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LPS was used to induce switching of B cells to IgG3 and, in the presence of TGF-β, to IgG2b and IgA. Switching to all three isotypes increased with division number according to a consistent relationship that was independent of time in culture. The mode of activation altered the relationship with division, as CD40 ligand increased switching to IgA and decreased switching to IgG2b and IgG3 when measured per division. This division-linked switching behavior could be described by Gaussian probability distributions centered around a mean division number. The divisions at which switching to IgG3 and IgG2b occurred overlapped, raising the possibility that the two switching mechanisms were linked. However, when IgG3+ and IgG3− B cells were sorted and placed back in culture, they switched to IgG2b at an equivalent rate, indicating that alternative switching decisions were made independently within a single cell. As a consequence, isotype switching could be predicted at the population level by standard probability laws. Therefore, division number provides a framework for a stochastic description of differentiation that may be widely applicable. The Journal of Immunology, 1999, 163: 4707–4714.

Immunoglobulin isotype switching by B lymphocytes is a biologically important feature of the humoral immune response. In mice, conventional B cells possess the ability to switch from IgM and IgD expression to any of six alternative heavy chain genes after activation. The mechanism of switching depends upon the initial expression of sterile germline transcripts of the target Ig heavy chain gene followed by deletional recombination, which leads to splicing of the Ag-recognizing VDJ unit adjacent to the selected heavy chain gene (1–5). B cells display an exquisite ability to control and target heavy chain switching. Cytokines such as IL-4, IFN-γ, and TGF-β (6, 7) play a crucial role in directing the switching machinery. The mode of B cell activation, whether T-independent, as invoked by LPS, or T-dependent after exposure to intact T cells or CD40 ligand (CD40L)3 also affects the outcome of cytokine stimulation, including the efficiency and direction of isotype switching (7–9). It remains of great interest to understand the “logic” of Ig isotypes, why they are induced by some stimuli and not others, and how they might be controlled to facilitate regulation of the immune system, including the class of Ab response elicited during natural infection or after vaccination.

Methods for inducing each of the murine isotypes in vitro have been developed. Switching to IgG1 and IgE with expression of γ1 and ε germline transcripts occurs as a result of activation of B cells with the T-independent stimulus LPS in the presence of IL-4 (10, 11) or with the T cell-dependent combination of CD40L and IL-4 (12). Addition of IFN-γ to LPS-stimulated cultures induces switching to IgG2a and the production of γ2a germline transcripts (13, 14). When TGF-β is added to the same culture system, the rate of switching to IgG2b and the steady-state levels of γ2b germline transcripts increase (15). Furthermore, several groups have demonstrated that TGF-β can induce LPS-stimulated membrane IgA+ B cells to secrete IgA (16, 17). One isotype to which switching does not appear to require a cytokine is IgG3. Thus, stimulation of B cells with LPS is sufficient on its own to induce switching to this isotype, with production of γ3 germline transcripts (18–20).

Further complexity in isotype switching results from the potential for sequential switching. It has been shown by analysis of DNA switch circles, and from the sequence of recombined switch regions, that the switch to IgE and to IgA sometimes proceeds through IgG intermediates, whereas on other occasions it is made directly from IgM (21–27). Sequential switching also takes place between IgG isoforms as shown, for example, in the case of IgG2b, where switching can occur via an IgG3 intermediate (28). The relative importance of direct vs sequential switching for the production of a given isotype remains unclear. For instance, although a high proportion of IgE+ cells switch via IgG1 (21), interference with the switch to IgG1 does not affect the rate of switching to IgE (1, 29). The mechanism of regulation underlying this diverse range of pathways therefore requires further investigation.

An unexpected feature that is shared by all isotype switching in vitro is its inefficiency. Under identical culture conditions involving activation of purified resting B cells in the presence of, for example, IL-4, only 20–50% and 2–25% of cells in culture switch to IgG1 and IgG3, respectively (21, 29–31). Recently, we provided an explanation for this phenomenon. B cells undergoing isotype switching increase in frequency by a measurable amount per division (32). However, B cell proliferation in vitro is highly asynchronous, as the cells that are recovered from culture encompass a wide range of division numbers (33). Thus, even though the cells are following a simple division-based rule, the net result appears complex due to variation in the division history of individual cells. These studies have also established the principle that control of the
division-based rate of switching (expressed as a probability per division) and the rate of proliferation (expressed in units of time) are independent events that can be distinguished and studied separately (32, 33). This is particularly important when studying the effect of individual cytokines, alone or in combination, as they frequently alter the rates of both switching and proliferation (33).

In this study, the relationship between division number and the isotypes IgG3, IgG2b, and IgA has been examined by culturing B cells in the presence of LPS, CD40L, and TGF-β.

Materials and Methods

**Mice**

Male and female CBA/H mice were obtained from the Animal Resources Centre (Canning Vale, Australia). Mice were maintained under specific pathogen-free conditions in the Centenary Institute animal facility and used between 8 and 12 wk of age.

**Reagents and Abs**

Percoll was obtained from Pharmacia Biotech AB (Uppsala, Sweden). 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR). LPS from *Salmonella typhosa* was obtained from Sigma (St. Louis, MO) and human recombinant TGF-β1 was acquired from Boehringer Mannheim (Mannheim, Germany). CD40L, prepared from the Sf9 insect cell line transfected with baculovirus vector containing the CD40L gene (34) was a generous gift of Dr. M. R. Kehry (Boehringer Ingehelm, Ridgefield, CT). Biotinylated rat anti-mouse IgG2b, IgG3, and IgA were purchased from Southern Biotechnological Associates (Birmingham, AL). Biotinylated rat anti-mouse IgG2a was obtained from Serotec (Oxford, U.K.). R1E4 (anti-mouse IgE) and AMS 15.1 (anti-mouse IgD) were conjugated to PE using succinimidyl 4-(p-maleimidophenyl) butyrate (Pierce, Rockford, IL). RS3.1 (anti-mouse IgG2a) was obtained from UCB (Brussels, Belgium). 5-iodo-2′-deoxyuridine (5-IUDr) was added to each well to a final concentration of 0.5 μm. Bead and live cell events were discriminated using side and forward scatter parameters acquired during flow cytometric analysis. The cytokine TGF-β1 was acquired from Boehringer Mannheim (Mannheim, Germany). The proportion of cells in each division was determined by setting gates around each peak using CellQuest software as described previously (32, 33).

**Results**

**LPS induces division-linked, time-independent switching to IgG3**

Small CFSE-labeled B cells were cultured with 50 μg/ml LPS for 4 days before being stained for each murine Ig isotype. As shown in Fig. 1B, cells divided asynchronously and were spread over a range of divisions, as noted previously after CD40L stimulation (33). Contour plots of CFSE vs Ig confirmed that LPS induced a proportion of cells to switch to IgG3 and caused a smaller fraction of cells to express IgG2b (18, 20) (Fig. 1). However, in the absence of exogenous cytokines, LPS stimulation failed to induce switching to the other four murine isotypes (Fig. 1). In addition, IgM and IgD were shown to display different division-based patterns of loss that resembled those observed after CD40L stimulation (33). IgM remained at a high level before diminishing, presumably as a result of switching to downstream isotypes. In contrast, IgD was lost progressively from dividing cells, irrespective of whether or not they underwent isotype switching.

LPS-stimulated CFSE+ cells were harvested on consecutive days from equivalent cultures, stained for IgG3, and examined by flow cytometry. The resulting CFSE histograms were analyzed as described previously (32, 33) with a view toward calculating both the proportion of total viable cells found in each division, and the proportion of IgG3+ cells in each division. Fig. 2 shows the proportion of cells in each division at each time for two separate experiments and indicates that B cells progressed on average ~1.5 divisions per day. However, when the proportion of IgG3+ cells in each division was plotted, the results were remarkably consistent, revealing a reproducible, time-independent, division-linked relationship for switching, as was observed for other isotypes (32, 33).

**Effects of TGF-β on B cell proliferation**

The cytokine TGF-β has been reported to promote switching to IgG2b and IgA and inhibit proliferation (37). If the TGF-β-induced switching to these isotypes is division-related, its effects on proliferation will complicate the interpretation of any influence it may exert on the switching process per se, as has been shown for
IgG1 and IgE (33). For this reason, it was important to separate the two potential actions of TGF-β. The proportion of live cells in each division was calculated through division slicing based on CFSE intensity. Pulsing LPS-stimulated B cells with TGF-β caused a potent inhibition of proliferation (Fig. 3) that was dependent upon the time of addition of the cytokine. When added in high concentrations (0.5 ng/ml) at the start of culture, cell proliferation was diminished to very low levels. However, as shown previously (38), a delay in addition of the cytokine by 24 or 48 h permitted significant numbers of cells to progress to later divisions. Therefore, for additional experiments, TGF-β was added at 24 h, to allow sufficient proliferation to occur so that its effects on isotype switching could be monitored.

TGF-β-induced switching to IgG2b is division-related and time-independent

The effect of TGF-β on switching to IgG2b was examined by stimulating B cells with LPS in the presence of 0.5 ng/ml TGF-β (added after 24 h). After harvesting of the cultures on day 5, IgG2b⁺ cells were found to comprise 20% of the total cell population (Fig. 4). When the proportion of IgG2b⁺ cells per division was calculated, switching to this isotype exhibited a division-related profile. Moreover, there was a low level of switching in the absence of exogenous TGF-β that also appeared to be division-dependent. According to Snapper et al. (39), this low level of “endogenous” IgG2b switching is due to secretion of small concentrations of TGF-β into culture by activated B cells.

To examine whether the switch to IgG2b proceeded independently of time in culture, a time course experiment was performed on four separate occasions (Fig. 5). Although the proportion of cells found in each division varied between experiments, suggesting some sensitivity to culture conditions (Fig. 5A), the pattern of division-related switching to IgG2b was clearly time-independent and was remarkably consistent between the four experiments (Fig. 5B).

Next, the effect of varying the dose of TGF-β on switching to IgG2b was examined (Fig. 6). As the concentration of TGF-β was reduced from 0.5 to 0.005 ng/ml, a progressive decrease in division-associated switching occurred, with 0.005 ng/ml equivalent to the background level. Furthermore, the reduced rate of division-linked switching seen at lower concentrations was again time-independent. By comparing the dose response curves for induction of switching to IgG2b and for inhibition of proliferation, the two...
responses were shown to occur over a similar concentration range (Fig. 6C).

Division-related switching to IgA is induced by TGF-β

In addition to promoting switching to IgG2b, TGF-β is known to enhance switching to IgA (16, 17). When cells were stimulated with LPS, ≈2% switched to IgA even in the presence of TGF-β (Fig. 7A). This low percentage resulted from a low rate of switching during later divisions (Fig. 7B). As IgA is a T cell-dependent isotype, cells stimulated with CD40L and TGF-β were also examined. Under these conditions, TGF-β inhibited CD40L-induced proliferation (data not shown) and once again resulted in expression of IgA by only a small percentage of the total cells (Fig. 7A). However, when plotted per division, it was apparent that a high proportion of the small number of cells reaching the later divisions had switched to IgA (Fig. 7A, 7B). The percentage of the total live cells in each division was determined. The upper panel shows an overlay of the results of three of the experiments indicated by either open symbols, shaded symbols, or filled symbols. Squares indicate cells harvested at day 3, diamonds indicate cells harvested at day 4, and circles indicated cells harvested at day 5. The percentage of IgG2b+ cells in each division was also calculated. The lower panel shows the mean ± SE of IgG2b expression taken from all four experiments harvested on days 3 (squares), 4 (diamonds), and 5 (circles).

Independent control of IgG2b and IgG3 switching

The above results revealed that TGF-β induced switching to IgG2b over the same range of divisions as IgG3 switching occurred in the absence of this cytokine (compare Fig. 2 with Fig. 5). These observations provided an opportunity to resolve the question of how individual B cells would behave when confronted with alternative switching influences.

Initially, the effect of TGF-β on LPS-induced switching to IgG3 was examined. Fig. 8A shows that TGF-β caused a decrease in the total percentage of cells expressing IgG3. When examined on a division basis, this reduction was clearly shown to be due to a decrease in the division-related appearance of IgG3+ cells in the later divisions (Fig. 8B). The number of double-positive cells expressing both IgG3 and IgG2b in the cultures was insignificant (data not shown), thereby raising the possibility that the inhibitory effect of TGF-β on the switch to IgG3 occurred as an indirect result of some cells switching to IgG2b. Because the γ2b constant region lies downstream of γ3, a B cell that has switched to IgG3 is still capable of switching to IgG2b, whereas an IgG2b+ cell is
incapable of switching to IgG3. Cell sorting was used to demonstrate that IgG3
1 cells could switch to IgG2b when placed back in culture (data not shown).

The above demonstration of sequential switching allowed the division-based association between IgG3 and IgG2b to be explored further. If the internal switching machinery for these two isotypes is shared within individual B cells, IgG3
1 cells might be expected to switch to IgG2b at a greater frequency than unswitched cells taken from the same divisions. Alternatively, the two isotypes may be regulated independently, in which case prior switching to IgG3 would not affect switching to IgG2b. To distinguish between these alternative predictions, B cells were stimulated with LPS and TGF-

\(\beta\) for several days before sorting IgM
2/IgG3
1 and IgM
1/IgG3
2 populations from divisions 4 – 8 (Fig. 9). The sorted cells were recultured with LPS and TGF-

\(\beta\) and examined for IgG2b expression after 24 h. As shown in Fig. 9C, both IgG3
1/IgM
2 and IgM
1/IgG3
2 cells switched to IgG2b at a similar frequency (~13%). In a second experiment, the sorted cells were recultured in the absence of TGF-

\(\beta\). Again, a similar proportion of the IgG3
1/IgM
2 and the IgM
1/IgG3
2 cells switched to IgG2b (data not shown).

**Discussion**

Recent studies in our laboratory have highlighted the crucial role played by division number in selection of the appropriate class of response by both T and B cells to a particular antigenic stimulus (32, 33, 40). For B cells, the outcome is switching from IgM to a downstream isotype. In our study, isotype switching to the T-independent isotypes IgG3 and IgG2b and the T-dependent, secretory isotype IgA was shown to display the same features as reported previously for IgG1, IgE, and IgG2a (32, 33, 41) (i.e., they are division number-related and independent of time in culture). Thus, division-based regulation can be regarded as a general mode of control for Ab responses of all classes.

Division-related control of the B cell response is influenced by both the type of activation stimulus and the cytokine milieu in which the B cells reside. As shown here, T-independent (LPS) and T-dependent (CD40L) stimuli directed B cells to follow alternate division-linked switching pathways (Fig. 7), thereby providing a mechanistic framework for earlier demonstrations of the importance of activation regime in determining the outcome of B cell isotype switching (7). For example, LPS on its own induced B cells to switch to IgG3 and in the presence of TGF-

\(\beta\) to IgG2b,
A, B cells were stimulated with 50 ng/ml TGF-β and harvested after 5 days. Contours of IgG3 expression plotted against CFSE intensity are shown, with the percentage of IgG3 cells given adjacent to the gated cells. The percentage of IgG3 cells in each division was also calculated for cells cultured with LPS (○) or LPS plus TGF-β (□). These data represent the mean of two experiments harvested at three timepoints each: days 3, 4, and 5. Therefore, the data points show the mean and SD of three to six separate determinations.

The division-based map of switching to IgG2b in the presence of TGF-β (Fig. 5B, IgG2b<sub>TGF</sub>), and the endogenous level of IgG2b induced in the absence of TGF-β (IgG2b<sub>lps</sub>), according to the following formula: (IgG3<sub>lps</sub> × [100 – IgG2b<sub>lps</sub>])/100 – IgG2b<sub>lps</sub>).

whereas little to no switching occurred under similar culture conditions when CD40L was added. By contrast, CD40L provoked a higher rate of division-based switching to IgA than did LPS. Thus, the mode of activation must have some influence over the as-yet unknown molecular mechanisms responsible for increasing the probability of isotype switching with progressive cell division.

The internal cellular processes responsible for influencing isotype switching are separate from the mechanisms that dictate the timing of division. In other words, the fastest and slowest dividing cells display an identical division-based isotype switching frequency. As a result of this time-independent behavior, division-based switching can be represented as a probability distribution which serves as a “map” of isotype transition with division number as the sole relevant variable. The construction of a probability map is illustrated in Fig. 10A for both IgG3 and IgG2b induced by LPS and LPS plus TGF-β, respectively. When plotted as a probability of switching per division, the distribution obtained for each isotype is approximated by a normal distribution (Fig. 10B).

The division-based switching maps for the two T-independent isotypes allow us to draw an important conclusion from the experiments described here. When TGF-β was introduced into an LPS culture, the IgG2b map covered a range of divisions that was similar to those for IgG3, raising the question of how cells respond when two switch decisions intersect. Because the gene for the IgG2b constant region is downstream of that for IgG3, switching in this sequential direction is possible, whereas the converse, namely switching from IgG2b to IgG3, is not, suggesting that IgG2b should be the dominant switch outcome. Here sequential switching from IgG3 to IgG2b was shown to occur by sorting IgG3<sup>+</sup> cells after 4–8 divisions and placing them back in culture, after which they continued to divide and switched to IgG2b. These data are consistent with previous reports that extrachromosomal DNA from LPS-stimulated splenocytes contains switch circles with segments of both the γ3 and γ2b switch regions arranged in a manner consistent with a sequential switch (28).

To assess whether there was an enhanced probability of IgG3<sup>+</sup> cells undergoing switching to IgG2b, IgM<sup>+</sup>/IgG3<sup>+</sup> cells were also sorted from the same divisions and placed back in culture. Strikingly, these cells not only continued to proliferate in an identical manner to IgG3<sup>+</sup> cells but switched to IgG2b at an identical frequency. The equal rate of switching to IgG2b suggests that an intermediate switch to IgG3 is neither obligatory nor detrimental for switching to IgG2b, and that the probabilities of switching to IgG2b and IgG3 are independent. In other words, cells switching to IgG3 will
In this situation, Ag specificity is thought to depend upon the ability of B cells specific for LPS to focus the mitogen to the cell surface, thereby increasing the effective level of stimulation (47). The consequence is that a range of high- and low-affinity B cells are likely to be activated early in the response to LPS, resulting in rapid division and IgM secretion. As Ab accumulates, however, it will progressively block the ability of the lower-affinity B cells to concentrate LPS at the cell surface, leaving only B cells of higher affinity to continue dividing and to switch to IgG3. Such a scenario illustrates how the link between division number and isotype switching leads to the selection of isotypes with the capacity to function more effectively as a bivalent Ab.

A similar role for sustained Ag-driven B cell proliferation can be postulated for the regulation of IgA, which is typically produced at sites draining mucosal surfaces (48). Thus, stimulation of B cells with LPS or CD40L in the presence of TGF-β yielded only a small percentage of cells expressing IgA (49, 50). However, the percentage of cells within the Peyer’s patches expressing IgA is much higher (51), which led McIntyre et al. to pose the question of how this high proportion of IgA+ cells arose if TGF-β induces such a low rate of switching (52). According to the results presented here, the expectation is likely to reflect sustained stimulation of B cells by continual exposure to gut-derived Ags. Under these conditions, reactive B cells would be driven into the later divisions despite the antiproliferative effects of TGF-β, thereby ensuring a high rate of switching. The cytokine TGF-β produced at sites of chronic Ag exposure should therefore act both as a damper of clonal expansion and as a facilitator of the switch to the secretory isotype.

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**References**


