Kinetics of T cell turnover following thymectomy

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Abstract. We develop a mathematical formula that provides the number of cells in an isolated population that have divided k times in n days ($0 \leq k \leq n$). This differential cell division formula is applied to the kinetics of peripheral T cells in the diabetes-prone BB rat following thymectomy. These rats received daily intraperitoneal injections of the DNA precursor, bromodeoxyuridine (BrdUrd), over a period of 12–13 days. As the cells divided, they incorporated BrdUrd progressively into their DNA molecules, and the differential formula provides a close prediction of the fraction of BrdUrd-positive T cells present each day during this 'pulse' phase. No further BrdUrd was administered after 13 days, and the diminishing fraction of BrdUrd-positive cells was recorded for several more weeks. The differential cell division formula was capable of describing the rather complex form of the retention curve as BrdUrd-tagged DNA molecules passed to progeny cells during this 'chase' phase. We believe that this simple formula may be found generally useful in describing cell kinetic data in mitotically active cells.

INTRODUCTION

We present here a mathematical model that describes our experimental data on the incorporation of bromodeoxyuridine (BrdUrd) into T cells, and the subsequent release of BrdUrd. The model requires only one parameter, the mean rate of cell division, and yet fits the observed data very closely. While the model was developed for a very specific purpose, it is quite a general representation of cell proliferation that can be used in many other contexts.

T lymphocytes exist in a dynamic state of equilibrium in the periphery of healthy adult animals. During early life, sustained output of T cells from the thymus builds up the peripheral pool of circulating T lymphocytes (PRL). Subsequently, the thymus atrophies and the thymic output of T cells is reduced substantially (Modigliani *et al.* 1994). The size of the PRL remains stable because more than 80% of the peripheral T cells are naïve 'antigen-inexperienced' cells, that can survive for extended periods of time in a quiescent state. The remaining 20% of the T cells consist mainly of 'antigen-experienced' memory T cells that have a slow rate of turnover and maintain the steady-state of the PRL (Tough & Sprent 1994). As a consequence, thymectomy at adulthood does not affect the size of the PRL in any significant manner. The mitotic activity of peripheral T cells is antigen-driven in the case of

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naïve T cells, while that of memory T cells can be triggered by cross-reactive antigens and non-T cell receptor mediated signals (Tanchot *et al.* 1997). The diabetes-prone BioBreeding (BB) rat exhibits a genetically determined peripheral T lymphopenia characterized by a 10-fold reduction in the number of peripheral T cells (Woda *et al.* 1986). The *lyp* mutation carried by the BB rat compromises the survival and persistence of mature T cells in a quiescent state (Ramanathan, Norwich & Poussier 1998). By labelling dividing cells with the DNA precursor, BrdUrd, a high rate of T cell turnover in the periphery of BB rat has been demonstrated (Ramanathan *et al.* 1998). However, the PRL does not increase in size because the progeny are also short-lived. The inability of T cells to accumulate in the periphery of BB rats makes it an excellent model to study the kinetics of T cell turnover. Evaluation of BrdUrd incorporation in peripheral T cells of animals with an intact thymus is complicated since T cells exiting the thymus would have incorporated the label at some undetermined time during their intrathymic development. Therefore, the studies on T cell turnover were performed in adult thymectomized rats. Age-matched non-lymphopenic Wistar Furth rats were used for comparison.

Pulse and *chase* experiments were performed in each of the two groups of rats, BB and WF. In the pulse phase, intraperitoneal injections of BrdUrd were made daily for several days. Accumulation of BrdUrd in T cells was monitored using a flow cytometer. The termination of the pulse phase marked the beginning of the chase phase of the experiment, during which the fate of BrdUrd⁺ cells was followed. BrdUrd was passed on from mother to daughter cells during DNA replication and was lost from the system as cells died. Critical to the analysis of data and, hence, for the understanding of T cell kinetics is the number of times a cell can divide and still register as BrdUrd⁺ on flow cytometry. We shall discuss this further below.

MATERIALS AND METHODS

Experimental

Age and sex-matched inbred Wistar Furth (WF) and diabetes-prone BB rats were purchased from Charles River (Frederick, MD, USA) and from the University of Massachusetts (Worcester, MA), respectively. All animals were housed in specific pathogen-free conditions. BB rats were tested three times a week for the presence of glycosuria and ketonuria. Once the animals became glycosuric, the diagnosis of diabetes was made on the basis of hyperglycaemia (blood glucose > 16.7 mM) for two consecutive days. Diabetic rats were treated with subcutaneous implants of insulin (LinplantTM, University of Toronto, Ontario, Canada). Thymectomy and sham thymectomy were performed as previously described (Poussier *et al.* 1992). T cell turnover 5-bromo-2'-deoxyuridine (BrdUrd, Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (PBS) (10 mg/ml) and 100 mg/kg body weight day⁻¹ was injected intraperitoneally as two injections given at 8:00 a.m. and 8:00 p.m. for various periods of time following thymectomy. The animals were rested for 1 week before the start of BrdUrd injections. During the pulse phase, groups of 2–3 rats received daily injections of BrdUrd for 12 or 13 days and were sacrificed at the time points indicated in Figure 1. During the chase phase, rats were sacrificed at various times as indicated in Figure 2.

BrdUrd incorporation into the DNA of peripheral T cells was assessed as follows (Carayon & Bord 1992; Tough & Sprent 1994). Mononuclear cells pooled from secondary lymphoid organs were incubated with PE-labelled anti-CD3e monoclonal antibody (PharMingen, San Diego, CA, USA). T cells were then sorted using a FACStar (Becton Dickinson). Sorted T

Figure 1. Pulse experiment. The large squares give the measured fraction of labelled cells that were BrdUrd⁺ at various times. Animals had been receiving daily injections of BrdUrd since zero time. The small diamonds indicate the fraction of BrdUrd⁺ cells calculated from equation 2. $\epsilon = 0.14 \text{ day}^{-1}$.

cells were washed and fixed by dropwise addition of 70% ice-cold ethanol. After a 30 min incubation period on ice, cells were washed with PBS, and incubated in PBS containing 1% paraformaldehyde and 0.01% Tween-20 for 30 min at room temperature. Cells were pelleted and exposed to 50 Kunitz units of DNase I (Sigma) in 1 ml 0.15 M NaCl, 4.2 mM MgCl₂, pH 5, for 15 min at room temperature. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated, anti-BrdUrd monoclonal antibody (Becton Dickinson) and analysed by fluoroflow cytometry.

We use the symbol BrdUrd⁺ to represent a cell that contains a measurable quantity of BrdUrd, and BrdUrd⁻ to represent a cell that does not.

Mathematical analysis

It seems quite possible to analyse the kinetics of BrdUrd with only a few assumptions, all of which are substantiated by knowledge of the biological and physical systems involved. We make the following four assumptions:

- 1 That removal of T cells from the system is unaffected by the presence of BrdUrd dye.
- 2 That a constant fraction, ϵ , of cells divide per day, and it is unlikely that any one cell will divide more than once per day.
- 3 That in a pulse experiment, each time a BrdUrd⁻ cell divides, it becomes BrdUrd⁺.
- 4 That in a chase experiment, a BrdUrd⁺ cell that divides for the first time after acquiring the dye produces two BrdUrd⁺ cells, but when each of these daughter cells divides a second time, the progeny cells become BrdUrd⁻. The latter assumption is consistent with the operation of the flowcytometer employed to detect the BrdUrd⁺ cells.

Figure 2. Chase experiment. The large squares give the measured fraction of labelled cells that were BrdUrd⁺ at various times. No BrdUrd had been injected since day zero. ♦ indicate the fraction of BrdUrd⁺ cells calculated from equation 6. $\epsilon = 0.14 \text{ day}^{-1}$. One calculation has been made for each day (each value of n).

Pulse phase

Suppose that we begin a pulse experiment with N'_0 cells, all of which are BrdUrd⁻. A fraction, ϵ , of these cells divide per day, and each cell division gives rise to two daughter cells. Since BrdUrd is present in excess quantities in the rat, each daughter cell will become BrdUrd⁺.

Supposing that, and we shall return to this matter later, no cells die or are removed from the ensemble. Then, after n days, the total number of cells will be equal to $N'_0(1 + \epsilon)^n$. But the number of cells *not* dividing in any one day is $N'_0(1 - \epsilon)$. So the number of cells not dividing for n successive days is equal to $N'_0(1 - \epsilon)^n$. Hence, the number of BrdUrd⁺ cells present after n days is equal to the total number of cells less the number that have not divided at all. That is

$$\text{Number of BrdUrd}^+ \text{ cells after } n \text{ days} = N'_0(1 + \epsilon)^n - N'_0(1 - \epsilon)^n \quad (1)$$

So the fraction, f , of BrdUrd⁺ cells after n days is given by

$$f = \frac{N'_0(1 + \epsilon)^n - N'_0(1 - \epsilon)^n}{N'_0(1 + \epsilon)^n} = 1 - \left(\frac{1 - \epsilon}{1 + \epsilon} \right)^n \quad (2)$$

We have developed expression 2 by stipulating that no cells die or are removed from the ensemble, which is not realistic. It is more realistic to suppose that the lifetime of a cell is governed by the geometric distribution, where the probability of cell removal per day is small

but constant. However, we recall from the first assumption listed above that cell removal is taken to be independent of the presence of BrdUrd. That is, the metabolism and ageing of the cell is unaffected by BrdUrd; the cell cannot recognize the presence of BrdUrd. Under these circumstances, the ratio of the number of either species to the total (*e.g.* the ratio of BrdUrd⁺ cells to total T cells), cannot change *as a result of the process of disappearance*. A similar result is obtained for the case of isotopically labelled molecules (Norwich 1977): specific activity cannot change through the process of biochemical degradation. This principle can be illustrated using a simple analogy. Consider an urn that contains a large number of small balls that are identical in every respect except in colour: some of the balls are red and some are blue. Balls are withdrawn randomly by reaching into the urn, say once each minute, and removing a handful. The colour of the balls that are removed cannot be observed. The ratio of the number of red balls in the urn to the total number of balls in the urn was r before any balls were removed. After m minutes (and m handfuls of balls have been removed), the ratio of red to total balls remaining in the urn will not differ systematically from the original ratio, r . There may, of course, be a random fluctuation in the value of r . If the red-blue distinction cannot be recognized by the withdrawing agent, then r will not change. In the same way, expression 2, which expresses the ratio of BrdUrd⁺ to total cells (*cf* red balls to total balls) for the case of zero cell removal, can be used to predict the shape of the BrdUrd curves of the type shown in Figure 1, where the rate of cell removal is not negligible, provided that BrdUrd (*cf* colour of balls) does not affect the removal process.

Only one parameter, ε , need be estimated from the data. We shall return to the question of parameter estimation later.

Chase phase

The chase phase begins just as the pulse phase ends. Suppose we begin with N_0 cells present at zero time. On day zero, we take the value of f , the fraction of BrdUrd⁺ cells, to be equal to 1.0, *i.e.* all cells are BrdUrd positive. This initial condition is valid for the case of the BB rats only (Fig. 1). On day 1, a fraction, ε , of cells have divided, giving rise to $2\varepsilon N_0$ BrdUrd⁺ cells that have divided once. There then remain $(1 - \varepsilon)N_0$ BrdUrd⁺ cells that have divided zero times. We could continue, in this way, to calculate the number of BrdUrd⁺ cells that have divided once or twice, and the number of BrdUrd⁻ cells that have divided more than once for each successive day of the chase phase. However, with only a little mathematical labour, we can construct an expression that will treat the general case.

Let us examine the expression $N_0[(1 - \varepsilon) + 2\varepsilon]^n$, where n is a positive integer. The k^{th} term of this binomial expansion is the familiar

$$N_0 \frac{n!}{(n-k)!k!} (1 - \varepsilon)^{n-k} (2\varepsilon)^k \quad (3)$$

We can show that expression 3 gives the number of cells that have divided k times by day n , where $k \leq n$

Proof: the number of cells that have divided on a particular set of k days and have failed to divide on the remaining $n - k$ days is equal to $N_0(1 - \varepsilon)^{n-k}(2\varepsilon)^k$. But the number of combinations of k and $n - k$ days is given by the binomial coefficient $n!/[(n-k)!k!]$. Multiplying these expressions gives expression 3 directly. Of course

$$\sum_{k=0}^n N_0 \frac{n!}{(n-k)!k!} (1 - \varepsilon)^{n-k} (2\varepsilon)^k = N_0[(1 - \varepsilon) + 2\varepsilon]^n = N_0(1 + \varepsilon)^n \quad (4)$$

which shows that summing the number of cells that have divided by all possible modes is just equal to the total number of cells present.

Analysis of the chase data is now quite simple, since the flowcytometer records only those cells that have divided zero times or once. Thus the fraction, f , of cells that register BrdUrd⁺ at n days is given by

$$f = \frac{N_0 \sum_{k=0}^1 \frac{n!}{(n-k)!k!} (1-\varepsilon)^{n-k} (2\varepsilon)^k}{N_0(1+\varepsilon)^n} \quad (5)$$

or simply

$$f = \frac{\sum_{k=0}^1 \frac{n!}{(n-k)!k!} (1-\varepsilon)^{n-k} (2\varepsilon)^k}{(1+\varepsilon)^n} \quad (6)$$

Clearly, by just changing the limits of summation 0 to j (instead of 0 to 1), the above expression gives the fraction of cells that have divided j times in n days, which makes it more generally useful. As in expression 2, the denominator in expression 5 comes from the $N_0(1+\varepsilon)^n$ cells that are present after n days, for the case of zero cell death or removal. Again, as in the pulse phase, the process of removal of cells does not change the fraction, f . Expression 6 gives the BrdUrd⁺ fraction as a function of time, and was used to generate the theoretical curves shown in Figure 2.

We selected a single value of the cell division rate, ε , to fit both pulse and chase. The value of ε providing the best overall fit to both pulse and chase data for the BB rats was 0.14 day^{-1} .

However, for WF rats, we must allow for the fact that at the beginning of the chase period only a fraction N_0/N_{TOTAL} of cells were found to be BrdUrd⁺, where N_0 is the number of BrdUrd⁺ cells and N_{TOTAL} is the total number of cells. Therefore, in the chase period, the total number of cells on day n , assuming zero removal, is equal to $N_{\text{TOTAL}}(1+\varepsilon)^n$. That is, expression 6 must be modified slightly. While the numerator in the fraction in expression 6 must be multiplied by N_0 , the denominator must be multiplied by N_{TOTAL} . Therefore, the more general form of expression 6 is

$$f = \frac{N_0}{N_{\text{TOTAL}}} \cdot \frac{\sum_{k=0}^1 \frac{n!}{(n-k)!k!} (1-\varepsilon)^{n-k} (2\varepsilon)^k}{(1+\varepsilon)^n} \quad (7)$$

For the case of BB rats, $N_{\text{TOTAL}} = N_0$, so that expression 7 reduces to expression 6. We did not analyse the WF rats numerically.

DISCUSSION

The problem we encounter is not modelling the pulse phase, but the chase phase. The pulse phase could be modelled quite satisfactorily by the usual differential equation

$$\frac{dN^+}{dt} = k(N_{\text{TOTAL}} - N^+) \quad (8)$$

where $N^+(t)$ is the number of BrdUrd⁺ cells at time, t . That is,

$$N^+(t) = N_{\text{TOTAL}}(1 - e^{-kt}) \quad (9)$$

With appropriate selection of the constant, k , expression 9 will fit the pulse data quite well.

However, the chase phase exhibits a characteristic sigmoid shape: flat for large and small times, and falling steeply for intermediate times. In order to understand this characteristic behaviour of the chase curve, it was necessary to take account of the binding and release of the BrdUrd molecule to and from the DNA molecule. This was accomplished using the binomial model.

We know, from extended observations of the BB rats post-thymectomy, that the cell kinetics are not perfectly stable: the total number of T cells is increasing gradually with time. However, we believe that the value of ε , which defines the cell cycling rate, is essentially constant during the pulse and chase studies reported herein.

Equation 7 is quite a general formula that will provide an estimate of the fraction of cells that have divided k times in n time intervals, where a 'time interval' is the mean or expected period of time between successive cell divisions.

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