following equation $C(t) - U(t) = 89{,}100 + 2{\cdot}841 \int_0^t R(\tau)\,d\tau$ with a standard error of the slope = 0·232. In this case P(0) is directly estimated from the value of the intercept with the ordinate (89,100); the upper 95% confidence limit of this intercept gives a maximum estimate of P(0) = 157,000.

Inset: diagrammatic representation of the function $k_2'[P(t) - P(0)]$ *v.* $P(t) - P(0).e^{-qt}$.

cells by means of autoradiographic techniques. Finally, the intercept of the straight line traced in Fig. 7 with the abscissa leads to an estimate of $P(0) = 6{\cdot}7 \times 10^4$ cells. This represents about 11% of the initial splenocyte population, a percentage not far from that previously reported for lectin stimulated mouse splenocytes (Jones, 1973). Knowing P(0), P(t) can be calculated using eqn 16, while by subtracting P(0) from C(0), we obtain Q(0) and thereby $Q(0).e^{-qt}$.

At this point the splenocyte cytokinetics in our system can be reconstructed as illustrated in Fig. 8. The cells responding to Con-A at time zero are a minor percentage (approximately 10%) of the initial population. Under the influence of the lectin, these cells [P(t)] undergo an intense proliferation leading to an increase of about eight-fold from their initial number in 100 hr, with a minimum doubling time of 20 hr between 30 and 60 hr. In keeping with previous reports (Gunther *et al.*, 1974; Toyoshima, Iwata & Osawa, 1976) this proliferation starts after a lag phase of 20 hr and proceeds, without substantial cell loss and with a time course represented by a sigmoid curve; cells practically stop growing at about 6×10^5 cells/well, after 100–120 hr of incubation. The average time spent in S by the cycling cells appears roughly constant (7·6 hr) at any stage of the culture, although the reservations previously pointed out concerning the possibility of weighing the late changes in t_s by $\bar{t}_s(t)$ computation also apply here. On the other hand, the Con-A-unresponsive cells [Q(t)], which are approximately 90% of the time zero population, undergo an exponential decrease, falling to less than 10% of their initial number after 100 hr. On the whole the initial ratio of Con-A-responsive *v.* unresponsive subpopulations appears completely inverted at the end of incubation, when the cells originated from P(0) represent nearly the totality of the population. The peculiar experimental trend of C(t) values is thus clearly explained. This, in fact,

practically coincides with the kinetics of $P(t) + Q(t)$ (dotted line in Fig. 8), being largely accounted for by the decrease in $Q(t)$ while $P(t)$ is relatively small, and approaching the $P(t)$ kinetics as $Q(t)$ progressively decreases and $P(t)$ expands.

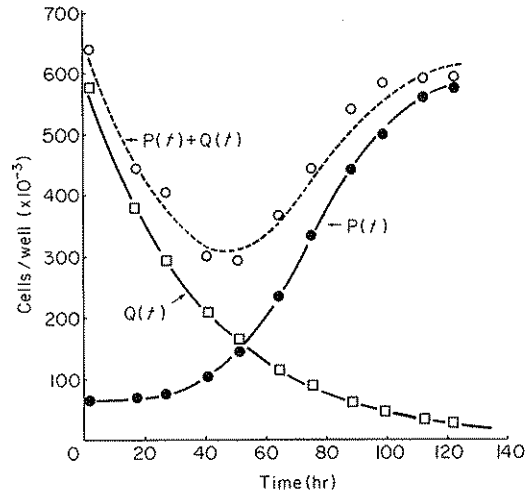

Fig. 8. Cytokinetics of Con-A-treated splenocytes in culture. ○, $C(t)$ experimental points, taken from Fig. 4, [$C(0) = 6{\cdot}4 \times 10^5$]; ●, $P(t)$ calculated from eqn 16 [$P(0) = 67{,}600$, calculated from Fig. 7]; □, $Q(t) = Q(0).e^{-qt}$ for $q = 0{\cdot}025$ (see eqn 10) [$Q(0) = C(0) - P(0) = 572{,}400$]. Dotted line, kinetics of $P(t) + Q(t)$.

Conclusions

This paper shows that the measurement of the cumulative kinetics of labelled thymidine incorporation [$\int_0^t R(\tau)\,d\tau$] is an important contribution to the cytokinetic analysis of normal and neoplastic cell populations. Such kinetics can be correctly determined only by integrating the area under the curve interpolating short labelling pulses [$R(t)$], as lengthy incubation in the presence of radioactive thymidine markedly perturbs the growth parameters of proliferating cells (Cleaver, 1967; Aherne, Camplejohn & Wright, 1977; Pollack, Bagwell & Irvin, 1979; Beck, 1981). The practical utility of knowing the $\int_0^t R(\tau)\,d\tau$ kinetics depends on whether there exists, within the population under study, a direct proportionality between $R(t)$ and the number of cells in S at the corresponding time (eqn 1). This proportionality, in turn, implies that other factors affecting the overall rate of thymidine incorporation by a cell population are negligible as compared to the number of DNA-synthesizing cells. When the above proportionality exists, $\int_0^t R(\tau)\,d\tau$ reflects the product of the sum of the cells which passed through S during the interval 0–t, and the weighted mean [$\bar{t}_s(t)$] of the periods spent in this phase by such cells during the same interval (eqns 3–6). In these conditions, knowing $\int_0^t R(\tau)\,d\tau$ and the number of cells generated in the interval 0–t makes it possible to estimate $\bar{t}_s(t)$. The latter is a useful cytokinetic index, as it allows a technically simple evaluation of the average time spent in S by all the cells passing through this phase from zero up to various times of incubation.

The validity of the kinetic approach summarized by eqns 3–6 can be easily assessed when, as for RI leukaemia cells, the whole population is recruited into growth at time zero and cell loss is practically negligible throughout the incubation. In this case, in fact, one can develop from eqns 3–6 the same relations between $\int_0^t R(\tau)\,d\tau$ and the total increase of cell number, as can be directly established from experimental data (eqn 2). However, as shown for Con-A-stimulated splenocytes, our kinetic approach can also be profitably applied to complex cell populations, in which only a fraction of the cells proliferate under the influence of a mitogen, while the remainder is not substantially affected by the same mitogen. These

conditions are not unlikely to occur in several experimental models, where heterogeneous cell populations are treated *in vitro* with a stimulatory factor specific for a particular cell line more or less widely represented within the whole initial population. Besides lectin or antigen stimulated lymphoid cells, examples of such populations are provided by haemopoietic cells under the influence of colony stimulatory factors or poietins (Quesenberry & Levitt, 1979). To work with these populations, not only eqn 1 must be experimentally proved, but it is also necessary to estimate the real growth of the mitogen responsive fraction. The latter may be difficult to assess directly, since the number of responsive cells within the initial poulation [P(0)] is usually unknown and, as in the case of mouse splenocytes, a concomitant decay of the unresponsive cells may occur, complicating the kinetics of the overall cell number [C(t)]. However, these difficulties may be overcome by determining, in addition to the C(t) kinetics, the time course of the overall cell number in the absence of the mitogen [U(t)] and plotting $\int_0^t R(\tau)\,d\tau$ against $C(t) - U(t)$ (Fig. 7). As far as the mathematical treatment proposed in this paper for Con-A-treated splenocytes can be applied to the cell population under study, this plotting leads to estimates of both $\bar{t}_s(t)$ and P(0) and hence to a complete understanding of the cytokinetics of the model.

Finally, it is worth pointing out some interesting analogies between Con-A-stimulated T blasts and syngeneic RI leukaemia cells which have emerged from the present work. Both types of cells grow in culture with similar sigmoid kinetics, with a plateau at about the same final number (6×10^5 cells/well). This arrest is mainly due to medium exhaustion, as partial renewal of the medium at the plateau of the growth curve is sufficient to make T blasts and leukaemia cells restart proliferation up to 10^6 cells per well (not shown). Furthermore, although with the reservations previously pointed out, estimates of $\bar{t}_s(t)$ suggest that both types of cells maintain the duration of S as constant throughout the different stages of growth, and show very similar values of the proportionality constant between R(t) and the number of cells in S at the corresponding times. These kinetic similarities emphasize the suitability of this experimental model for comparative studies aimed at discovering analogies and differences in the cell cycle regulation of normal and malignant blasts.

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REFERENCES

AGRAWAL, B.B.L. & GOLDSTEIN, I.G. (1967) Protein-carbohydrate interaction. VI. Isolation of Concanavalin A by specific absorption on cross-linked dextran gel. *Biochim. Biophys. Acta*, **147**, 262.

AHERNE, W.A., CAMPLEJOHN, R.S. & WRIGHT, N.A. (1977) In: *An introduction to cell population kinetics*, p. 42. Edward Arnold Publisher, London.

ANDERSSON, J., GRÖNVIK, K.O., LARSSON, E.L. & COUTINHO, A. (1979) Studies on T lymphocyte activation. I. Requirements for the mitogen-dependent production of T cell growth factors. *Eur. J. Immunol.* **9**, 581.

BASERGA, R. (1976) Multiplication and division in mammalian cells. Marcel Dekker, New York.

BECK, H.P. (1981) Radiotoxicity of incorporated [^{3}H]thymidine as studied by autoradiography and flow cytometry. Consequences for the interpretation of FLM data. *Cell Tissue Kinet.* **14**, 163.

CLEAVER, J.E. (1967) Thymidine metabolism and cell kinetics. North Holland Publishing Co., Amsterdam.

COUTINHO, A., LARSSON, E.L., GRÖNVIK, K.O. & ANDERSSON, J. (1979) Studies on T lymphocyte activation. II. The target cells for Concanavalin A-induced growth factors. *Eur. J. Immunol.* **9**, 587.

GELFANT, S. (1977) A new concept of tissue and tumor cell proliferation. *Cancer Res.* **37**, 3845.

GUNTHER, G.R., WANG, J.L. & EDELMAN, M. (1974) The kinetics of cellular commitment during stimulation of lymphocytes by lectins. *J. Cell Biol.* **62**, 366.

HABU, S. & RAFF, M.C. (1977) Accessory cell dependence of lectin-induced proliferation of mouse T lymphocytes. *Eur. J. Immunol.* **7**, 451.

HARRIS, J.W., SHON, B. & MENESES, J. (1973) Relationship between growth and radiosensitivity in P388 murine leukemia. *Cancer Res.* **33**, 1780.

HEWITT, H.B. (1962) The radiosensitivity of tumor cells *in vivo* as studied by cell transplantation techniques. In: *10th International Congress of Radiology*, Abstracts, No. 1980, p. 30. Montreal, Quebec, Canada.

JANIK, P. & STEEL, G.G. (1972) Cell proliferation during immunological perturbation in three transplanted tumours. *Br. J. Cancer*, **26**, 108.

JONES, G. (1973) The number of reactive cells in mouse lymphocyte cultures stimulated by phytohemagglutinin, concanavalin A or hystocompatibility antigen. *J. Immunol.* **111**, 914.

LARSSON, E.L. & COUTINHO, A. (1979) The role of mitogenic lectins in T-cell triggering. *Nature*, **280**, 239.

MAURER, H.R. (1981) Potential pitfalls of [^{3}H]thymidine techniques to measure cell proliferation. *Cell Tissue Kinet.* **14**, 111.

NICOLINI, C. (1975) The discrete plan of the cell cycle: autoradiographic, physical and chemical evidences. *J. Nat. Cancer Inst.* **55**, 821.

O'LEARY, J.J., MEHTA, C., HALL, D.J. & ROSENBERG, A. (1980) Quantitation of [^{3}H]thymidine uptake by stimulated human lymphocytes. *Cell Tissue Kinet.* **13**, 21.

OLIVOTTO, M., BODDI, V. & DELLO SBARBA, P. (1979) Biological and mathematical aspects of growth resumption by resting cells. In: *Biological and mathematical aspects in population dynamics* (Ed. by R. de Bernardi), p. 135. Mem. Ist. Ital. Idrobiol. Supplement **37**.

OLIVOTTO, M. & PAOLETTI, F. (1980) Studies on the kinetics of initial cycle progression *in vitro* of ascites tumour cells subsequent to isolation from ascites fluid. *Cell Tissue Kinet.* **13**, 605.

POLLACK, A., BAGWELL, C.B. & IRVIN, G.L. (1979) Radiation from tritiated thymidine perturbs the cell cycle progression of stimulated lymphocytes. *Science*, **203**, 1025.

QUESENBERRY, P. & LEVITT, L. (1979) Hematopoietic stem cells. *New Engl. J. Med.* **301**, 819.

STEEN, H.B. & LIDMO, T. (1978) The effect of colchicine and colcemid on the mitogen-induced blastogenesis of lymphocytes. *Eur. J. Immunol.* **8**, 667.

STEWART, C.C. & HAMILL, B. (1977) Factors affecting the measurement and interpretation of *in vitro* lymphocyte reactivity. In: *Regulatory mechanisms of lymphocyte activation* (ed. by D. O. Lucas), p. 461. Academic Press, New York.

TICE, R., THORNE, P. & SCHNEIDER, E.L. (1979) Bisack analysis of the phytohaemagglutinin-induced proliferation of human peripheral lymphocytes. *Cell Tissue Kinet.* **12**, 1.

TOYOSHIMA, S., IWATA, M. & OSAWA, T. (1976) Kinetics of lymphocyte stimulation by Concanavalin A. *Nature*, **264**, 447.

WILSON, D.B., BLYTH, J.L. & NOWELL, P.C. (1968) Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of the response. *J. Exp. Med.* **128**, 1157.