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Estimating average cellular turnover from 5-bromo-2′-deoxyuridine (BrdU) measurements

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Cellular turnover rates in the immune system can be determined by labelling dividing cells with 5-bromo-2′-deoxyuridine (BrdU) or deuterated glucose (^{2}H-glucose). To estimate the turnover rate from such measurements one has to fit a particular mathematical model to the data. The biological assumptions underlying various models developed for this purpose are controversial. Here, we fit a series of different models to BrdU data on $CD4^+$ T cells from SIV^- and SIV^+ rhesus macaques. We first show that the parameter estimates obtained using these models depend strongly on the details of the model. To resolve this lack of generality we introduce a new parameter for each model, the 'average turnover rate', defined as the cellular death rate averaged over all subpopulations in the model. We show that very different models yield similar estimates of the average turnover rate, i.e. *ca*. 1% day^{-1} in uninfected monkeys and *ca*. 2% day^{-1} in SIV-infected monkeys. Thus, we show that one can use BrdU data from a possibly heterogeneous population of cells to estimate the average turnover rate of that population in a robust manner.

Keywords: 5-bromo-2′-deoxyuridine; parameter fitting; labelling; T lymphocyte; cellular proliferation; AIDS

1. INTRODUCTION

Several techniques for determining cellular turnover rates in the immune system rely on the incorporation of labelled molecules into the newly synthesized DNA of dividing cells. The base analogue 5-bromo-2′-deoxyuridine (BrdU) can be administered via drinking water and substitutes for thymidine in newly synthesized DNA. $BrdU^+$ cells can be easily detected by flow cytometry, and BrdU labelling has been widely used to study lymphocyte kinetics in animals (Gratzner 1982; Penit & Vasseur 1988; Forster *et al.* 1989; Forster & Rajewsky 1990; Rocha *et al.* 1990; Schittek & Rajewsky 1990; Tough & Sprent 1994, 1998; Dolbeare 1995; Zimmerman *et al.* 1996; Mohri *et al.* 1998; Bonhoeffer *et al.* 2000; Kovacs *et al.* 2001). Cellular turnover rates in man have also been studied by the administration of deuterated glucose (^{2}H-glucose), which leads to deuterium being incorporated into the DNA of dividing cells (Macallan *et al.* 1998; Hellerstein 1999; Hellerstein *et al.* 1999; McCune *et al.* 2000; Mohri *et al.* 2001; Ribeiro *et al.* 2002*a*,*b*).

For systems at steady state, i.e. those in which the total number of cells remains approximately constant, the total rates of cellular production and of cell loss have to be equal. The death rate at steady state is conventionally called the 'turnover' rate. To determine the cellular turnover rate in a certain subpopulation of the immune system, for example $CD4^+$ T cells, one typically records the accumulation of labelled cells in the presence of BrdU, and the loss of labelled cells afterwards. After BrdU withdrawal a rapid loss of the labelled cells has been observed in SIV-infected macaques (Mohri *et al.* 1998; Bonhoeffer *et al.* 2000). This was surprising because the progeny of labelled T cells remain labelled until the label becomes so dilute that it can no longer be detected. To account for the rapid loss of BrdU-labelled T cells in a system at steady state, Mohri *et al.* (1998) found that a source of unlabelled cells that dilutes or 'washes out' labelled cells was important.

Although labelling techniques are widely used to determine the rates of cellular turnover, the measurements are difficult to interpret in the absence of an appropriate mathematical model (Bonhoeffer *et al.* 2000). The methods employed for interpreting the data include: determining the time at which a certain fraction of the cells are $BrdU^+$ (Rocha *et al.* 1990), as a measure for the average cellular half-life; estimating a single turnover parameter, i.e. a replacement rate (Hellerstein *et al.* 1999); fitting a mathematical model with source, proliferation and death parameters to data (Mohri *et al.* 1998; Bonhoeffer *et al.* 2000); and the use of more complex models incorporating clonal expansion and cell heterogeneity (Grossman *et al.* 1999; Bonhoeffer *et al.* 2000). Which model to use to interpret BrdU data is controversial (Grossman *et al.* 1999; Rouzine & Coffin 1999), and existing BrdU data can be fitted to a variety of models, each based upon different assumptions (Grossman *et al.* 1999; Bonhoeffer *et al.* 2000). Here, we show that the parameter estimates obtained by fitting a particular mathematical model to the data strongly depend on the assumptions underlying the model.

One problem with developing appropriate models, and hence with interpreting the data, is that, in the immune system, cell populations are heterogeneous. Populations typically consist of phenotypically different cells, for example $CD45RA^+$ and $CD45RO^+$, and the rate of cell division and cell death may be different not only for

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phenotypically different subpopulations but also for cells in a phenotypically homogeneous population. For lymphocytes, it has been suggested that much of the proliferation is confined to a small subpopulation of cells recently triggered by antigen, and that most of the deaths occur in cells that have recently divided (Grossman *et al.* 1999). Since current measurements provide only the fraction of labelled cells in the whole population (or the fraction of labelled DNA in the whole population in the case of ^{2}H-glucose; Hellerstein 1999), it is infeasible to estimate the individual parameters that characterize the cellular kinetics of each subpopulation.

By comparing the outcomes of different mathematical models fitted to the same BrdU data, we show that the estimates for the activation, proliferation and death rates, as revealed by a nonlinear parameter-fitting procedure, depend, crucially, on the assumptions of the model and the way it accounts for the heterogeneity of the population. When sampling from a possibly heterogeneous population at steady state by the BrdU technique, one should at least be able to estimate the *average* death rate of that population. We will show that averaging death rates over all subpopulations within a model yields an estimate of the turnover rate that seems reasonably independent of the particular model used. Hence, this procedure should provide a more reliable estimate of the true average turnover rate of the population. We find that SIV infection tends to double the average death rate.

2. ONE-COMPARTMENT MODELS

A simple one-compartment model was proposed by Mohri *et al.* (1998) to explain BrdU data. Here, we rewrite this model considering two subpopulations of 'resting' cells, R, with a slow turnover and 'activated' cells, A, with a more rapid turnover, as suggested earlier (Grossman *et al.* 1999; Bonhoeffer *et al.* 2000). The activated cells have a source of s cells per day from elsewhere, and proliferate and die at per-cell rates ρ and δ, respectively,

$$\mathrm{d}A/\mathrm{d}t = s + (\rho - \delta)A. \tag{2.1}$$

The nature of the source remains unspecified, and should be thought of as a generalized 'production term', which could represent the production of cells in the thymus and/or an inflow of proliferating activated cells from a compartment of resting cells (Mohri *et al.* 1998; Bonhoeffer *et al.* 2000). We will elaborate on the latter interpretation and write models where the source represents activation and clonal expansion of resting cells. However, we prefer a more general interpretation of the source because: (i) our main conclusion will be that for estimating the average turnover it makes little difference what model one uses; and (ii) biologically, activation and clonal expansion of resting cells need not be the major component of the source we estimate from labelling experiments. For instance, rapid clonal expansion of labelled cells can lead to BrdU dilution, which would also appear as a source of unlabelled cells during the delabelling phase. The 'resting' cells are considered hardly to pick up BrdU on the time-scale of these experiments.

Without loss of generality, one can scale the steady state total number of cells to one, i.e. $R + A = 1$. Because, at steady state, the total number of activated cells remains constant, i.e. $\mathrm{d}A/\mathrm{d}t = 0$, one obtains for the source $s = (\delta - \rho)A$. Let L be the fraction of cells labelled with BrdU, and U be the fraction of unlabelled activated cells. Initially, no cells are labelled ($L(0) = 0$). During the labelling period, unlabelled cells are lost by death and by proliferation (since they acquire label during the S-phase). Assuming that during the labelling period the source yields labelled cells, and that labelling is 100% efficient so that upon division each progeny acquires a label, one obtains from equation (2.1) (Mohri *et al.* 1998; Bonhoeffer *et al.* 2000):

$$\frac{\mathrm{d}L}{\mathrm{d}t} = s + 2\rho U + (\rho - \delta)L, \tag{2.2}$$

where the factor two appears because an unlabelled cell divides into two daughter cells.

Using $s = (\delta - \rho)(1 - R)$ and $U = 1 - L - R$, this has the solution

$$L(t) = \alpha(1 - \mathrm{e}^{-(\delta+\rho)t}), \tag{2.3}$$

where $\alpha = 1 - R$ is the maximum that the fraction of labelled cells would approach if BrdU were given indefinitely. If labelling is less than 100% efficient, we are underestimating ρ during the labelling period (Mohri *et al.* 1998; Bonhoeffer *et al.* 2000). However, in the experiments of Mohri *et al.* (1998), bone-marrow aspirations performed on each macaque at week 3 showed that the rapidly proliferating myeloid cells were, on average, 87% BrdU$^+$, which suggests an adequate uptake of BrdU in proliferating cells.

In the period shortly after BrdU administration has ended, division of labelled cells yields labelled daughter cells (Mohri *et al.* 1998). Assuming that during this period the source provides only unlabelled cells (Mohri *et al.* 1998), which seems a fair assumption if the source represents activation and clonal expansion of resting cells, one obtains from equation (2.1)

$$\frac{\mathrm{d}L}{\mathrm{d}t} = (\rho - \delta)L, \tag{2.4}$$

with the solution

$$L(t) = L(t_e)\mathrm{e}^{-(\delta-\rho)(t-t_e)}, \tag{2.5}$$

where $L(t_e)$ is the fraction of labelled cells at the time, t_e, that BrdU administration ends.

From the model, one can easily extract the quantities $\rho + \delta$, characterizing the initial slope, or up-slope, of the labelling curve, $\delta - \rho$, characterizing the rate of decay of labelled cells after labelling has ended, or down-slope, and α, the asymptote of the labelling curve. By fitting the model to the data, one can generally estimate the three independent parameters ρ, δ and α. Although the model presented by Mohri *et al.* (1998) was slightly more restricted because α was formally not a free parameter, the parameter estimates in that paper were actually obtained by treating α as an independent parameter. Thus, the original analysis (Mohri *et al.* 1998) was performed with a model that is mathematically identical to the one presented above.

Table 1. Fitting the one-compartment model with three free parameters to the $CD4^+$ T-cell BrdU data of SIV-infected and uninfected rhesus macaques.
(Infected monkeys are divided into two groups with high (H) and low (L) viral loads. Uninfected monkeys are identified by a U. All parameters have been multiplied by 100, and can hence be interpreted as percentages. The 95% confidence limits were calculated by a bootstrap method (Efron & Tibshirani 1986) and are shown in parentheses. Monkey H1292 was removed from the analysis because there was only one datum in the period it was drinking enough $BrdU^+$ water, and one cannot estimate either α or $\rho + \delta$ from a single point. (i.d., identification number; SSR, residual sum of squares.))

i.d.	$\rho \times 100$ day^{-1}	$\delta \times 100$ day^{-1}	$\alpha \times 100$	$\hat{\rho} \times 100$ day^{-1}	$\hat{\delta} \times 100$ day^{-1}	SSR
H1284	0.0 (0.0–3.7)	2.2 (1.8–5.8)	95 (38–100)	0.0 (0.0–1.5)	2.1 (1.5–2.4)	92
H1296	10.7 (2.6–246.5)	14.6 (7.0–250.7)	15 (12–19)	1.6 (0.5–32.9)	2.1 (1.2–33.5)	32
H1314	0.0 (0.0–2.0)	1.4 (0.7–3.8)	72 (25–100)	0.0 (0.0–0.5)	1.0 (0.7–1.3)	55
H1316	2.5 (0.4–5.1)	4.4 (2.5–6.8)	44 (34–76)	1.1 (0.3–1.8)	1.9 (1.7–2.4)	31
H1348	5.0 (0.9–14.5)	9.3 (5.7–18.3)	34 (28–48)	1.7 (0.4–4.1)	3.1 (2.4–5.4)	77
H1442	0.0 (0.0–4.4)	2.9 (2.2–7.0)	77 (35–93)	0.0 (0.0–1.6)	2.2 (1.6–2.7)	117
mean ± s.d.	3.0 ± 4.3	5.8 ± 5.1	56 ± 30	0.7 ± 0.8	2.1 ± 0.7	
L1294	0.1 (0.0–0.4)	1.1 (0.6–1.3)	100 (100–100)	0.1 (0.0–0.4)	1.1 (0.6–1.3)	86
L1324	1.3 (0.2–2.5)	3.1 (2.0–4.2)	38 (29–61)	0.5 (0.1–0.7)	1.2 (1.1–1.3)	5
L1380	0.9 (0.0–3.4)	3.0 (1.9–5.4)	27 (17–46)	0.2 (0.0–0.6)	0.8 (0.7–1.0)	8
L1394	2.2 (0.0–6.6)	4.2 (2.1-8.4)	17 (13–37)	0.4 (0.0–0.9)	0.7 (0.6–1.1)	9
L1436	0.0 (0.0–4.2)	4.3 (3.2–7.8)	62 (35–73)	0.0 (0.0–1.5)	2.7 (2.1–3.2)	112
mean ± s.d.	0.9 ± 0.9	3.1 ± 1.3	49 ± 33	0.2 ± 0.2	1.3 ± 0.8	
U1372	0.3 (0.2–0.3)	0.5 (0.3–0.6)	100 (100–100)	0.3 (0.2–0.3)	0.5 (0.3–0.6)	3
U1426	0.0 (0.0–0.2)	0.9 (0.7–1.0)	100 (100–100)	0.0 (0.0–0.2)	0.9 (0.7–1.0)	13
U1458	0.1 (0.0–0.2)	0.9 (0.7–1.0)	100 (100–100)	0.1 (0.0–0.2)	0.9 (0.7–1.0)	12
U1466	0.0 (0.0–2.5)	1.6 (1.2–3.8)	83 (30–100)	0.0 (0.0–0.7)	1.3 (1.0–1.4)	20
mean ± s.d.	0.1 ± 0.1	1.0 ± 0.5	96 ± 8	0.1 ± 0.1	0.9 ± 0.3	

(a) *Fitting the data*

Table 1 gives the estimates for the three parameters in the model for the $CD4^+$ T-cell data published by Mohri *et al.* (1998). Averaging the parameter estimates over the groups of monkeys with a high viral load, those with a low viral load, and uninfected monkeys suggests that SIV infection increases the proliferation rate, ρ, more than 10-fold, and the death rate, δ, almost sixfold (see table 1 and Mohri *et al.* (1998) for a statistical comparison). Because the estimated proliferation rate is typically much smaller than the death rate, the production of $CD4^+$ T cells is in most monkeys largely accounted for by the source (see table 1). Because in adult monkeys the thymus is expected to have a minor contribution to the total-body production of $CD4^+$ T cells (Steinmann *et al.* 1985), the parameter estimates in table 1 and Mohri *et al.* (1998) imply a non-thymic nature of the source.

There are several problems with fitting the three-parameter Mohri *et al.* (1998) model to these data. First, the 95% confidence limits (shown in parentheses) are large, and for several monkeys the proliferation rate, ρ, is statistically indistinguishable from zero and the fraction of activated cells, α, is indistinguishable from one. Second, the fraction of activated cells, α, tends to be larger in uninfected monkeys than in infected monkeys. This is unlikely to be true, and is probably the result of the fact that the percentage of labelled cells increases so slowly in uninfected monkeys that the asymptotic value α is not yet approached, and hence cannot be estimated reliably. Third, there is a trade-off between α and ρ: in infected monkeys we find non-zero proliferation rates only when α is small. Similarly, death rates tend to be high when α is small.

The α column in table 1 shows that each particular monkey has a different value for the fraction of cells that can maximally become labelled (if BrdU were given indefinitely). Whenever $\alpha < 1$, the model allows for a non-zero subpopulation of resting cells, R, that is biologically 'inert' to BrdU labelling because of their slow turnover. Thus, the estimates for the proliferation and death rates given in table 1 are valid only for the fraction α of the population that is capable of becoming labelled by BrdU, and are not valid averages for the whole $CD4^+$ T population. Importantly, this implies that the biological interpretation of ρ and δ depends on the estimate of α. When $\alpha = 1$, the estimates for the proliferation and death rates reflect the population average, but when $\alpha < 1$, ρ and δ reflect the kinetics of the activated subpopulation. Unfortunately, the estimate of α defining the two subpopulations is inaccurate because the labelling period is far too short to allow an approach to the asymptote.

To minimize the impact of inaccuracies in estimating α, we define an 'average death rate' of the whole population in the one-compartment model as $\hat{\delta} \simeq \alpha\delta$. Similarly, one can define an 'average proliferation rate' as $\hat{\rho} = \alpha\rho$. The averaging procedure not only corrects the difference in interpretation of the parameters, but also compensates for the high variability in α, i.e. monkeys with a low estimate for α implicitly obtained high estimates for δ, so that the differences in $\hat{\delta}$ between monkeys with a high viral load and uninfected monkeys decrease considerably. The average proliferation rate differs approximately sevenfold and the average death rate differs about twofold between uninfected and infected monkeys (see table 1). Moreover, the average death rates of 0.9% day^{-1} in uninfected monkeys and 2.1% day^{-1} in infected monkeys are in good agreement with estimates obtained in humans with a variety of techniques (Clark *et al.* 1999).

The average proliferation rate remains statistically indistinguishable from zero for several of the monkeys (table 1). In the three-parameter Mohri *et al.* (1998) model both the

Table 2. Fitting the $\rho = 0$ two-parameter simplification of the basic model to the $CD4^+$ T-cell data. (i.d., identification number; SSR, residual sum of squares.)

i.d.	$\delta \times 100$ day^{-1}	$\alpha \times 100$	$\hat{\delta} \times 100$ day^{-1}	SSR
H1284	2.2 (1.8–3.0)	94 (79–100)	2.1 (1.8–2.5)	92
H1296	5.9 (3.7–8.0)	25 (22–34)	1.4 (1.1–2.0)	58
H1314	1.4 (0.8–2.9)	71 (42–100)	1.0 (0.7–1.3)	55
H1316	2.2 (1.9–2.8)	95 (83–100)	2.1 (1.9–2.4)	51
H1348	5.2 (3.7–6.8)	54 (46–65)	2.8 (2.2–3.4)	128
H1442	2.9 (1.9–4.1)	76 (62–100)	2.2 (1.8–2.7)	117
mean ± s.d.	3.3 ± 1.8	70 ± 27	1.9 ± 0.6	
L1294	1.2 (1.0–1.4)	100 (100–100)	1.2 (1.0–1.4)	89
L1324	1.9 (1.6–2.2)	70 (64–79)	1.4 (1.3–1.5)	8
L1380	2.3 (1.7–2.8)	41 (37–50)	0.9 (0.8–1.0)	8
L1394	2.2 (1.6–2.9)	36 (32–49)	0.8 (0.7–1.0)	12
L1436	4.4 (3.3–6.0)	61 (51–72)	2.7 (2.2–3.3)	111
mean ± s.d.	2.4 ± 1.2	62 ± 25	1.4 ± 0.8	
U1372	0.8 (0.7–0.9)	100 (100–100)	0.8 (0.7–0.9)	12
U1426	0.9 (0.8–1.0)	100 (100–100)	0.9 (0.8–1.0)	13
U1458	1.0 (0.9–1.1)	100 (100–100)	1.0 (0.9–1.1)	13
U1466	1.5 (1.2–2.0)	83 (70–100)	1.3 (1.2–1.4)	20
mean ± s.d.	1.0 ± 0.3	96 ± 8	1.0 ± 0.2	

Table 3. Fitting the $\alpha = 1$ two-parameter simplification of the basic model to the $CD4^+$ T-cell data. (i.d., identification number; SSR, residual sum of squares.)

i.d.	$\rho = \hat{\rho} \times 100$ day^{-1}	$\delta = \hat{\delta} \times 100$ day^{-1}	SSR
H1284	0.0 (0.0–0.3)	2.0 (1.6–2.3)	95
H1296	0.0 (0.0–0.3)	0.7 (0.2–0.9)	220
H1314	0.0 (0.0–0.3)	0.9 (0.4–1.1)	59
H1316	0.0 (0.0–0.2)	2.1 (1.7–2.3)	53
H1348	0.0 (0.0–0.5)	1.9 (0.8–2.2)	549
H1442	0.0 (0.0–0.3)	2.0 (1.3–2.2)	164
mean ± s.d.	0.0 ± 0.0	1.6 ± 0.6	
L1294	0.1 (0.0–0.4)	1.1 (0.6–1.4)	86
L1324	0.0 (0.0–0.2)	1.2 (0.9–1.3)	32
L1380	0.0 (0.0–0.3)	0.7 (0.3–0.8)	47
L1394	0.0 (0.0–0.0)	0.6 (0.5–0.7)	45
L1436	0.0 (0.0–0.4)	2.0 (1.0–2.3)	375
mean ± s.d.	0.0 ± 0.0	1.1 ± 0.6	
U1372	0.3 (0.1–0.3)	0.5 (0.4–0.6)	3
U1426	0.0 (0.0–0.2)	0.9 (0.7–1.0)	13
U1458	0.1 (0.0–0.2)	0.9 (0.7–1.0)	12
U1466	0.0 (0.0–0.0)	1.2 (1.1–1.3)	25
mean ± s.d.	0.1 ± 0.1	0.9 ± 0.3	

source and the proliferation terms account for the *de novo* production of $CD4^+$ T cells. We therefore examined the consequences of forcing all production to be from the source (by fixing $\rho = 0$, see table 2). Although this simplified version of the model has only two parameters, i.e. α and δ, the fits to the data are almost as good as those of the three-parameter model shown in table 1. Moreover, for several monkeys in table 2 the 95% confidence limits on the α parameter include $\alpha = 1$. Thus, the data from these monkeys can also be successfully fitted to a one-parameter simplification of the original model where we fix $\alpha = 1$ and $\rho = 0$ (not shown). For these monkeys, the death rate estimated with this one-parameter simplification is comparable to the average death rate, $\hat{\delta}$, estimated by the two- and three-parameter versions of the model (not shown). Note that the turnover rate of this one-parameter simplification is similar to the replacement rate estimated by Hellerstein *et al.* (1999). We also fitted the data to the other two-parameter version of the model where we fix $\alpha = 1$ (table 3). This allows for reasonable fits in all uninfected monkeys and in a subset of the infected monkeys. Note that almost all proliferation rates now become statistically indistinguishable from zero.

In summary, most of the data can be fitted to a variety of models. Whenever different models yield similar residual sums of squares (SSR), the fits are statistically indistinguishable (which was confirmed by visual inspection of the fits of the different models). The biological interpretations of the parameter estimates provided by the different models are quite different. The conclusion that SIV infection increases the proliferation rate more than 10-fold (or the average proliferation rate sevenfold) is

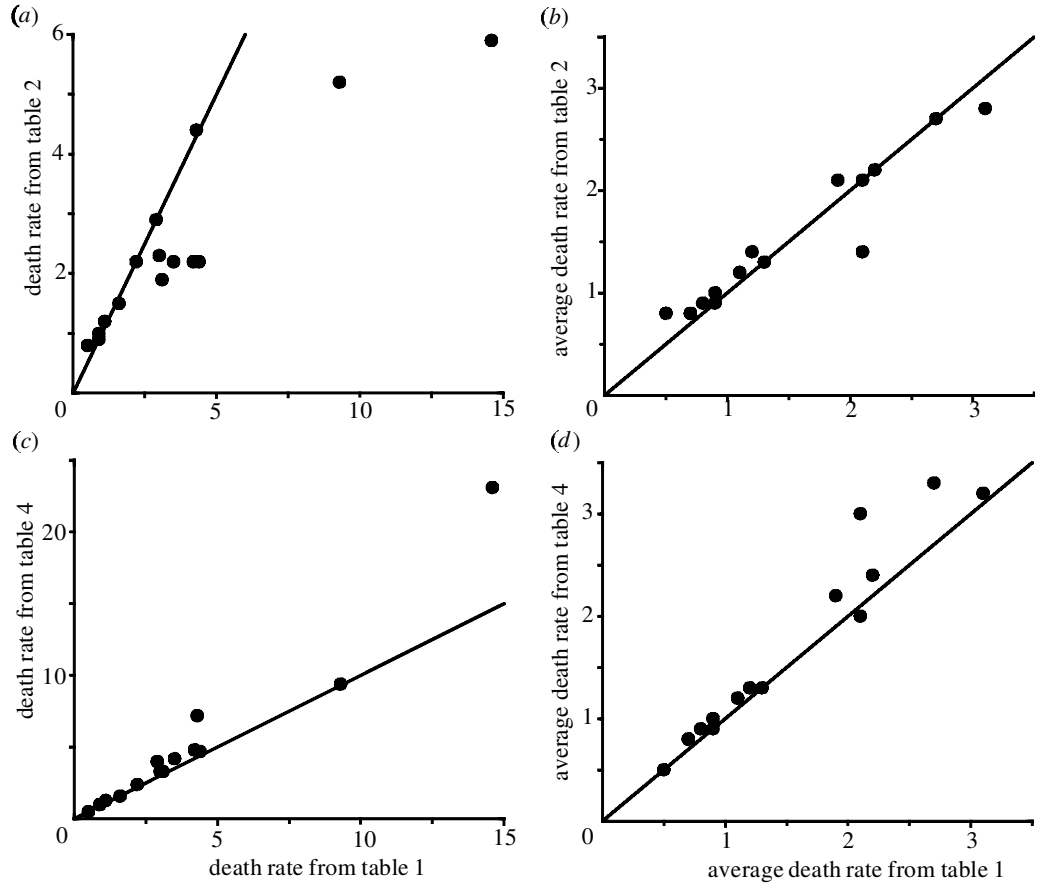

Figure 1. Comparing the death rates and the average death rates of the different models. The symbols in the panels depict $100 \times \delta$ in tables 2 and 4 as a function of its value in table 1.

obviously irrelevant for the $\rho = 0$ simplification of the model where all production is accounted for by the source. Because the fits of the $\rho = 0$ simplification are of comparable quality to those with a free ρ parameter, the estimated increases in the proliferation rate (Mohri *et al.* 1998) are unreliable. Similarly, drawing conclusions about the size of the activated compartment remains unreliable.

The only parameter that differs little between the different models is the average death rate, $\hat{\delta}$ (see figure 1). Also, note that the average death rates are considerably smaller than the death rates (figure 1), which is a natural result because the death rate applies only to a more active sub-population. Thus, the conclusion that SIV infection increases the death rate of the average $CD4^+$ T cell from 1% to *ca.* 2% seems reliable because it is independent of the biological assumptions. The five monkeys with a low viral load have an intermediate average death rate. This twofold difference between uninfected monkeys and monkeys with a high viral load is considerably smaller than the sevenfold increase in δ, and than the more than 10-fold increase in ρ in table 1 (and that in Mohri *et al.* (1998)), but is in good agreement with human data (Clark *et al.* 1999). We conclude that these BrdU data allow one to estimate reliably only the average rate of death. The other parameter estimates depend too much on the underlying modelling assumptions.

3. TWO-COMPARTMENT MODEL

Grossman *et al.* (1999) criticized the Mohri *et al.* (1998) model by arguing that labelled cells should, on average, have a shorter lifespan than unlabelled cells. Labelled cells are cells that have recently divided and hence should be more prone to apoptosis than unlabelled cells. In an attempt to resolve this issue, we developed a new model in which we explicitly state the nature of the source.

Consider a compartment of activated cells A that divide (at rate ρ), and are generated from resting cells R upon activation (at rate γ). Activated cells die (at rate δ), or revert to a resting state (at rate r). Resting cells are defined as cells that never divide; they die at rate μ, and may be produced by the thymus at rate σ, i.e.

$$\frac{\mathrm{d}A}{\mathrm{d}t} = c\gamma R + \rho A - (\delta + r)A, \tag{3.1}$$

$$\frac{\mathrm{d}R}{\mathrm{d}t} = \sigma + rA - (\mu + \gamma)R. \tag{3.2}$$

Here, c is a clonal expansion parameter: if $c > 1$, each activated resting cell gives rise to a clone of c activated cells. Antigenic stimulation of naive $CD4^+$ T cells is indeed known to trigger a cascade of cell divisions (Bajenoff *et al.* 2002; Foulds *et al.* 2002; Lee *et al.* 2002). The total number of cells, $T = A + R$, is again scaled to one. The total numbers of resting and activated cells should each remain constant during a BrdU experiment; we therefore let $\mathrm{d}A/\mathrm{d}t = \mathrm{d}R/\mathrm{d}t = 0$ (see Appendix A). This model is similar to the one developed by Bonhoeffer *et al.* (2000). However, they allowed resting cells to divide slowly, and ignored the return of activated cells to a resting state.

Table 4. Fitting the two-compartment model to the $CD4^+$ T-cell data.
(Since in adult monkeys the thymic source of resting cells should be small we set $\sigma = 0$. Because the main point of this table is that the variation in the original parameters of the model is unrealistically large, i.e. several orders of magnitude, we do not calculate averages and standard deviations for these parameters. (i.d., identification number; SSR, residual sum of squares.))

i.d.	c	ρ	$\delta \times 100$	μ	γ	r	$\hat{A} \times 100$	$\hat{\delta} \times 100$	SSR
H1284	443	2×10^{-3}	2.4	2×10^{-9}	3×10^{-4}	5×10^{-5}	84	2.0	93
H1296	25	5×10^{-2}	23	1×10^{-3}	1×10^{-3}	2×10^{-2}	13	3.0	7
H1314	6	2×10^{-5}	4.2	5×10^{-4}	2×10^{-3}	1×10^{-2}	19	0.8	67
H1316	399	2×10^{-2}	4.7	4×10^{-3}	6×10^{-5}	5×10^{-3}	42	2.2	25
H1348	310	5×10^{-2}	9.4	2×10^{-4}	7×10^{-5}	6×10^{-4}	33	3.2	77
H1442	339	6×10^{-6}	4.0	4×10^{-3}	2×10^{-4}	3×10^{-3}	56	2.4	127
mean ± s.d.							41 ± 26	2.3 ± 0.9	
L1294	818	1×10^{-11}	1.3	5×10^{-3}	2×10^{-4}	4×10^{-4}	93	1.2	86
L1324	29	7×10^{-3}	3.3	3×10^{-3}	6×10^{-4}	6×10^{-3}	35	1.3	3
L1380	6	1×10^{-8}	3.3	2×10^{-5}	2×10^{-3}	7×10^{-3}	25	0.9	5
L1394	5	1×10^{-2}	4.8	4×10^{-4}	2×10^{-3}	1×10^{-2}	15	0.8	7
L1436	386	9×10^{-8}	7.2	5×10^{-3}	1×10^{-4}	7×10^{-3}	42	3.3	106
mean ± s.d.							42 ± 40	1.5 ± 1.0	
U1372	139	3×10^{-3}	0.5	3×10^{-9}	1×10^{-3}	2×10^{-5}	99	0.5	3
U1426	607	3×10^{-10}	1.0	2×10^{-3}	3×10^{-4}	9×10^{-5}	95	0.9	13
U1458	279	1×10^{-10}	1.0	4×10^{-3}	7×10^{-4}	2×10^{-4}	95	1.0	12
U1466	225	2×10^{-9}	1.6	2×10^{-4}	3×10^{-4}	1×10^{-4}	82	1.3	20
mean ± s.d.							93 ± 7	0.9 ± 0.3	

Although the model given by equations (3.1) and (3.2) has too many parameters for accurate parameter estimation, we have fitted it to the data and show the parameter estimates in table 4. Parameters indeed differ by orders of magnitude between the different monkeys (and the confidence limits are huge (not shown)). The steady-state fraction of activated cells, $\hat{A}$, is similar to the α estimates of the previous models, and again tends to be higher in uninfected monkeys. Having two explicit compartments, it is now straightforward to define the average death rate as $\hat{\delta} = \mu\hat{R} + \delta\hat{A}$, where $\hat{R}$ and $\hat{A}$ are the steady-state fractions of resting and activated cells and are given by equations (A 1) and (A 2) in Appendix A, respectively. For most monkeys, the average death rate is quite similar to that estimated with the one-compartment models (see table 4 and figure 1*d*). The most notable exception is H1296, whose estimated average death rate, $\hat{\delta} = 3\%\ \text{day}^{-1}$, is considerably higher than the $\hat{\delta} = 2.1\%\ \text{day}^{-1}$ estimate obtained with the three-parameter one-compartment model (table 1). The reason for this difference is that the two-compartment model has a much better fit to the data for this monkey (i.e. the SSR drops from 32 to 7; see table 4 and figure 2). The fact that only the average death rates, $\hat{\delta}$, have remained similar in this new model further supports our main result that the BrdU data allow one to estimate only average death rates.

We estimate a considerable clonal expansion, c, for all monkeys in table 4, and in some monkeys very little proliferation, ρ. The large estimates for the clonal expansion are in good agreement with recent data (Bajenoff *et al.* 2002; Lee *et al.* 2002), but should be interpreted with caution because one can also fit the data while fixing the clonal expansion at much lower values, provided $c > 1$. Allowing for only three divisions, i.e. $c = 8$, works well (not shown). Having clonal expansion, the model resembles the Grossman *et al.* (1999) model. Because we find that $\delta > \mu$, we naturally conclude that labelled cells, which predominate in the activated compartment, have a shorter expected lifespan than unlabelled cells, which was the main assumption of Grossman *et al.* (1999). The model also resembles the one-compartment model because it consists of a compartment of cells that tend to become labelled, and a second compartment of cells that hardly pick up labelling (i.e. the reversal parameter r tends to be small). As a consequence, the $c\gamma R$ term corresponds to the 'source' term in the one-compartment model (see below).

The loss of labelled cells after BrdU withdrawal with this model requires that $c > 1$. If one sets $c = 1$ in equation (3.1) (and $\sigma = 0$ in equation (3.2)), the proliferation term ρA is the only production term (which can be checked by adding equations (3.1) and (3.2)). Because, at steady state, the total production has to balance total death, i.e. $\rho\hat{A} \simeq \delta\hat{A} + \mu\hat{R}$, one obtains $\rho > \delta$. By equation (A 6) in Appendix A, $\rho > \delta$ would imply that the fraction of labelled cells expands after BrdU is withdrawn, which is not the case. The finding that $c > 1$ could suggest that much of the proliferation occurs in cascades in which resting cells, after being activated, expand into a clone of activated cells. Thus, the fact that one typically fits a low proliferation rate and a large source (Mohri *et al.* 1998), provides hardly any information on the total proliferation (or production) in the $CD4^+$ T-cell population.

Finally, we show that the one-compartment model that we started with is consistent with a two-compartment interpretation. The two-compartment model may be simplified by assuming that resting cells do not become labelled, i.e. we assume $R_L = 0$ (see Appendix A). We therefore treat R as a constant, and ignore the reversion of activated cells to rest. Equation (3.1) can then be rewritten as:

$$\frac{\mathrm{d}A}{\mathrm{d}t} = aR + (\rho - \delta)A, \tag{3.3}$$

where $a = c\gamma$ combines activation and clonal expansion. Assuming that in the presence of BrdU all cells undergo-

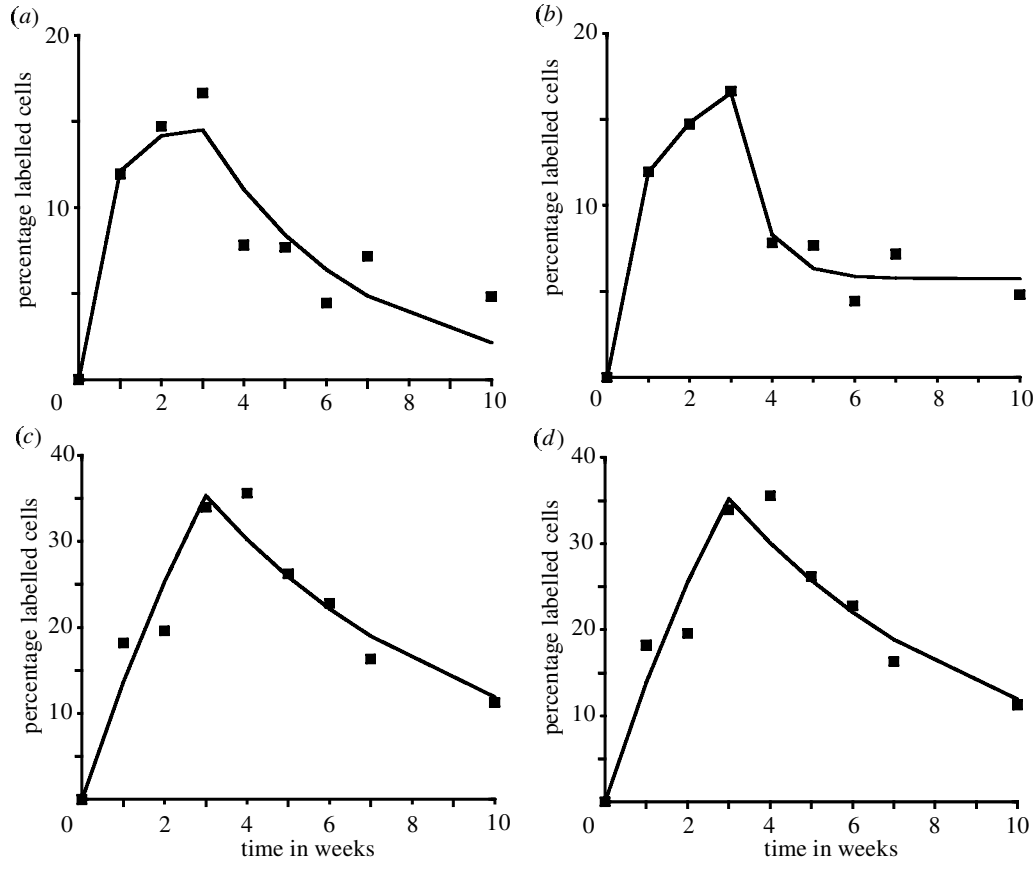

Figure 2. Comparing the fits of the basic model with the two-compartment model. (*a*,*c*) The fits of the full one-compartment model (see table 1). (*b*,*d*) The fits of the two-compartment model (see table 4). (*a*,*b*) The atypical monkey H1296 where the two-compartment model fits a biphasic decline. (*c*,*d*) The more typical H1484 monkey where both models fit a single exponential after BrdU withdrawal.

ing clonal expansion become labelled, one obtains for the fraction of labelled activated cells:

$$\frac{\mathrm{d}L}{\mathrm{d}t} = aR + 2\rho U + (\rho - \delta)L, \tag{3.4}$$

where $U = 1 - L - R$ is the fraction of unlabelled activated cells. Since $A + R = 1$, and at steady state $\mathrm{d}A/\mathrm{d}t = 0$, one can eliminate the parameter a by using the steady-state condition $a = (\delta - \rho)(1 - R)/R$. Thus, the solution of equation (3.4), with the initial condition $L(0) = 0$, is the same as equation (2.3) of the one-compartment model. Under this interpretation of the source parameter, the source should indeed yield labelled cells during BrdU administration and yield largely unlabelled cells in the absence of BrdU.

This interpretation of the source is not the only one. For instance, one could also argue that in the absence of BrdU, labelled activated cells may undergo considerable clonal expansion and appear as a 'source' of unlabelled cells (Rouzine & Coffin 1999). Summarizing, many different models can fit the data, and our most important conclusion remains that the average death rate can be estimated independently of the precise biological assumptions.

4. MANY-COMPARTMENT MODELS

In the two-compartment model the proliferation cascade induced by the activation of a resting cell is instantaneous, i.e. the model ignores delays caused by having several rounds of cell division. Grossman *et al.* (1999) argued that such delays are crucial because the cells, having picked up BrdU in the proliferation cascade, would be expected to die rapidly by apoptosis once they approach the end of the cascade. Grossman *et al.* (1999) developed a model involving two populations of resting cells, R and R_o, three populations of activated cells A_i corresponding to three rounds of cell division, and effector cells E. They showed that this model was able to explain BrdU data for $CD4^+$ T cells. One can easily define an average death rate for the Grossman *et al.* (1999) model by averaging the death rates of their six subpopulations at steady state, i.e. $\hat{\delta} = \delta_0 R_0 + \delta_r R + b_1 A_1 + b_2 A_2 + b_3 A_3 + \delta_E E$. Computing this average turnover rate for the parameters of the uninfected U1372 and the infected H1316 macaques shown in Grossman *et al.* (1999), we find $\hat{\delta} = 0.004\ \mathrm{day}^{-1}$ and $\hat{\delta} = 0.028\ \mathrm{day}^{-1}$, respectively. These values are in good agreement with the average turnover rates obtained with the four models analysed above (where we found average death rates of 0.005 and 0.02 day^{-1}, respectively). This confirms our main result that our procedure of estimating the average death rate is reliable and fairly independent of the choice of model.

5. DISCUSSION

We have shown that many different mathematical models can be fitted to the BrdU data obtained from $CD4^+$

T cells in SIV-infected and uninfected rhesus macaques. Since each model yields its own particular parameter estimates, it seems difficult, if not impossible, to determine which estimates reflect the true kinetic parameters of $CD4^+$ T cells. Similar concerns about how the choice of the model affects data interpretation have been raised in a recent paper (Asquith *et al.* 2002) that appeared after the submission of this paper. We have demonstrated that some of these problems can be solved by computing an 'average turnover rate' by averaging the death rates of all subpopulations in the model. In all of the models studied here, we estimate similar average turnover rates when the different models are fitted to the same data. Because the average turnover rate seems independent of the precise assumptions of each model, we suggest a conclusive estimate for the average turnover of $CD4^+$ T cells of *ca*. 1% in uninfected monkeys and *ca*. 2% in chronically infected monkeys. Moreover, these particular estimates for the average death rates are in good agreement with estimates for T-cell lifespans obtained in humans by a variety of techniques (Michie *et al.* 1992; Clark *et al.* 1999; Nowak & May 2000). Although one can also compute an average proliferation rate, the numerical value of such a proliferation parameter provides little information on the total proliferation in the population. A small proliferation rate rather suggests that most of the proliferation occurs by clonal expansion of resting cells, and not by proliferative renewal of activated cells.

Conventional statistical tests provide a rational basis for limiting the complexity of the model employed for fitting data. For example the partial *F*-test allows one to test whether the extension of a model with additional parameters significantly improves the quality of the fit (Armitage & Berry 1994; Borghans *et al.* 1999; De Boer *et al.* 2003). However, the problem that the biological interpretation of the death rate, δ, depends on the value of the asymptote, α, is not solved by such statistical procedures. By defining a novel parameter, $\hat{\delta}$, averaging the death rates whenever the fitting requires the existence of two subpopulations, we have obtained a procedure in which all data are fitted under the same interpretation.

All models require a source of unlabelled cells to account for the loss of labelled cells after BrdU withdrawal. What does this tell us about the nature of $CD4^+$ T-cell production? One interpretation of the source is clonal expansion of resting cells (see our two-compartment models, and Grossman *et al.* (1999) and Bonhoeffer *et al.* (2000)). For uninfected monkeys this seems an intuitive explanation because one expects that on a timescale of weeks, different specificities of resting cells are triggered to proliferate. For macaques chronically infected with SIV this explanation may not be the whole story, since some clones (e.g. SIV-specific ones) may be persistently activated. An alternative process, whereby labelled cells could be replaced by unlabelled cells, is a strong dilution of their BrdU content by rapid clonal expansion (Rouzine & Coffin 1999). If labelled cells go through many divisions between the weekly BrdU measurements, their progeny would be unlabelled owing to the strong dilution of the BrdU. As such a scenario would hardly affect the relative distribution of BrdU intensities in the flow histograms, and mainly decrease the fraction of $BrdU^+$ cells (Rouzine & Coffin 1999), this remains in agreement with the data (see note 13 in Mohri *et al.* (1998)). However, the fact that one finds a similar rapid loss of label in human studies with ^{2}H-glucose (Mohri *et al.* 2001), which cannot be the result of dilution of label (Hellerstein 1999), suggests that the rapid loss of $BrdU^+$ cells is probably not caused by extensive clonal expansion of labelled cells.

A source is not always required to explain BrdU data. Studies into the fate of memory cells after lymphocyte choriomeningitis virus (LCMV) infection in mice (Zimmerman *et al.* 1996) revealed a slow loss of $BrdU^+$ memory cells. If these data were fitted to the one-compartment model, one would either find a limited turnover in this compartment, as suggested by Zimmerman *et al.* (1996), or find a scenario with more rapid turnover where memory-cell renewal balances memory-cell death (i.e. $\rho \simeq \delta$ in the basic model).

We are unable to go beyond estimating only an average death rate from BrdU data because of two main problems. First, BrdU experiments are typically too short to obtain a good estimate of the asymptotic fraction of labelled cells, α, that would be approached if the experiments were carried out over a longer time-scale. Having an unreliable estimate for α, we have so little knowledge of the heterogeneity of the population that it is infeasible to estimate parameters of sub-populations. Second, there are two forms of T-cell proliferation. Activated cells are continuously involved in a slow-renewal type of proliferation (Ahmed & Gray 1996), in which each division halves the BrdU intensity of the daughter cells. Another form of proliferation is a rapid clonal expansion after a short antigenic stimulation (Grossman *et al.* 1999, 2002; Bajenoff *et al.* 2002; Lee *et al.* 2002). In the models discussed here we have attempted to account for the renewal with the proliferation term, and for the clonal expansion with the source term. The rapid loss of $BrdU^+$ cells and the fact that BrdU intensity distributions show little evidence for dilution (Mohri *et al.* 1998) seem to argue that most of the T-cell production should have the form of clonal expansion of resting cells activated by antigen. One should remain cautious, however. Better BrdU data may allow one to estimate better the relative contributions of clonal expansion and renewal to the total production of T cells.

Notwithstanding our limited understanding of the biology explaining the loss of labelled cells during chronic infection, we have demonstrated that one can get a reliable estimate for the average turnover rate within a population sampled for $BrdU^+$ cells by averaging the estimated death rate(s). We would therefore suggest the initiation of an analysis of BrdU data using a general model with an independent asymptote, α, a fully labelled source during BrdU administration and an unlabelled source afterwards, a renewal type of proliferation, ρ, and death, and then statistically test what subset of parameters is required to fit the data (De Boer *et al.* 2003). Different datasets will require different subsets (De Boer *et al.* 2003). The Mohri *et al.* (1998) data with the rapid loss of $BrdU^+$ cells can be fitted with $\rho = 0$ (see table 2). The data from several monkeys can also be fitted with the one-parameter simplification of $\rho = 0$ and $\alpha = 1$. Datasets in which the percentage of $BrdU^+$ cells declines slowly (Zimmerman *et al.* 1996) probably require $\rho > 0$. Provided that one represents the

parameter estimates in terms of the average turnover, these different choices fortunately hardly affect the results.

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APPENDIX A

In order to find equations for the fractions of labelled and unlabelled cells in the two-compartment model, we obtain from equation (3.2) the fraction of resting cells at steady state:

$$\hat{R} = \frac{\sigma + r}{r + \mu + \gamma}, \tag{A 1}$$

and from equation (3.1) that of activated cells:

$$\hat{A} = \frac{c\gamma}{c\gamma + \delta + r - \rho} = 1 - \hat{R} = \frac{\mu + \gamma - \sigma}{r + \mu + \gamma}. \tag{A 2}$$

From equation (A 2), we determine a parameter constraint for having the steady state $\mathrm{d}R/\mathrm{d}t = \mathrm{d}A/\mathrm{d}t = 0$ as:

$$\delta = \rho + \frac{r((c-1)\gamma + \sigma - \mu) + c\sigma\gamma}{\mu + \gamma - \sigma}. \tag{A 3}$$

Restricting ourselves to the case $c > 1$ we write, for the fractions of unlabelled activated cells, A_{U}, and resting cells, R_{U}, during BrdU labelling:

$$\frac{\mathrm{d}A_{\mathrm{U}}}{\mathrm{d}t} = -(\rho + \delta + r)A_{\mathrm{U}}, \tag{A 4}$$

$$\frac{\mathrm{d}R_{\mathrm{U}}}{\mathrm{d}t} = (1 - f)\sigma + rA_{\mathrm{U}} - (\mu + \gamma)R_{\mathrm{U}}, \tag{A 5}$$

where f is again a fixed fraction of the source of resting cells yielding labelled resting cells. The initial condition is $A_{\mathrm{U}}(0) = \hat{A}$ as given by equation (A 2), and $R_{\mathrm{U}}(0) = \hat{R}$ as given by equation (A 1). After the labelling period, the equations for the labelled activated cells, A_{L}, and labelled resting cells, R_{L}, are:

$$\frac{\mathrm{d}A_{\mathrm{L}}}{\mathrm{d}t} = c\gamma R_{\mathrm{L}} + (\rho - \delta - r)A_{\mathrm{L}}, \tag{A 6}$$

$$\frac{\mathrm{d}R_{\mathrm{L}}}{\mathrm{d}t} = \sigma + rA_{\mathrm{L}} - (\mu + \gamma)R_{\mathrm{L}}, \tag{A 7}$$

with $A_{\mathrm{L}}(0) = \hat{A} - A_{\mathrm{U}}(t)$ and $R_{\mathrm{L}}(0) = \hat{R} - R_{\mathrm{U}}(t)$.

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