B Cell Differentiation and Isotype Switching Is Related to Division Cycle Number

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Summary

The mature, resting immunoglobulin (Ig) M, IgD$^+$ B lymphocyte can be induced by T cells to proliferate, switch isotype, and differentiate into Ig-secreting or memory cells. Furthermore, B cell activation results in the de novo expression or loss of a number of cell surface molecules that function in cell recirculation or further interaction with T cells. Here, a novel fluorescent technique reveals that T-dependent B cell activation induces cell surface changes that correlate with division cycle number. Furthermore, striking stepwise changes are often centered on a single round of cell division. Particularly marked was the consistent increase in IgG1$^+$ B cells after the second division cycle, from an initial level of <3% IgG1$^+$ to a plateau of ~40% after six cell divisions. The relationship between the percentage of IgG1$^+$ B cells and division number was independent of time after stimulation, indicating a requirement for cell division in isotype switching. IgD expression became negative after four divisions, and a number of changes centered on the sixth division, including the loss of IgM, CD23, and B220. The techniques used here should prove useful for tracking other differentiation pathways and for future analysis of the molecular events associated with stepwise differentiation at the single cell level.

After activation by T cells, mature B lymphocytes proliferate and develop into Ig-secreting or memory cells (1). T cell–activated B cells also alter their functional characteristics such as Ig isotype, and expression of cell surface markers in response to cell contact and cytokine-mediated signals. The best studied example is the response to the cytokine IL-4, which induces the activated B cell to switch isotype from IgM to IgG1 and IgE (2, 3). As B cell differentiation is usually associated with mitosis, it has been difficult to directly assess whether cell division is required for differentiation, although a number of previous reports have linked the isotype switching mechanism with cell division (4–8). A technique for simultaneously tracking the division cycle history of stimulated cells and examining the cell surface phenotype has been developed by Lyons and Parish (9). Here, we use this method to track the relationship between division cycle number and B cell Ig isotype expression induced by the combination of plasma membranes from activated T cells and T cell–derived lymphokines (10). The results indicate that expression of surface IgG1 and other markers of B cell activation correlate with cell division number, making possible further molecular analysis of the process.

Materials and Methods

Preparation and Stimulation of B Cells. Small, dense resting B cells were prepared by Percoll density gradient from anti-Thy1, -CD4, -CD8, and complement-treated CBA/H mouse spleens as described (10). B cells to be labeled with carboxyfluorescein, diacetate succinimidyl ester (CFSE; Molecular Probes, Inc., Eugene, OR) cells were washed twice in PBS containing 0.1% BSA and resuspended in this solution at 10^7 cells/ml. CFSE was then added to a final concentration of 10 μM and the suspension incubated at 37°C for 10 min. Labeled cells were washed into B cell culture medium (RPMI 1640 containing 10% FCS, 5×10^5 M 2-ME, 10 mM Hepes, pH 7.3, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate [11]). B cells were then stimulated with optimal dilutions of Th membrane from Con A–stimulated H66.61 T cell clone (11) and the supernatant from Con A–activated D10G4.1, yielding a final concentration of 140 U/ml IL-4 and 31 U/ml IL-5 in culture. These conditions reproduced CD40-dependent Th2 T cell–induced B cell proliferation and differentiation to IgM, IgG1, and IgE secretion, as described (12). At various time points, cells were harvested from the wells and disaggregated by vigorous pipetting before being washed in PBS/0.1% BSA and divided into groups for staining with antibodies and analysis by flow cytometry.

Cell Labeling and Flow Cytometry. B cells harvested from cul-

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ture were incubated at 4°C for 30 min with biotinylated antibodies directed against cell surface markers or surface Ig. Antibodies used were IM7 (anti-CD44), 1G10 (anti-B7-1), GL1 (anti-B7-2) (PharMingen, San Diego, CA); AMS 9.1 (anti-IgDa), RS3.1 (anti-IgMa) goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL); and 281-2 (anti-syndecan 1), B3B4 (anti-CD23), and 2.4G2 (anti-FcγII) (gifts from P. Lalor, Walter and Eliza Hall Institute, Meboume, Australia). Cells were then washed twice in PBS/BSA and incubated for a further 30 min with either streptavidin-peridinin chlorophyll protein-cap (Serotec, Kidlington, Oxford, UK) or streptavidin-peridinin chlorophyll protein (Becton Dickinson & Co., Mountain View, CA). For staining CD45R, a direct conjugate of antibody RA3-6B2 with PE was used (PharMingen). Labeled B cells were analyzed on a FACScan® using Cellquest software (Becton Dickinson & Co.). Two-dimensional data representations are 10% linear contour plots with two smoothing cycles and 5% threshold. Displayed events are gated to fall within established viable cell forward and side scatter parameters and to eliminate B cell aggregates. Control cultures of unlabeled cells stimulated with membrane and sn were analyzed for autofluorescence to determine the background. Labeled B cells in culture catabolize CFSE rapidly over the first few days and then more slowly between days 3 and 5 (9). To determine the mean fluorescence of undivided cells, labeled B cells were cultured in IL-4 containing sn only. These cells displayed similar intrinsic fluorescence decay characteristics and a proportion remained viable without actually dividing. The autofluorescent level of stimulated cells and the mean fluorescence of undivided control cells was used to determine the position of the division guide lines indicated for each time point.

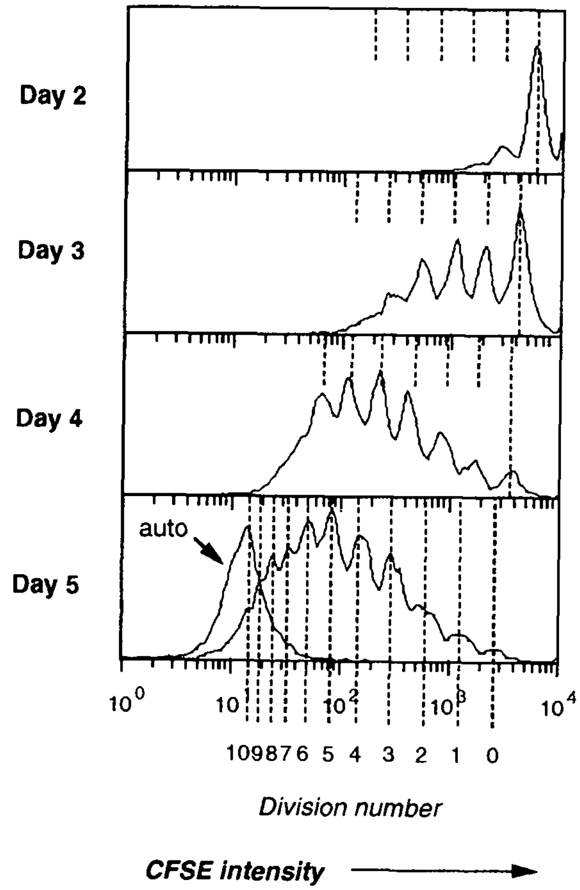

Figure 1. Tracking B cell division with CFSE. CFSE-labeled small, dense B cells were stimulated with Th membrane and sn for 2, 3, 4, and 5 d before being harvested and analyzed by flow cytometry. (*Dashed lines*) Division cycle number for each panel. (*Arrow*) The autofluorescence level of stimulated but unstained cells.

Results

Asynchronous B Cell Division Tracked with CFSE. Resting B lymphocytes were labeled with CFSE according to the method of Lyons and Parish (9). CFSE-labeled cells exhibit a single sharp log-normal distribution of fluorescence intensity, the mean of which decays with time in culture but is not altered by activation or cell enlargement and is diluted twofold with each consecutive cell division (9). Comparisons of this technique and bromodeoxyuridine incorporation confirm that fluorescence decay is due to cell division (9). Fig. 1 shows a time course of B cells stimulated to divide by incubation with Th cell membrane and Th2 cell supernatant containing IL-4 and IL-5. The first cell divisions occurred at 48 h and continued to the final time point at day 5. During this period, a marked asychrony of B cell division in culture was observed. Thus, at day three some B cells remained undivided, whereas others had divided five to six times. The similar standard deviation of consecutive log-normal division peaks for up to five or six divisions indicated a remarkable fidelity in the distribution of label between daughter cells during division. The total number of divisions that can be tracked in these experiments, however, is set by the autofluorescence level of unlabeled B cells which increases upon activation and is shown for the day 5 panel (Fig. 1). Dividing cells approach this limit asymptotically as the later division peaks compress together because of the addition of the constant background fluorescence to the geometrically decreasing CFSE level. Thus, it becomes difficult to separate cell divisions beyond seven to eight division cycles. Highly asynchronous division is not unique to Th membrane stimulation as it is also a feature of B cell proliferation induced by LPS and the combination of anti-IgM and IL-4 (data not shown).

B Cell Differentiation Is Linked to Division Cycle Number. Small resting B cells express a number of surface molecules that are lost after stimulation with Th membrane whereas others, such as IgG1, are acquired. These changes in cell surface phenotype are unaffected by CFSE labeling (data not shown), indicating that the relationship between cell division cycle number and differentiation can be determined. In Fig. 2 *A*, a time course of B cell activation is presented showing a histogram of division revealed by CFSE dilution, and contour plots of CFSE level versus expression of CD44, IgD, IgM, and IgG1. These results revealed distinct patterns of association between division cycle number and cell surface expression for different markers. CD44 is a glycoprotein involved in B cell recirculation, expressed on activated B cells (13, 14). Resting B cells were low for CD44 but, before dividing, became positive and retained high level expression for more than eight divisions. In contrast, IgD was initially expressed at a high level on undivided cells but decreased to low levels in two steps linked to cell division. After the first division, expression was reduced to an intermediate amount that was retained for another two divisions. The majority of cells then underwent a transition around the fourth cycle from intermediate to negative expression, so that by division five most B cells had lost surface IgD. IgM followed a different expression sequence with cells retaining high levels of IgM until a transition to low expression around division cycles five and six. Clearly, the pattern of IgM and IgD loss was not consistent with simple dilution of the initial surface Ig with each division. Furthermore, these data indicated that the level of IgD/IgM expression correlated with cell division number rather than the length of time after stimulation.

Of particular interest was the expression of IgG1, which requires an isotype switch event for surface expression. As observed for IgD and IgM, the cell surface phenotype showed a remarkable relationship with division cycle number and not with time after stimulation. B cells were uniformly negative for IgG1 until the third division cycle, when a small proportion of cells became positive, with this number increasing with further division rounds (Fig. 2 *A*). In Fig. 2 *B* the proportion of cells expressing IgG1 in each division round was calculated for each time point. This plot revealed very clearly that, despite large changes in the proportion of cells in each division cycle after different incubation times (Fig. 2 *B*), the percentage of IgG1$^+$ cells comprised a fixed proportion of the cells in each division round. A similar relationship to that shown in Fig. 2 *B* was observed if high IL-4 concentrations (up to 5,000 U/ml) were used, indicating that IL-4 was not limiting the switching process (data not shown). Furthermore, the pattern observed in Fig. 2 *A* was reproduced in five independent experiments, including one where B cells were stimulated by LPS and IL-4 rather than Th membrane (data not shown). The consistency of the proportion of IgG1$^+$ cells at each division cycle indicates that isotype switching is not affected by extraneous time-dependent events such as the development of larger cell aggregates, the accumulation of additional secreted factors, or the possible presence of IgG1 committed precursor cells with different intrinsic rates of division. If it is assumed that IgG1$^+$ cells have division rates similar to those of IgG1$^-$ cells, then the probability of switching at each division can be calculated from Fig. 2 *B*. Thus, cells after dividing three times have an ~4% probability of expressing IgG1, whereas cells at either the fourth, fifth, or sixth division cycle times have an ~10% probability. After the sixth division, few cells appear to express IgG1 de novo.

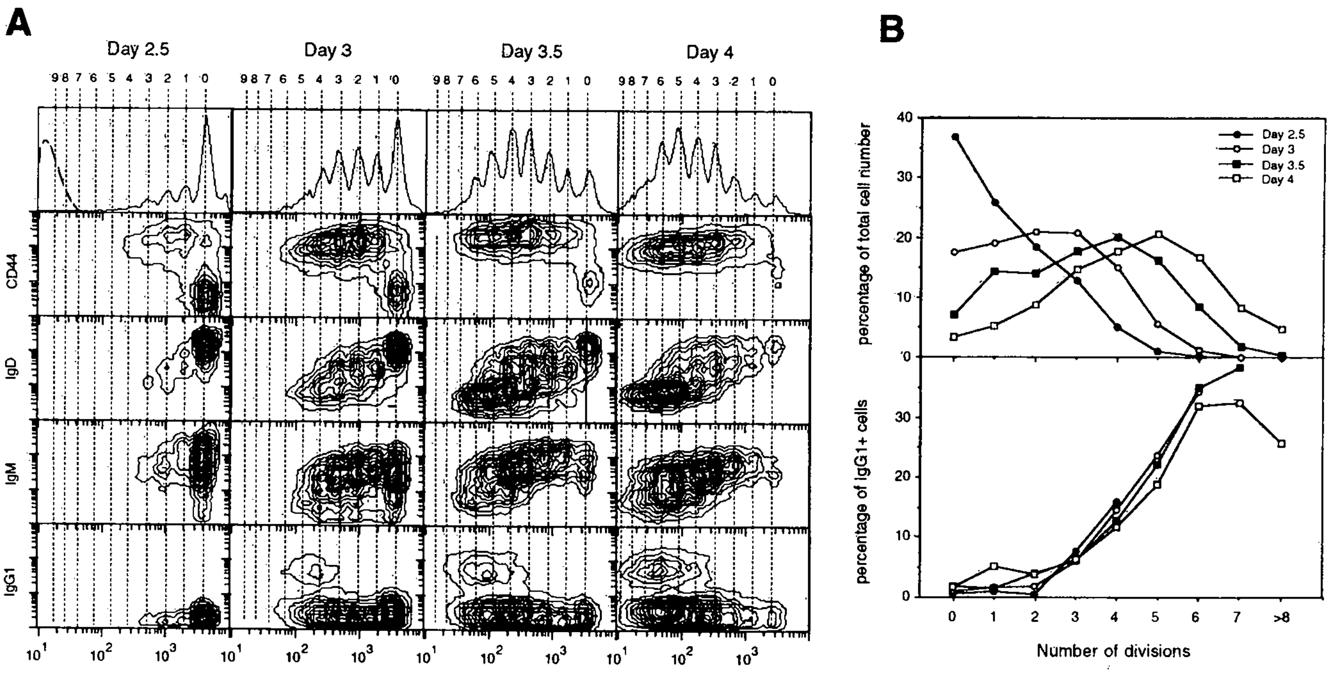

Figure 2. Tracking B cell division, isotype switching, and differentiation using CFSE. CFSE-labeled small, dense B cells were stimulated with Th membrane and sn for various times before being harvested and stained. (*A*) (*Top*) CFSE fluorescence histograms for activated B cells taken at days 2.5, 3, 3.5, and 4 after the start of culture. The progression of cell division with time is indicated by sequential twofold reductions in CFSE intensity. Dashed guidelines on each graph indicate the position of each division cycle number. Underneath each histogram are two-dimensional contour plots of CFSE versus surface labeling with CD44, IgD, IgM, and IgG1. (*B*) Data from *A* were gated to calculate the proportion of total viable cells in culture found in each division cycle at each time point (*top*). (*Bottom*) The proportion of cells in each division cycle that were IgG1$^+$ at each time point. Adjacent gates were set using Cellquest software to accommodate sequential division rounds centered on the mean for each division.

Together, these experiments suggest that T cell help initiates an isotype switching program that requires a number of division cycles before being completed. This is consistent with previous evidence that isotype switching requires cell division and occurs in the S phase of the cell cycle (4–8). In view of the requirement for up to six cell divisions for IgG1 expression, isotype switching in vivo may require prolonged recruitment of T cell help. Therefore, the availability of antigen for representation by dividing B cells to Th cells will be an important variable in the ratio of IgG1 and IgM produced.

Expression of other Markers Is Also Linked to Division Cycle Number. The expression of a further six B cell surface molecules of relevance to B cell differentiation and function was also determined. The asynchrony of B cell division in 4-d cultures allows the relationship between division cycle number and surface expression to be evaluated at a single time point (Fig. 3). The common B cell isoform of CD45, B220, which is usually expressed at high levels on resting and activated B cells, was lost abruptly at the sixth division in these experiments (Fig. 3 *a*). The CD28 receptor B7.1 was only weakly expressed (Fig. 3 *b*), whereas B7.2, was expressed at moderate levels only in the early divisions (Fig. 3 *c*), suggesting that the ability of the B cell to acquire T cell help will diminish with differentiation. The proteoglycan syndecan 1, a marker of Ig-secreting plasma cells in vivo (15), was found on cells that had divided five to six times and was, like IgG1, only expressed on a proportion of B cells (Fig. 3 *d*). IgG1 and syndecan expression was mutually exclusive (Fig. 4). Expression of CD23, a low affinity IgE receptor, closely followed that of IgM, becoming negative after six divisions (Fig. 3 *e*), whereas the low affinity Ig receptor FcγRII was only weakly expressed on dividing cells. Thus, there were three patterns of division-related cell phenotype change. CD44 behaved as an activation marker, with expression unrelated to cell division (pattern 1). IgD, IgM, CD23, and B220 underwent a pattern of change that was followed by most dividing cells in the population. The consistency of these patterns implies that cell division–dependent differentiation proceeds from one transition stage to the next with a high probability of success (pattern 2). In contrast, IgG1 and syndecan were acquired by only a proportion of cells, a pattern more consistent with a stochastic differentiation step (pattern 3). Because each form of differentiation occurred simultaneously, three lineages arose in these cultures after six to seven division cycles. Each cell was $CD44^{+}$, $B220^{-}$, $CD23^{-}$, $Fc\gamma RII^{-}$, IgD^{-}, IgM^{-}, and $B7^{-}$, however, 25% were $synd^{+}/IgG1^{-}$, 20% were $synd^{-}/IgG1^{+}$, and 55% were $synd^{-}/IgG1^{-}$ (Figs. 3 and 4). The relationship between these three cell types and plasma cells, memory cells, and cells that have switched to other Ig isotypes is currently being determined.

Collectively the data strongly suggest that T cell–stimulated B cells progress through a program of both predetermined and stochastic changes laid in place during consecutive division rounds. The mechanism by which the cell coordinates differentiation to division is of great interest and is now accessible to experiment. The method employed here, coupled with cell sorting and molecular techniques, has the potential to allow the generation of comprehensive differentiation maps for lymphocytes and stem cells undergoing development in vitro and in vivo.

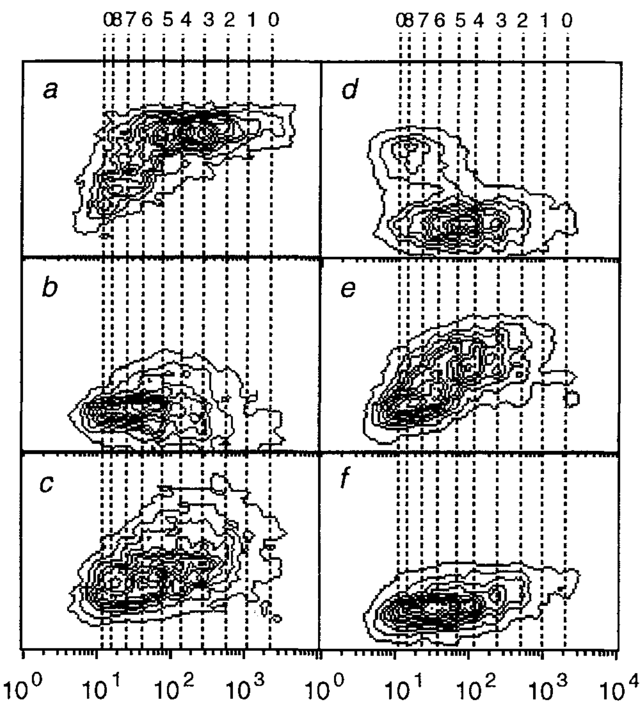

Figure 3. B cell surface changes correlate with division cycle number. CFSE-labeled small, dense B cells were incubated at 2 × 10^4 cells/ml with Th cell membrane and sn as described in Fig. 1. After 4.5 (*a–c*) or 5 d (*d–f*) in culture, cells were harvested and stained for either (*a*) CD45R (RA3-6B2), (*b*) B7.1, (*c*) B7.2 (GL-1), (*d*) syndecan (281-2), (*e*) CD23 (B3B4), or (*f*) FcγRII (24G2) (PharMingen). Plots *a–e* are two-dimensional linear contour diagrams with CFSE level on the abscissa and surface molecule expression as the ordinate.

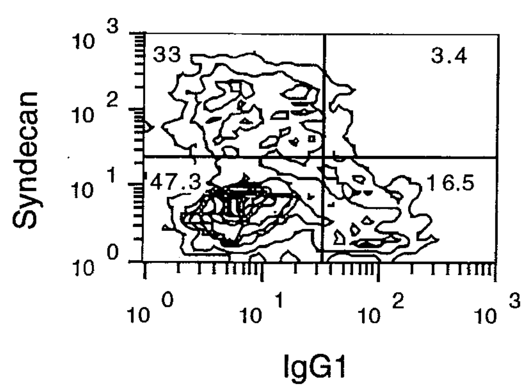

Figure 4. Syndecan and IgG1 are expressed on different B cells. CFSE-labeled small, dense B cells were incubated at 2 × 10^4 cells/ml with Th cell membrane and sn for 5 d. Cells were washed and stained with anti-syndecan 1-biotin followed by streptavidin-Tricolour and goat anti-mouse IgG1-PE. Cells shown had divided more than four times.

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