Quantifying lymphocyte kinetics *in vivo* using carboxyfluorescein diacetate succinimidyl ester (CFSE)

Becca Asquith^{1,2,*}, Christophe Debacq³, Arnaud Florins³, Nicolas Gillet³, Teresa Sanchez-Alcaraz³, Angelina Mosley¹ and Luc Willems³

¹Department of Immunology, Imperial College London, Norfolk Place, London W2 1PG, UK

²Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

³Molecular and Cellular Biology, FUSAG, B5030 Gembloux, Belgium

The cytoplasmic dye carboxyfluorescein diacetate succinimidyl ester (CFSE) is used to quantify cell kinetics. It is particularly important in studies of lymphocyte homeostasis where its labelling of cells irrespective of their stage in the cell cycle makes it preferable to deuterated glucose and BrdU, which only label dividing cells and thus produce unrepresentative results. In the past, experiments have been limited by the need to obtain a clear separation of CFSE peaks forcing scientists to adopt a strategy of *in vitro* labelling of cells followed by their injection into the host. Here we develop a framework for analysis of *in vivo* CFSE labelling data. This enables us to estimate the rate of proliferation and death of lymphocytes *in situ*, and thus represents a considerable advance over current procedures. We illustrate this approach using *in vivo* CFSE labelling of B lymphocytes in sheep.

Keywords: CFSE; kinetics; lymphocytes; homeostasis

1. INTRODUCTION

The measurement of *in vivo* lymphocyte kinetics is crucial to understanding the mechanisms underlying lymphocyte homeostasis in health and disease. The kinetics of peripheral lymphocytes can be estimated using labels, such as BrdU or deuterated glucose (Gratzner 1982; Hellerstein 1999), that are incorporated into the DNA of dividing cells. This technique has been widely applied to investigate *in vivo* cell dynamics in a range of diseases including HIV-1, leukaemia and infectious mononucleosis where it has produced invaluable information (Mohri *et al.* 1998; Debacq *et al.* 2002; Ribeiro *et al.* 2002; De Boer *et al.* 2003b; Macallan *et al.* 2003); however, a major drawback of DNA labels is that they only label dividing cells. Consequently, although it is possible to measure the average proliferation rate of the whole lymphocyte population, it is not possible to measure the average death rate: only the death rate of the labelled subpopulation can be measured (Asquith *et al.* 2002; De Boer *et al.* 2003a). The labelled subpopulation is frequently only 1–2% of the whole population and, moreover, is atypical, consisting of cells that have recently divided: its kinetics are therefore unlikely to be representative of the whole population (Asquith *et al.* 2002). An understanding of changes in the magnitude of a lymphocyte population requires quantification of the average death rate as well as the average proliferation rate of the population of interest, so that the balance between the two can be determined. Questions such as, why do CD4+ cell numbers

inexorably decline in HIV-1 infection? why do lymphocyte counts rise in leukaemia? or, what maintains normal lymphocyte counts in healthy individuals? are therefore difficult to answer with DNA labelling techniques.

One way to assess the kinetics of the whole lymphocyte population is to use the cytoplasmic dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Lyons & Parish 1994). In contrast to DNA labels, all cells in contact with a sufficient concentration of CFSE will become labelled regardless of their stage in the cell cycle. When a CFSE labelled cell divides, CFSE is apportioned equally between the two daughter cells. Provided that the initial cell labelling is relatively homogeneous, this leads to a characteristic flow cytometric profile where a number of peaks of progressively halving CFSE fluorescence intensity are observed. By analysing these data the average proliferation rate and the average death rate of the lymphocyte population can be estimated (Gett & Hodgkin 2000; Veiga-Fernandes *et al.* 2000; Pilyugin *et al.* 2003; Leon *et al.* 2004). Importantly, because CFSE labels cells regardless of their division state, kinetics obtained from CFSE labelling pertain to the whole peripheral blood lymphocyte population and not just the minority of cells that have recently divided. However, CFSE labelling is not without problems. The need to obtain a clear resolution of CFSE peaks means that the initial cell labelling must be fairly homogeneous. This is achieved by CFSE labelling *in vitro*. That is, the cells of interest are extracted, CFSE labelled *in vitro* and then injected into the host animal. However, this protocol is controversial, since extraction of cells and *in vitro* manipulation is likely to alter their kinetics: the very parameter we are trying to estimate. Alternatively, CFSE can be directly injected into animals, thus labelling cells *in vivo*. This is preferable to *in vitro*

* Author for correspondence (b.asquith@ic.ac.uk).

The electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2005.3432> or via <http://www.journals.royalsoc.ac.uk>.

Figure 1. Schematic diagram to illustrate the difference between CFSE labelling *in vitro* and *in vivo*. (a) CFSE labelling *in vitro* typically produces homogeneous labelling so that (b) subsequent division peaks are easily resolvable. (c) CFSE labelling *in vivo* produces more heterogeneous labelling that (d) masks subsequent division peaks.

labelling since it is less likely to disrupt cell kinetics, but it leads to heterogeneous labelling of cells (figure 1) making it impossible to interpret and analyse the data using existing techniques.

Here we present a new approach that enables the analysis of *in vivo* CFSE labelling data. We illustrate this approach by analysing CFSE labelled B lymphocytes in sheep; the biological interpretation of this data is published elsewhere (Debacq *et al.* submitted; Florins *et al.* in preparation). In addition, we show that loss of CFSE labelled cells is due to a number of physiological factors of which cell death is only a small component and we quantify these multiple causes of label loss. We also show that cell kinetics derived from *in vivo* CFSE labelling pertain almost exclusively to the poorly recirculating lymphocyte pool. This information is crucial for the correct interpretation of *in vivo* CFSE labelling data.

2. MATERIAL AND METHODS

(a) *Fluorescent labelling of peripheral blood mononuclear cells and lymph collection*

The sheep were kept under controlled conditions at the Veterinary and Agrochemical Research Centre (Machelen, Belgium). CFSE (25 ml) was dissolved in 4 ml of dimethylsulphoxide (DMSO) containing 40 µl of heparin (1.0 U ml⁻¹) and injected in the jugular vein (Ristevski *et al.* 2003). At regular time-intervals, blood was collected by jugular venipuncture and peripheral blood mononuclear cells were isolated by Percoll gradient centrifugation. Cannulae were surgically inserted into intestinal or prescapular efferent lymphatics allowing sampling of lymph at 0.01, 0.08, 0.25, 0.50, 0.92, 1.08, 1.25, 1.92, 2.92, 3.92 and 4.92 days (Young & Hay 1995). Lymph circulating through the cannulae was collected in sterile bottles containing heparin. Cells were harvested by centrifugation at 1000g for 10 min and their viability was estimated by trypan blue dye exclusion. Spleens were removed surgically after ligation of the splenic vein and artery. This study was approved by the Gembloux review committee.

(b) *Immunophenotyping of cell populations*

Peripheral blood and lymphatic cells were labelled with monoclonal antibodies directed against surface immunoglobulin M (1H4 and Pig45A2) and CD11b (CC125). Cells were then labelled either with phycoerythrin or with a peridinin chlorophyll protein-antibody conjugate (Becton Dickinson Immunocytometry Systems). Labelled cells were analysed by flow cytometry on a Becton Dickinson FACScan flow cytometer.

(c) *Mathematical model*

Before formulating a mathematical model to describe CFSE labelling it was necessary to determine n , the number of divisions that an average CFSE-positive (CFSE+) cell immediately after labelling (0.00 days) would have to undergo to become CFSE-negative (CFSE-). This was calculated for all four sheep using the following formula, which estimates the number of halvings of fluorescence before a CFSE+ cell becomes CFSE-:

$$n = \log \left[\frac{\text{MFI of CFSE+ population}}{\text{MFI of CFSE- population}} \right] / \log[2].$$

In this equation, the mean fluorescence intensity (MFI) can be defined for both the CFSE+ and CFSE- population (see, for example, figure S1 of the electronic supplementary material).

The number of divisions, n , was found to be five in each case. This does not mean that all CFSE labelled cells will become negative after five divisions (some will take more, some less), but that on average it will take five divisions.

The model developed is given below (figure 3):

$$\begin{aligned} \dot{x}_0 &= -(p+d)x_0, \\ \dot{x}_1 &= 2px_0 - (p+d)x_1, \\ \dot{x}_2 &= 2px_1 - (p+d)x_2, \\ \dot{x}_3 &= 2px_2 - (p+d)x_3, \\ \dot{x}_4 &= 2px_3 - (p+d)x_4, \\ \dot{x}_5 &= 2px_4 + (p-d)x_5 + \lambda, \end{aligned}$$

Figure 2. CFSE labelling of peripheral blood B cells in four sheep. CFSE labelling of peripheral blood B cells as a function of time since the CFSE injection. (a) The proportion of B cells that are CFSE+. (b) The ratio of the MFI of CFSE+ cells to the MFI of CFSE- cells. Insets show change in proportion and ratio over the first 4 days. Closed diamonds, sheep 4533; closed squares, sheep 4534; closed triangles, sheep 4535; crosses, sheep 4536.

where x_i is the proportion of B cells that have undergone i divisions since CFSE labelling. Cells in the x_5 category are CFSE- (either because they have divided five or more times since labelling and therefore their CFSE fluorescence is below the level of detection, or because they were not labelled by the initial injection). The average proliferation rate of cells is p , the average disappearance rate is d and the average replacement rate is λ . These equations were solved analytically and used to find expressions for I , the ratio of the MFI of the CFSE+ population to the CFSE- population, and P , the proportion of CFSE+ cells. It was found that

$$I = \frac{4(24 + 24pt + 12p^2t^2 + 4p^3t^3 + p^4t^4)}{3 + 6pt + 6p^2t^2 + 4p^3t^3 + 2p^4t^4}, \quad (2.1)$$

$$P = F e^{-(p+d)t} (1 + 2pt + 2p^2t^2 + 4/3p^3t^3 + 2/3p^4t^4), \quad (2.2)$$

where F is the proportion of peripheral blood B cells labelled by the initial injection.

The MFI of CFSE in undivided cells ($\mathcal{F}$ in figure 3) appeared in both the numerator and denominator of the expression for I , and hence has cancelled out: it is therefore not necessary to estimate $\mathcal{F}$ in order to interpret the data. To simplify the expression, we have constrained the population to be of constant size and thus eliminated λ ; however, this is not necessary and λ can be left as a free parameter. Elimination of λ does not mean that it has been assumed to be negligible, it means that the average rate of replacement, λ , is constrained to balance the difference between proliferation and death so that the lymphocyte population remains of constant size.

These expressions were then fitted to the experimental data using nonlinear regression to estimate the lymphocyte kinetic parameters.

3. RESULTS

(a) Basic experimental data

The approach that we have developed to analyse *in vivo* CFSE data is illustrated using CFSE labelling of B lymphocytes in sheep. A bolus of CFSE was injected into the jugular vein of four sheep and the fluorescence intensity of CFSE label in peripheral blood B cells was measured by flow cytometry at regular time-intervals during the subsequent 11 weeks. Representative data is shown in figure S1 of the electronic supplementary material. The intensity of CFSE labelling was highly heterogeneous making it impossible to resolve individual division peaks (figure 1). This was expected, since the fluorescence intensity of labelled cells is directly proportional to the local concentration of CFSE (Lyons *et al.* 2001), which will vary throughout the body because a single injection will not lead to instantaneous uniform label distribution. Since the CFSE labelling was heterogeneous, we did not attempt to identify how many times cells had divided based on their CFSE intensity (as is required with current methods of CFSE analysis), instead we simply classified cells as CFSE+ or CFSE- (see figure S1 of the electronic supplementary material).

During the experiment the absolute value of the MFI of CFSE labelling of the CFSE- population fluctuated. To correct for this variation in background labelling, the ratio of the MFI of CFSE+ cells to the MFI of CFSE-

Figure 3. Schematic of the model to describe CFSE labelled cells. Peripheral blood lymphocytes were assumed to proliferate at an average rate p , to disappear at an average rate d and to be replaced at an average rate λ . On division, the fluorescence intensity (initially J) was assumed to halve. After five divisions, CFSE fluorescence intensity was so low that it fell below the threshold of detection and the cell was considered to be unlabelled.

cells was used. We repeated our analysis using just the MFI of the CFSE+ population to ensure that our background correction did not alter our conclusions. The experimental data is summarized in figure 2.

(b) Toxicity of CFSE/DMSO

To assess the toxicity of CFSE/DMSO to B cells *in vivo*, we quantified the absolute number of B cells in the splenic vein and in the jugular vein following CFSE/DMSO injection in two sheep. The absolute number of B cells in both the splenic and jugular vein decreased approximately twofold within the first hour after injection but recovered to pre-injection levels by 4 h. Importantly, the proportion of cells that were CFSE+ in the splenic and jugular vein followed very different kinetics from the absolute number of B cells: the proportion of CFSE+ cells decreased more slowly and in a monotonic fashion, so that the decrease in the proportion of labelled cells over the first day was far in excess of the decrease in absolute numbers. This suggests that toxicity associated with CFSE/DMSO was very short-lived and could not explain the decrease in CFSE+ cells observed.

(c) Basic model

Two pieces of data can be reliably extracted from *in vivo* CFSE labelling experiments: I , the ratio of the MFI of CFSE+ cells to the MFI of CFSE- cells, and P , the proportion of CFSE+ cells. We constructed a model (figure 3; §2) to describe the relationship between I and P and the underlying cell kinetics. By fitting this model to the data using nonlinear regression, we estimated three kinetic parameters of B lymphocytes: the average proliferation rate, the average disappearance rate from the bloodstream and the average rate of entry of unlabelled lymphocytes into the bloodstream. Initially, before equilibrium has been reached between labelled cells in blood and lymphoid tissue, a net loss of labelled cells by circulation out of the bloodstream will contribute to label disappearance. Unlabelled lymphocytes will enter the bloodstream upon recirculation from the lymph system and also be produced *de novo* by the bone marrow and/or Peyer's patches (Reynolds *et al.* 1991). After equilibration the only net source of unlabelled lymphocytes will be *de novo* production; recirculation will not contribute to the disappearance of labelled lymphocytes.

(d) Rapid loss of labelled B cells in first day

In all four sheep the rate of loss of CFSE labelled peripheral blood B cells in the first few days post-injection was considerably faster than at later time points (figure 2).

The median rate of loss of labelled cells in the first day after the injection was 0.93 d^{-1} (falling from an average of 70% of cells labelled at time 0.01 day to 29% by 0.92 day). CFSE labelled cells may be lost due to multiple rounds of division, cell death and/or the circulation of labelled cells out of the blood. We quantified what proportion of the labelled cell loss in the first day was attributable to these factors. The contribution of cell proliferation and cell death was estimated by assuming that the rate of cell proliferation and death in the first day was comparable with proliferation and death at later time points.

(i) Contribution of cell proliferation

Cell division ultimately leads to a loss in the proportion of labelled cells (due to the intensity of CFSE fluorescence falling below the detection threshold). To estimate labelled cell loss in the first day due to cell division, we assumed that the rate of proliferation of B lymphocytes in the first day was the same as in subsequent days (the median rate of lymphocyte proliferation estimated to occur from day 1.92 onwards was 0.03 d^{-1}). We substituted this proliferation rate into equation (2.2) with $d=0$ and $t=1$; this showed that

$$\begin{aligned} \text{proportion of CFSE+ cells at } t=1 (P) \\ = 1.03 \times \text{proportion of CFSE+ cells at } t=0 (F), \end{aligned}$$

i.e. proliferation in the first day actually increased the number of labelled cells by 3% so we conclude that cell division was very unlikely to contribute to early labelled cell loss.

(ii) Contribution of cell death

The loss of labelled cells due to cell death was also low: of the order of 0.09 d^{-1} (see §3e).

Cell proliferation and death did not contribute significantly to the loss of labelled cells: this suggested that the majority of labelled cell loss was due to the circulation of labelled cells from the blood to the peripheral lymphoid organs. The circulation of CFSE labelled cells from the blood to the lymphoid organs will only result in a net loss of label ('dilution') if the proportion of labelled cells circulating out of the blood is higher than the proportion of labelled cells entering the blood from the lymphoid organs. Once equilibrium is attained between blood and lymphoid organs there will be no net loss of label to these compartments. The extent to which recirculating lymphocytes dilute label in the bloodstream will depend on two factors: the number of B cells entering the bloodstream and the proportion of these cells that are CFSE-. These factors are not known, in

Figure 4. Best fit of the model to the data. The graphs show the experimentally observed data from $t=1.92$ onwards. Closed squares, proportion (P) of CFSE+ B cells experimental data; closed triangles, intensity (I) of CFSE labelling experimental data; dotted and unbroken lines, best theoretical fit.

particular, it is not known how extensively cells in the various lymphoid organs will be labelled by a single CFSE injection.

(iii) Contribution of lymph node dilution

We investigated dilution of CFSE in the lymph by cannulating an efferent lymph duct in each of the four sheep and measuring the proportion of CFSE+ B cells over time. No CFSE labelled cells were detectable immediately after the CFSE injection ($t=10$ min), indicating that a single CFSE injection was insufficient to directly label cells of the efferent lymph. In contrast, peripheral blood B cells from a small vessel in the foot and from the carotid artery were almost entirely CFSE+ 1 min after the CFSE injection. This was consistent with the rapid and effective labelling of peripheral blood lymphocytes previously reported (Ristevski *et al.* 2003). The proportion of CFSE+ B cells in the efferent lymph increased over time to reach an equilibrium of about 4% after 1 day (Debacq *et al.* submitted). Two subpopulations of peripheral blood B cells have been described in sheep based on their CD11b phenotype (Gupta *et al.* 1998). It has been shown that CD11b- cells recirculate via the lymph nodes and that CD11b+ cells, which recirculate poorly through the lymph nodes, are almost entirely restricted to the blood and spleen. The proportion of CFSE+ cells in poorly recirculating CD11b+ B lymphocytes will not be altered by recirculation. Even if the proportion of CFSE+ cells in recirculating B cells drops to zero (due to the large number of unlabelled cells in this subpopulation), the total proportion of CFSE+ cells in the blood will remain high as recirculating CD11b- cells only constitute a small fraction of blood B lymphocytes. We found that the median proportion of CD11b- B lymphocytes in the peripheral blood was 14% and that the median proportion of labelled cells in the blood 15 min after the CFSE injection was 72%. Therefore, if dilution by unlabelled recirculating cells from the lymph was the only factor contributing to labelled cell loss in the peripheral blood, the proportion of CFSE+ peripheral blood B cells following equilibration between blood and

lymph would be:

proportion of peripheral blood B cells that are CFSE+ ($t=1$ d) = proportion of peripheral blood B cells that are CD11b- $\times$ proportion of CD11b- B cells that are CFSE+ ($t=1$ d) + proportion of peripheral blood B cells that are CD11b+ $\times$ proportion of CD11b+ B cells that are initially CFSE+ = $14 \times 0.04 + 86 \times 0.72 = 62.5$.

The lowest that the proportion of CFSE+ cells in the blood could drop to due to unlabelled cells from the lymph was therefore 62.5%. We repeated this calculation using individual data from each sheep, and found that the predicted proportion of labelled B cells in the blood at day 1, if dilution was the only factor causing label loss, was 61.6, 52.4, 61.4 and 69.4% for sheep 4533, 4534, 4535 and 4536, respectively. These figures represented 15.2, 11.1, 27.1 and 11.4% of the actual fall observed: that is, a median of about 13% of the total loss of label could be explained by dilution due to unlabelled cells from the lymph.

(iv) Contribution of splenic dilution

Having shown that the early rapid loss of labelled cells could not be explained simply by death, proliferation and dilution due to recirculating cells from the lymph, we proceeded to investigate the impact of unlabelled cells from the spleen. This was done by splenectomizing two of the sheep (4534 and 4535) and then labelling them with CFSE as before (Florins *et al.* in preparation). The early drop in the proportion of labelled cells was almost entirely abolished by splenectomy: post-splenectomy, the proportion of CFSE labelled cells fell at a rate of about 0.09 d^{-1} in the first day, 10-fold less than pre-splenectomy. Splenectomy will alter lymphocyte kinetics, so results are approximate, but we conclude that the majority of early labelled cell loss was due to the exit of a large number of unlabelled B cells from the spleen to the blood stream. In summary, the initial drop in the proportion of labelled cells was due to unlabelled cells from the spleen (76%), unlabelled cells from the lymph (13%) and labelled cell death (11%) (electronic supplementary material; figure S2).

(e) Fitting the model to the data

During the first day following the CFSE injection there was rapid equilibration of label between blood and the lymphoid organs, particularly between the blood and the spleen. If the model were fit to this early data, then the lymphocyte disappearance rate calculated would largely reflect the rate of exit of labelled lymphocytes from the blood due to recirculation. Since we wished to measure the death rate of B lymphocytes rather than the recirculation rate, we ignored pre-equilibrium data points and only fitted time points from 1.92 days onwards. Only fitting data from 1.92 days onwards also ensures that any early rapid drop in MFI of CFSE staining due to the turnover of labelled short half-life proteins or to the leeching of unbound but fluorescent CFSE from cells (Lyons *et al.* 2001) will not impact on our estimates of B cell kinetics.

The model fitted the data well (figure 4), yielding a median death rate of 0.09 d^{-1} and a median proliferation rate of 0.03 d^{-1} . Importantly, despite equilibration between blood and lymph, the proportion of labelled B cells in efferent lymph at day 2 (median 4%) was considerably lower than that in the bloodstream (median 32%). This indicates that most of the labelled B cells in the bloodstream were poorly recirculating (consistent with their CD11b+ phenotype): the readily recirculating fraction having rapidly reached a much lower equilibrium due to extensive dilution by unlabelled recirculating cells. Parameters derived from this CFSE labelling protocol will therefore be for poorly recirculating B cells. The kinetics of the readily recirculating fraction could be captured by more extensive labelling (e.g. by a continuous CFSE infusion or multiple injections).

(f) Finite cell cycle length

An important paper by Pilyugin *et al.* (2003; later extended in Ganusov *et al.* 2005) challenged the validity of existing CFSE models arguing that it was necessary to model a deterministic, finite time for a cell to progress through the cell cycle. In the method that we have presented here, a longer time to progress through the cell cycle, following the commitment to divide, will be manifest as a slower proliferation rate (see figure S3 of the electronic supplementary material). Unfortunately, Pilyugin's method, which was used to analyse transgenic cells that were labelled with CFSE *in vitro*, can only be applied to data where individual peaks can be resolved. Furthermore, Pilyugin's method introduces an extra free parameter—the length of the cell cycle—reducing the accuracy with which other parameters can be independently estimated. Although Pilyugin's method cannot be applied to our data, we were concerned that the omission of a finite cell cycle time from our model would lead to errors in the parameter estimates. We therefore experimented with a model with an explicit deterministic cell cycle phase. The model that we used was the Smith–Martin model of the cell cycle (Smith & Martin 1973); this is the model on which Pilyugin's method was based. We used the Smith–Martin model with a range of parameter values to generate a range of theoretical CFSE 'data'. That is, we asked the question, if dividing cells obeyed the Smith–Martin model, what would the CFSE data obtained from labelling such cells look like for a number of known values of the proliferation rate and the

death rate in the physiological range? We then fitted our model to this data to see how well it predicted the known values of the proliferation rate and death rate. That is, we analysed whether the fact that our model did not explicitly include a finite cell cycle would lead to significant errors in the estimates of the cell kinetics. The agreement between the true values of the proliferation and death rate and our estimated values (see figure S4 of the electronic supplementary material) was extremely good and we conclude that our decision not to explicitly model a finite cell cycle time had not adversely affected our parameter estimates.

4. DISCUSSION

Peripheral blood lymphocytes are highly heterogeneous with varying migratory capacity and kinetics depending on their exposure to cognate antigen, activation status and phenotype. It is often implicitly assumed that labelled cells are representative of the entire peripheral lymphocyte population and therefore that the kinetics obtained from labelling experiments pertain to the whole lymphocyte population. This is an oversimplification. DNA labels such as BrdU or deuterated glucose preferentially label rapidly dividing lymphocyte subpopulations and therefore kinetics estimated using these methods pertain to recently divided lymphocytes. CFSE labels all lymphocytes regardless of their division kinetics and thus avoids one of the major drawbacks of DNA labels. The probability of a cell becoming labelled can also depend on its location in the body at the time of labelling and the label used. The proportion of dividing cells that are BrdU positive in varying compartments throughout the body has been studied in rodents (Rocha *et al.* 1990), but the distribution of lymphocytes that incorporate deuterated glucose or CFSE is unknown. We begin to address these questions here.

In summary, we show that CFSE data can be usefully interpreted even if CFSE peaks cannot be resolved and therefore that *in vitro* labelling protocols which could drastically alter cell kinetics are unnecessary. In addition, we make two observations central to the interpretation of *in vivo* CFSE experiments. First, during the first few days post-labelling, the majority of labelled cell loss is not due to cell death as is often assumed but the exit of unlabelled cells from the spleen. Second, kinetics obtained from CFSE labelling experiments will pertain to poorly recirculating lymphocytes and thus could usefully be used in parallel to BrdU labelling, which will pertain mainly to cells in lymphoid tissue. This work opens the way for utilizing CFSE in the physiological setting to estimate lymphocyte kinetics *in situ*.

This work was supported by the Leverhulme Trust (B.A.) and the Belgian Foundation against Cancer. We thank Pierre Kerkhofs (CERVA, Uccle, Belgium), G. Jean (FUSAG, Gembloux, Belgium), J. Hay (University of Toronto, Canada), Isabelle Schwartz and Michel Bonneau (INRA, Jouy-en-Josas, France) for sharing experimental data on lymphatic cannulation and splenectomy of sheep. N.G. (Télévie Fellows), A.F. (Research Fellow), C.D. (Postdoctoral researcher) and L.W. (Research Directors) are members of the 'Fonds national de la recherche scientifique'.

REFERENCES

- Asquith, B., Debacq, C., Macallan, D. C., Willems, L. & Bangham, C. R. 2002 Lymphocyte kinetics: the interpretation of labelling data. *Trends Immunol.* **23**, 596–601. (doi:10.1016/S1471-4906(02)02337-2)
- De Boer, R. J., Mohri, H., Ho, D. D. & Perelson, A. S. 2003a Estimating average cellular turnover from 5-bromo-2'-deoxyuridine (BrdU) measurements. *Proc. R. Soc. B* **270**, 849–858. (doi:10.1098/rspb.2002.2299)
- De Boer, R. J., Mohri, H., Ho, D. D. & Perelson, A. S. 2003b Turnover rates of B cells, T cells, and NK cells in simian immunodeficiency virus-infected and uninfected rhesus macaques. *J. Immunol.* **170**, 2479–2487.
- Debacq, C. *et al.* Submitted. Peripheral blood B cell death compensates for excessive proliferation in lymphoid tissues and maintains homeostasis in an ovine model of leukemia. *J. Immunol.*
- Debacq, C., Asquith, B., Kerkhofs, P., Portetelle, D., Burny, A., Kettmann, R. & Willems, L. 2002 Increased cell proliferation, but not reduced cell death, induces lymphocytosis in bovine leukemia virus-infected sheep. *Proc. Natl Acad. Sci. USA* **99**, 10 048–10 053. (doi:10.1073/pnas.142100999)
- Ganusov, V. V., Pilyugin, S. S., de Boer, R. J., Murali-Krishna, K., Ahmed, R. & Antia, R. 2005 Quantifying cell turnover using CFSE data. *J. Immunol. Methods* **298**, 183–200. (doi:10.1016/j.jim.2005.01.011)
- Gett, A. V. & Hodgkin, P. D. 2000 A cellular calculus for signal integration by T cells. *Nat. Immunol.* **1**, 239–244. (doi:10.1038/79782)
- Gratzner, H. G. 1982 Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* **218**, 474–475.
- Gupta, V. K., McConnell, I., Dalziel, R. G. & Hopkins, J. 1998 Two B cell subpopulations have distinct recirculation characteristics. *Eur. J. Immunol.* **28**, 1597–1603. (doi:10.1002/(SICI)1521-4141(199805)28:05<1597::AID-IMMU1597>3.0.CO;2-Y)
- Hellerstein, M. K. 1999 Measurement of T-cell kinetics: recent methodologic advances. *Immunol. Today* **20**, 438–441. (doi:10.1016/S0167-5699(99)01529-7)
- Leon, K., Faro, J. & Carneiro, J. 2004 A general mathematical framework to model generation structure in a population of asynchronously dividing cells. *J. Theor. Biol.* **229**, 455–476. (doi:10.1016/j.jtbi.2004.04.011)
- Lyons, A. B. & Parish, C. R. 1994 Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* **171**, 131–137. (doi:10.1016/0022-1759(94)90236-4)
- Lyons, A. B., Hasbold, J. & Hodgkin, P. D. 2001 Flow cytometric analysis of cell division history using dilution of carboxyfluorescein diacetate succinimidyl ester, a stably integrated fluorescent probe. *Methods Cell Biol.* **63**, 375–398.
- Macallan, D. C. *et al.* 2003 Rapid turnover of T cells in acute infectious mononucleosis. *Eur. J. Immunol.* **33**, 2655–2665. (doi:10.1002/eji.200324295)
- Mohri, H., Bonhoeffer, S., Monard, S., Perelson, A. S. & Ho, D. D. 1998 Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* **279**, 1223–1227. (doi:10.1126/science.279.5354.1223)
- Pilyugin, S. S., Ganusov, V. V., Murali-Krishna, K., Ahmed, R. & Antia, R. 2003 The rescaling method for quantifying the turnover of cell populations. *J. Theor. Biol.* **225**, 275–283. (doi:10.1016/S0022-5193(03)00245-5)
- Reynolds, J. D., Kennedy, L., Peppard, J. & Pabst, R. 1991 Ileal Peyer's patch emigrants are predominantly B cells and travel to all lymphoid tissues in sheep. *Eur. J. Immunol.* **21**, 283–289.
- Ribeiro, R. M., Mohri, H., Ho, D. D. & Perelson, A. S. 2002 *In vivo* dynamics of T cell activation, proliferation, and death in HIV-1 infection: why are CD4+ but not CD8+ T cells depleted? *Proc. Natl Acad. Sci. USA* **99**, 15 572–15 577. (doi:10.1073/pnas.242358099)
- Ristevski, B., Young, A. J., Dudler, L., Cahill, R. N., Kimpton, W., Washington, E. & Hay, J. B. 2003 Tracking dendritic cells: use of an *in situ* method to label all blood leukocytes. *Int. Immunol.* **15**, 159–165. (doi:10.1093/intimm/dxg016)
- Rocha, B., Penit, C., Baron, C., Vasseur, F., Dautigny, N. & Freitas, A. A. 1990 Accumulation of bromodeoxyuridine-labeled cells in central and peripheral lymphoid organs: minimal estimates of production and turnover rates of mature lymphocytes. *Eur. J. Immunol.* **20**, 1697–1708.
- Smith, J. A. & Martin, L. 1973 Do cells cycle? *Proc. Natl Acad. Sci. USA* **70**, 1263–1267.
- Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A. & Rocha, B. 2000 Response of naive and memory CD8+ T cells to antigen stimulation *in vivo*. *Nat. Immunol.* **1**, 47–53. (doi:10.1038/76907)
- Young, A. J. & Hay, J. B. 1995 Rapid turnover of the recirculating lymphocyte pool *in vivo*. *Int. Immunol.* **7**, 1607–1615.