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Isotype Switching by Human B Cells Is Division-Associated and Regulated by Cytokines

Stuart G. Tangye, Anthea Ferguson, Danielle T. Avery, Cindy S. Ma, and Philip D. Hodgkin

Isotype switching by murine B cells follows a pattern whereby the proportion of cells undergoing switching increases with division number and is regulated by cytokines. Here we explored whether human B cells behaved in a similar manner. The effect of IL-4, IL-10, and IL-13, alone or in combination, on Ig isotype switching by highly purified naive human CD40 ligand (CD40L)-activated B cells was measured against division number over various harvest times. Switching to IgG was induced by IL-4 and, to a lesser extent, IL-13 and IL-10. The combination of IL-10 with IL-4, but not IL-13, induced a higher percentage of cells to undergo switching. Isotype switching to IgG by human CD40L-activated naive B cells was found to be linked to the division history of the cells: IgG+ cells appeared in cultures of B cells stimulated with CD40L and IL-4 after approximately the third cell division, with the majority expressing IgG1, thus revealing a predictable pattern of IgG isotype switching. These results reveal a useful quantitative framework for monitoring the effects of cytokines on proliferation and isotype switching that should prove valuable for screening Ig immunodeficiencies and polymorphisms in the population for a better understanding of the regulation of human humoral immune responses. The Journal of Immunology, 2002, 169: 4298–4306.

A n important function of B cells during an immune response is to produce high affinity Ag-specific Ig which facilitates the eradication of infectious pathogens. Following antigenic stimulation, IgM+ IgD+ naive B cells can undergo isotype switching to produce IgG, IgA, or IgE Abs while retaining their Ag specificity (1). Thus, switching enables B cells to alter their effector function, thereby contributing diversity to the humoral immune response (1). It is generally observed in vitro that isotype switching by B cells stimulated by T-dependent signals requires both ligation of CD40 and a second instructive signal provided by a T cell-derived cytokine (2–10). Consequently, activated T cells play a critical role in the process of Ig isotype switching by providing these signals to the B cell via transient expression of CD40 ligand (CD40L)4 and production of various cytokines (6, 7, 9, 10). In both mice and humans, unique cytokines are thought to specifically induce particular isotypes. For example, CD40-stimulated naive human B cells undergo isotype switching to IgG4 and IgE in the presence of IL-4 and IL-13 (11–13) and to IgG1 and IgG3 in the presence of IL-10 (14–16). IL-10 is also thought to be required, in combination with TGF-β, for switching to IgA1 and IgG2a (17–19). By contrast, the specific signal required for switching to IgG2 remains elusive. Although IFN-γ or combinations of Staphylococcus aureus Cowan strain I, anti-CD40 mAb, and IL-10 have been found to induce expression of transcripts of the γ2 heavy chain region gene (20–22), CD40-stimulated human B cells failed to secrete IgG2 following culture with any known recombinant cytokine (21, 23). These B cells, however, did secrete IgG2 when cultured with crude supernatant from activated T cell clones (23, 24), suggesting that T cells are the source of an as yet unidentified factor.

In most studies Ig isotype switching by human B cells is assessed by measuring the amount of Ig present in 7- to 12-day culture supernatants of B cells stimulated under various conditions. However, Ig secretion is the net result of multiple facets of B cell activation, including efficiency of activation, proliferation rate, isotype switching, differentiation to an Ig-secreting cell, and the rate of cell death in culture. As a result of this complexity, the precise contribution made by individual cytokines to switching can be unclear, as they may exert differential effects on some, or all, of these different parameters of B cell activation (25). This problem has been recently highlighted further by studies in mice that show that switching to all Ig isotypes by naive B cells increases in frequency with consecutive cell division (26–29). Specifically, naive murine B cells stimulated with CD40L and IL-4 switched to IgG1 with much greater frequency after three divisions, while switching to IgE or IgG2a in the presence of IL-4 or IFN-γ, respectively, increased markedly after approximately five or six divisions (26, 27, 29). Similarly, isotype switching to IgG3, and to IgG2b and IgA in the presence of TGF-β, by LPS- or CD40L-stimulated murine B cells was also division-linked (28). Thus, treatments that promote B cell proliferation alone may increase the number of switched cells without being true switching factors. As human B cell switching cytokines, such as IL-4, IL-10, and IL-13, can also promote proliferation (11–14, 25, 30–32), the question of whether they are true switch factors by this new procedure needs to be resolved. Here, we have examined the extent to which a division-linked differentiation program applies to isotype switching by human B
cells and how individual cytokines simultaneously affect both isotype switching and proliferation.

Materials and Methods

Reagents

Biotinylated mouse anti-human IgG1, IgG2, IgG3, IgG4, IgA, and IgE mAb; PE-conjugated anti-human IgM, IgD, IgG, and CD27 mAb; and isotype control mAb were purchased from BD Pharmingen (San Diego, CA). PE-conjugated mouse anti-human CD19 mAb and mouse IgG1 isotype control were purchased from BD Biosciences (San Jose, CA). Streptavidin conjugated to Tricolor (SA-TC) and FITC (SA-FITC) were purchased from Caltag Laboratories (Burlingame, CA). The source of recombinant human CD40L was membranes prepared from the SF21 insect cell line infected with baculovirus vector containing the CD40L cDNA (provided by Dr. M. Kehry, Boehringer Ingeheim, Ridgefield, CT) (33). Human recombinant IL-4 and IL-10 were provided by Dr. R. de Waal Malefyt (DNAX, Palo Alto, CA). IL-13 was purchased from PeproTech (Rocky Hill, NJ).

Cells

Normal human spleens were obtained from trauma victims undergoing splenectomies (Royal Prince Alfred Hospital, Sydney, Australia) or from organ donors (Australian Red Cross Blood Service, Sydney, Australia). Mononuclear cells were prepared by slicing splenic tissue into small pieces and disrupting the capsule by forcing the tissue through a filter mesh. RBC were lysed, and the remaining cells were washed twice and cryopreserved in liquid nitrogen until required. Cord blood was obtained from King George V Memorial Hospital for Mothers and Babies (Sydney, Australia). Mononuclear cells were isolated by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Total human B cells were isolated using CD19 Dynabeads (Dynal Biotech, Oslo, Norway) (34). The resulting cell population was >98% CD19+/IgD-. Naive CD27- B cells (34–36) were purified by labeling total splenic B cells with anti-CD27 mAb MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and passing through a column held in a magnetic field (Miltenyi Biotec). Naive (undeclared, CD27+) B cells were collected in the flow-through and contained >98% IgD+ B cells and <1% CD27+ IgD/ IgE+ B cells (34). Naive splenic B cells were also isolated by sorting using a FACStar Plus (BD Biosciences) by labeling with PE-conjugated anti-CD27 mAb and biotinylated anti-IgG, IgA and IgE, followed by SA-FITC. Gates were set to collect CD27- B cells. On reanalysis, the sorted B cells were >99% CD27- (see Fig. 3, a and b).

CFSE labeling and in vitro B cell cultures

Purified naive human B cells were labeled with CFSE (Molecular Probes, Eugene, OR) as previously described (37, 38). Briefly, B cells were resuspended at 1 × 10^6/ml in PBS containing 0.1% BSA. CFSE, dissolved in DMSO, was added at a final concentration of 5 μM. The cells were vortexed for 10 s and then incubated at 37°C for 10 min. Labelled cells were washed three times containing 0.1% BSA in PBS, and resuspended in culture medium. Naive CFSE-labeled naive B cells (2 × 10^4/500 μl) were cultured in 48-well plates (BD Biosciences, Franklin Lakes, NJ) with CD40L (final dilution, 1/250) in the absence or the presence of IL-4 (400 U/ml), IL-10 (100 U/ml), or IL-13 (10 ng/ml), alone or in combination. The specificity of the response induced by CD40L expressed by insect cells was confirmed in control experiments in which insect cells expressing a control protein failed to induce any proliferation of naive human B cells alone or in combination with exogenous cytokines (data not shown). B cells were cultured in RPMI 1640 containing 1-glutamine (Life Technologies, Grand Island, NY), 10% FCS (CSL, Parkville, Australia), 10 mM HEPES (pH 7.4; Sigma-Aldrich, St. Louis, MO), 0.1 mM nonessential amino-acid solution (Sigma-Aldrich), 1 mM sodium pyruvate (Life Technologies), 60 μg/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml amphotericin (Sigma-Aldrich). All cultures were conducted at 37°C in a humidified atmosphere containing 5% CO2.

Immunofluorescent staining

In vitro-activated naive B cells were harvested from culture wells, and nonspecific binding sites were blocked by preincubation with normal mouse IgG. The cells were then incubated with PE-conjugated isotype control, anti-IgM, -IgD, or -IgG mAb on ice for 20 min. To determine the expression of Ig light chains, cells were incubated on ice for 20 min with biotinylated anti-IgG1, -IgG2, -IgG3, or -IgG4 mAb; bound Ab was revealed by the addition of SA-TC. The cells were analyzed on a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Surface staining was measured on a logarithmic scale. Cells present in different divisions were characterized by “division slicing.” Gates were drawn around each of the peaks present in histograms of CFSE-labeled B cells, representing cells in different divisions. The proportion of cells within each gate or the expression of different surface Ig isotypes by these cells was determined by backgating and analyzing the differently divided populations, defined by CFSE dilution, using the analysis tools of CellQuest (BD Biosciences).

Ig ELISAs

Ninety-six-well microtiter plates (Dynex, Chantilly, VA) were precoated with goat anti-human IgM or IgG polyclonal antisera (Southern Biotechnology Associates, Birmingham, AL), and nonspecific binding sites were blocked with 2% FCS prepared in PBS. Culture supernatants and Ig standards were added to the wells and incubated for 2 h at 37°C before addition of biotin-conjugated anti-human IgM or IgG antisera (Southern Biotechnology Associates). Bound Ab was detected by addition of SA-conjugated HRP (Jackson ImmunoResearch, West Grove, PA) and was visualized with HRP substrate ABTS (Sigma-Aldrich; 1 mg/ml) prepared in citrate buffer (pH 4.5) containing 0.03% H2O2.

Results

IL-4, IL-10, and IL-13 differentially enhance division of CD40L-activated naive B cells

To develop an assay for precisely quantifying the proliferative effects of cytokines that induce Ig isotype switching in human B cells, CFSE-labeled B cells were cultured with CD40L in the absence or the presence of cytokines, and after 6 days their division profiles were determined. In the presence of CD40L alone, the greatest proportion of harvested cells (~30%) was found in the undivided fraction; the remaining cells were spread throughout the divisions, with the percentage of cells decreasing with each subsequent division (Fig. 1, a and g). When cultures of CD40L-stimulated B cells were supplemented with IL-4, IL-10, or IL-13, the proportion of harvested cells that remained undivided was significantly reduced (~10%), while that of cells in later divisions was increased (Fig. 1, b–d and h–j). Under these conditions, the greatest percentage of cells was found in the second to fourth divisions, suggesting that addition of these cytokines induced a greater proportion of activated B cells to undergo additional rounds of cell division. The effects of IL-4 and IL-10 on the distribution of CD40L-activated B cells across division were comparable. In contrast, although IL-13 enhanced the proliferation of CD40L-activated B cells, it was less effective than IL-4 and IL-10 in inducing the same proportion of B cells to enter the later divisions (Fig. 1, b–d and h–j). The addition of IL-10 to B cells cultured with CD40L and IL-4 induced greater proliferation than either signal alone and was accompanied by a further decrease in the percentage of undivided cells, with the greatest percentage of cells being found in the fourth or fifth divisions (Fig. 1, a and k). In contrast, proliferation in response to the combination of IL-10 and IL-13 did not greatly differ from that in response to IL-10 alone (Fig. 1, f and l).

Induction of isotype switching and Ig secretion by activated naive B cells

Human naive B cells were cultured with CD40L in the absence or the presence of IL-4, IL-10, or IL-13, alone or in combination. After 6 days the cells were harvested, and the expression of surface IgG and the amounts of secreted IgM and IgG in culture supernatants were determined. Very few IgG+ cells (~0.8%) were detected in cultures of naive B cells stimulated with CD40L alone. In contrast, variable percentages of IgG-expressing cells were evident in cultures of B cells stimulated with CD40L in combination with the different cytokines. When the effects of individual cytokines were assayed in isolation, IL-4 induced the greatest proportion of

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B cells to undergo isotype switching. However, significant proportions of IgG⁺ B cells were also generated from CD40L-activated B cells in the presence of IL-10 and IL-13 (Table I). Although the frequency of IgG-expressing cells appearing in cultures containing IL-10 was quite low, it was consistently higher than that observed in cultures of B cells stimulated with CD40L alone. The combination of IL-4 and IL-10 caused a 2- to 7-fold increase in the proportion of IgG⁺ B cells compared with cultures of CD40-activated B cells containing either cytokine on their own (Table I). In a parallel manner to the absence of an additive or synergistic effect on proliferation, the combination of IL-10 and IL-13 did not appreciably increase the frequency of IgG⁺ B cells compared with cultures containing IL-10 or IL-13 alone (Table I).

Naive B cells stimulated with CD40L alone secreted negligible amounts of IgG, although they did secrete significant levels of IgM (Table II). Addition of IL-4 or IL-10 resulted in the appearance of low, but detectable, amounts of IgG, and the combination of IL-4 and IL-10 usually increased the amount produced (Table II). Consistent with the data for expression of IgG, IL-13 appeared to be less efficient than IL-4 in inducing IgG secretion (Table II). Although IgG could be detected, the levels observed here were several orders of magnitude less than those reported by other investigators (31, 39–41) (see Discussion). The effects of these cytokines on IgM production by CD40L-stimulated naive B cells were quite variable; they either increased or decreased secretion in separate experiments (Table II).

### Table 1. Ig isotype switching by activated human naive B cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>% IgG⁺ B Cells (mean ± SD)</th>
<th>Range (n experiments)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40L</td>
<td>0.74 ± 0.45</td>
<td>0.3–1.2 (10)</td>
</tr>
<tr>
<td>CD40L, IL-4</td>
<td>6.7 ± 3.78</td>
<td>3.4–15.0 (10)</td>
</tr>
<tr>
<td>CD40L, IL-10</td>
<td>1.86 ± 0.82</td>
<td>1.0–3.6 (9)</td>
</tr>
<tr>
<td>CD40L, IL-13</td>
<td>3.95 ± 1.83</td>
<td>2.4–6.5 (4)</td>
</tr>
<tr>
<td>CD40L, IL-4, IL-10</td>
<td>14.15 ± 6.05</td>
<td>4.5–22.9 (11)</td>
</tr>
<tr>
<td>CD40L, IL-10, IL-13</td>
<td>4.68 ± 1.35</td>
<td>2.9–6.3 (5)</td>
</tr>
</tbody>
</table>

* Purified naive human B cells (4 × 10^5/ml) were cultured with CD40L alone or in the presence of IL-4 (400 U/ml), IL-10 (100 U/ml), IL-13 (10 ng/ml), IL-4 and IL-10, or IL-10 and IL-13. After 6 days, the percentage of cells expressing IgG was determined by immunofluorescence.

Isotype switching by human naive B cells is linked to cell division

Ig isotype switching in murine naive B cells has been found to be linked to cell division (26–29). When isotype switching of CD40L-stimulated naive B cells was analyzed with respect to cell division, very few if any switched cells were present in any division (Fig. 2, a and g). However, by comparing IgG expression by human naive B cells activated with CD40L and cytokines to CFSE intensity, it was apparent that the IgG⁺ B cells accumulated in populations of B cells that had undergone the greatest number of divisions (Fig. 2, a–f). Thus, in the presence of IL-4 or IL-13, B cells expressing IgG were detected after approximately the third or fourth division (Fig. 2, b and d, and h and j). The percentage of IgG⁺ cells increased with each successive division, such that 10–20% of CD40L- and IL-4- or IL-13-stimulated B cells in the fifth to seventh divisions were IgG⁺ (Fig. 2, h and j). Consistent with the finding that IL-13 induced fewer cells to switch to IgG than IL-4 at the bulk population level (Table I), the rate of IgG isotype switching per division was less in the presence of IL-13 than IL-4 (compare Fig. 2, b and h). This cannot be attributed to IL-13-inducing fewer cells to reach the later divisions, because there was a reduced percentage of IgG⁺ cells per division in cultures containing IL-13 than IL-4 (Fig. 2, h and j). A low level of switching to IgG could also be demonstrated in the presence of IL-10 after the cells had undergone five or more divisions (Fig. 2i), which is in agreement with the data shown in Table I. The increased percentage of IgG⁺ cells evident at the population level in the presence of IL-4 and IL-10 (Table I) was occasionally observed on a per division basis; in other words, a greater proportion of IgG⁺ B cells were detected per division in the presence of both IL-4 and IL-10 than in cultures containing either cytokine alone (Fig. 2k). Under these conditions, 20–40% of cells in divisions 5–7 were IgG⁺ (Fig. 2k). In contrast, the combination of IL-10 and IL-13 did not appreciably increase the proportion of IgG⁺ B cells in each division compared with IL-13 alone (Fig. 2f).

Highly purified naive B cells can undergo isotype switching in a division-linked manner

The total human B cell population contains both naive B cells and memory B cells, which can be distinguished by the differential...
expression of CD27 (34–36). The memory (CD27+) population also contains nonswitched and isotype-switched cells (34) (Fig. 3a). These populations can be resolved phenotypically, with naïve B cells being IgM+/D/G/A/E− CD27−, nonswitched memory B cells being IgM+/D+/G/A/E− CD27−, and isotype switched memory B cells being IgM−D/G/A/E− CD27+ (Fig. 3a and data not shown) (34). Although phenotypic analysis of MACS-purified B cell populations suggested an absence of residual CD27+ IgG/A/E− memory cells, a contribution to IgG isotype switching from a very small population of contaminating cells could not be completely excluded. To check this, naïve B cells were purified by sorting CD19+CD27+ IgG/A/E− B cells. The resulting naïve B cells (>99.5% pure; Fig. 3b) were cultured with CD40L, IL-4, and IL-10, and after 6 days their pattern of isotype switching to IgG was determined compared with that of B cells isolated from the same donor by MACS beads and cultured under similar conditions. For both MACS- and sort-purified naïve B cell cultures, IgG+ cells appeared after approximately the third or fourth division, with the percentage of IgG+ cells increasing with each successive division (Fig. 3c), thereby making a contribution from residual memory B cells very unlikely.

To further exclude any possible effect of residual memory B cells on Ig isotype switching, experiments were performed using cord blood and infant peripheral blood as a source of naïve B cells, both of which are devoid of CD27+ expressing B cells (34, 42, 43). When these naïve B cells were cultured with CD40L only, <0.5% IgG+ B cells were detected (data not shown). However, in the presence of IL-4, 5–10% of cells harvested expressed IgG (Fig. 4, a and b). Similarly, IL-10 induced IgG expression by ~1–2% of cells (not shown). When analyzed on a per division basis, cord blood and infant B cells stimulated with CD40L and IL-4 switched to IgG following the third cell division. The proportion of positive cells continued to increase with each subsequent division, reaching a maximum of 15–20% IgG+ B cells in divisions 5 and greater (Fig. 4, c and d). Moreover, IgG+ B cells were detected in the later divisions of cultures of cord blood and infant B cells stimulated with CD40L and IL-10 (Fig. 4, c and d). Although the proportion of IgG+ B cells generated in the presence of IL-10 was quite low, it always exceeded the proportion of these cells in cultures of CD40L alone (Fig. 4, c and d). The rate of division-based switching exhibited by cord blood and infant naïve B cells therefore was comparable to that of adult splenic naïve B cells (see Fig. 2). Taken with the data from cultures of sorted adult naïve splenic B cells (Fig. 3c), it would appear that the contribution of any contaminating pre- or nonswitched memory B cells to isotype switching observed here was minimal, and the switching results obtained reflected bona fide molecular events occurring in naïve B cells.

Table II. Ig secretion by activated human naïve B cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
<th>Expt. 4</th>
<th>Expt. 5</th>
<th>Expt. 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD40L</td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>+IL-4</td>
<td>760</td>
<td>&lt;1.0</td>
<td>162</td>
<td>&lt;1.0</td>
<td>2597</td>
<td>3.9</td>
</tr>
<tr>
<td>+IL-10</td>
<td>1298</td>
<td>75.9</td>
<td>551</td>
<td>6.0</td>
<td>633</td>
<td>14.8</td>
</tr>
<tr>
<td>+IL-13</td>
<td>2420</td>
<td>23.3</td>
<td>1009</td>
<td>&lt;1.0</td>
<td>2614</td>
<td>31.3</td>
</tr>
<tr>
<td>+IL-4, IL-10</td>
<td>2719</td>
<td>251</td>
<td>1317</td>
<td>43.1</td>
<td>751</td>
<td>30.3</td>
</tr>
<tr>
<td>+IL-4, IL-10, IL-13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>978</td>
<td>48.6</td>
</tr>
</tbody>
</table>

* Naïve CD19+CD27+ human B cells (4×10^4/ml) purified from different donor spleens (Expts. 1–5) or from cord blood (Expt. 6) were cultured with CD40L alone or in the presence of IL-4 (400 U/ml), IL-10 (100 U/ml), IL-13 (10 ng/ml), IL-4 and IL-10, or IL-10 and IL-13. After 6 days, the amounts of IgM and IgG present in the culture supernatants were determined by a H chain, specific immunoassay. ND, not done.

FIGURE 2. Ig isotype switching by human naïve B cells increases with cell division. CFSE-labeled naïve B cells were cultured for 6 days with CD40L alone (a and g) or in the presence of IL-4 (b and h), IL-10 (c and i), IL-13 (d and j), IL-4 and IL-10 (e and k), or IL-10 and IL-13 (f and l). a–f. The division history of the cells as well as the expression of sIgG were determined flow cytometrically by plotting CFSE intensity vs IgG expression. The contour plots are representative of 4–11 independent experiments. g–l. The percentage of sIgG+ cells in each division was determined by division slicing. Each point represents the mean ± SEM of up to 11 experiments using cells from four to six different donors.
Naive B cells undergoing division-linked isotype switching predominantly express IgG1

Isotype switching by activated human naive B cells to the four IgG subclasses was next investigated. In the presence of CD40L, IL-4, and IL-10, naive B cells were induced to switch to IgG1, IgG2, and IgG3 (Fig. 5). The majority of isotype-switched B cells (~70%) were IgG1⁺, with the remainder expressing IgG2 or IgG3. In contrast, there was negligible switching to IgG4 (Fig. 5, a–e), with the percentage of IgG4⁺ cells being approximately equal to that of cells cultured in the presence of CD40L alone (data not shown). When analyzed on a per division basis, the pattern of switching to the IgG subclasses was similar to that for switching to total IgG, with cells expressing IgG1, IgG2, or IgG3 appearing after the third or fourth division, and the percentage of positive cells increasing with each subsequent division (Fig. 5f). Thus, the cells comprising the total IgG⁺ population were predominantly IgG1⁺, with IgG2 and IgG3 being equally expressed (Fig. 5f). Importantly, the sum of the percentage of cells expressing IgG1, IgG2, and IgG3 per division equaled the percentage of total IgG⁺ cells per division, demonstrating the reliability and specificity of the reagents used. CD40L and IL-4 also induced switching to IgG1, IgG2, and IgG3, but to a lesser extent than the combination of CD40L, IL-4, and IL-10 (data not shown). No switching to IgG1 was induced by CD40L alone, in agreement with the finding that these culture conditions did not induce switching to total IgG (data not shown, and Table I and Fig. 2).

Down-regulation of IgM and IgD by CD40L-activated B cells expressing IgG is division linked

When naive B cells undergo isotype switching, the expression of IgM and IgD is down-regulated (25). The expression of IgM and IgD on CFSE-labeled human naive B cells cultured with CD40L, IL-4, and IL-10 was therefore examined. In an equivalent manner to purified naive B cells, undivided B cells as well as B cells that had undergone one or two divisions continued to express a high level of both IgM and IgD (Fig. 6, a and b). After about three divisions, however, a population of cells expressing reduced levels of IgM and IgD was detected. This population continued to increase with each successive division (Fig. 6, a and b). To determine the phenotype of cells expressing reduced levels of IgM, the cells were double stained with mAbs specific for IgM and IgG. It was found that all IgG⁺ cells were indeed IgMlow (Fig. 6c), consistent with isotype-switched B cells being unable to express multiple Ig isotypes. By quantitating the proportions of IgMlow and IgG⁺ cells in each division it was found that down-regulation of IgM occurred over the same divisions as isotype switching to IgG (Fig. 6d). Thus, loss of IgM and IgD from activated human B cells proved to be division-linked, as was observed for isotype switching.

Division-linked isotype switching to IgG is independent of time in culture

Ig isotype switching by murine B cells has been found to be independent of time in culture (26–29). That is, although the proportion of cells in each division differs at different times, the percentage of switched cells in the corresponding divisions remains constant, suggesting that the proportion of B cells undergoing isotype switching during each cell division is determined by the number of times the cells divide (i.e., the division history of the cells) rather than by the time of in vitro culture or exposure to a stimulus. As switching to IgG by human B cells was found to be division-linked, it was necessary to determine whether isotype switching was also independent of time in culture. To achieve this, CFSE-labeled human naive B cells were cultured with CD40L, IL-4, and IL-10 and then harvested on sequential days, after 5 and 6 days of
stimulation. At these different times (i.e., 5 and 6 days), the greatest percentage of cells was found in the third and fifth divisions, respectively, thereby demonstrating that the cells had undergone several division cycles during this 24-h period (Fig. 7a). Although the distribution of cells across division differed for these two times, the percentage of IgG-positive cells in each division remained the same (Fig. 7b). Thus, like mouse B cells (26), isotype switching by human B cells is independent of time of culture. This also indicates that this process is not affected by time-dependent events, such as production of endogenous factors by activated B cells that may accumulate over time or the asynchronous rate of proliferation.

Discussion
Many studies of Ig isotype switching by activated human B cells have relied on Ig secretion as a read-out of this process. However, Ig secretion represents the culmination of a series of events, including B cell proliferation, production and expression of germline and mature Ig heavy chain constant region gene transcripts, differentiation into Ig-secreting cells, and, ultimately, Ig secretion. Thus, like mouse B cells (26), isotype switching by human B cells is independent of time of culture. This also indicates that this process is not affected by time-dependent events, such as production of endogenous factors by activated B cells that may accumulate over time or the asynchronous rate of proliferation exhibited by activated naive B cells.

FIGURE 5. Induction of the expression of IgG subclasses by IL-4 and IL-10. CFSE-labeled naive B cells were cultured for 6 days with CD40L, IL-4, and IL-10. The expression of sIgG (a), IgG1 (b), IgG2 (c), IgG3 (d), and IgG4 (e) by dividing B cells was determined by flow cytometry; each value represents the percentage of cells expressing the indicated IgG isotype. f, The percentage of cells in each division expressing total IgG (■), IgG1 (□), IgG2 (○), IgG3 (△), or IgG4 (○) was determined by division slicing. These results are representative of at least three independent experiments using cells from different donors.

FIGURE 6. Coordinated down-regulation of IgM and IgD with IgG isotype switching by CD40L-activated B cells. CFSE-labeled human naive B cells were cultured with CD40L, IL-4, and IL-10 for 6 days. The expression of IgM (a) and IgD (b) by dividing B cells was determined by labeling the cells with PE-conjugated mAb. c, The expression of IgM and IgG was determined by labeling the cells with PE-conjugated anti-IgG and biotinylated anti-IgM mAb, followed by SA-TC. d, The percentages of IgMlow (■) and IgG+ (○) cells in each division were determined by division slicing. These results are representative of at least three independent experiments using cells from different donors.
Recombinant human CD40L was shown to induce human B cells to proliferate in the absence of additional cytokines. The proliferative response, however, was augmented by addition of IL-4, IL-10, or IL-13 (Fig. 1). The greater degree of proliferation of cytokine-stimulated B cells, revealed by dilution of CFSE staining, not only confirmed the results from earlier studies using [3H]thy- midine incorporation to monitor cell proliferation (13, 30, 31, 39, 40), but indicated that the increase reflects the ability of IL-4, IL-10, and IL-13 to promote a greater proportion of cells to 1) enter division and 2) undergo further rounds of division (Fig. 1). Furthermore, although the proportions of CD40L-stimulated B cells in each division in cultures containing IL-4 or IL-10 were similar, IL-13 induced fewer activated B cells to undergo a similar number of divisions. This is consistent with previous reports showing that [3H]thyymidine incorporation by CD40L-stimulated B cells cultured with IL-4 or IL-10 was comparable (30, 31, 40), whereas incorporation by IL-13-stimulated B cells was less than that by IL-4- or IL-10-stimulated B cells (13, 31, 45). Thus, even though IL-4 and IL-13 share many biological functions (46), IL-13 is a less efficient promoter of B cell growth than IL-4.

In addition to promoting proliferation, IL-4, IL-10, and IL-13 induced CD40L-activated naive B cells to switch to IgG in a division-linked, time-independent manner, as well as to secrete low amounts of IgG (Figs. 2 and 7 and Tables I and II). The appearance of IgG cells coincided with the appearance of IgM low/− B cells, thereby showing that down-regulation of IgM (and IgD) was also division-linked (Fig. 6). There appeared to be a hierarchy to cytokine-mediated isotype switching to IgG, with the rate being greatest in the presence of IL-4, less with IL-13 (in agreement with data from previous studies (13, 31, 32, 47)), and very low in the presence of IL-10 (Fig. 2). The reason for the difference in potency between IL-13 and IL-4 is unclear, but does not appear to reflect differences in the expression of receptors for these cytokines, because naïve B cells have been reported to express abundant levels of the IL-13 receptor α-chain (45, 48). The proportion of IgG cells at the bulk population level, and for some donors on a per division basis as well, was greater in the presence of IL-4 and IL-10 than with either cytokine alone (Table I and Fig. 2), similar to the effect of the combination on B cell proliferation (Fig. 1). This accorded with earlier data of Punnonen et al. (49), who reported that IL-10 synergized with IL-4 to induce IgG secretion by PBMC activated by anti-CD40 mAbs. However, because their B cell population contained memory as well as naïve B cells, it was unclear whether the increase in secreted IgG resulted from 1) a synergistic effect of IL-4 and IL-10 on isotype switching, 2) induction of Ig secretion by the memory B cells present in the PBMC, or 3) both. By contrast, the use of highly purified naïve B cells here allowed us to conclude that IL-4 and IL-10 can function synergistically to induce Ig isotype switching in these cells.

When IgG subclasses were examined, IL-4 alone or in combination with IL-10 induced CD40L-stimulated naïve B cells to express IgG1, IgG2, and IgG3, but little IgG4. These IgG isotypes appeared in a similar division-linked pattern to that of total IgG (Fig. 5). The majority (∼70%) of IgG cells expressed IgG1, with fewer cells expressing IgG2 or IgG3, and very few if any expressing IgG4 (Fig. 5). When naïve IgD+ B cells were cultured with an activated T cell clone rather than a defined cytokine, the percentages of total secreted IgG of IgG1, IgG2, IgG3, or IgG4 isotypes were ∼70, 15, 10, and 5%, respectively (24). Since IgG1 is the most abundant IgG subclass present in human serum (∼60–65% of total IgG) (50), these in vitro data point to a greater rate of switching to this isotype in vivo than to the other IgG subclasses.

Our experiments with defined naïve B cells yielded several unexpected results that contrasted with the findings of previous investigations. For example, IL-10 has been reported to induce isotype switching to IgG1 and IgG3 (14–16), and IL-4 to induce isotype switching to IgG4 (11–13, 51). Furthermore, none of the known recombinant cytokines has been shown to induce the secretion of IgG2, although IFN-γ or combinations of S. aureus Cowan strain I, anti-CD40 mAb, and IL-10 induced the expression of germline transcripts of the y2 heavy chain constant region gene (18, 20, 21). Thus, our observation that IL-4 induced expression in CD40L-stimulated B cells of IgG2 as well as IgG1 and IgG3 was surprising. One explanation for these discrepancies lies in the use of different experimental procedures. First, our studies employed recombinant trimeric CD40L, which is known to provide a stronger stimulus to B cells than divalent anti-CD40 mAb, which was used in previous studies (52). Consequently, this improved signaling may have influenced the expression of different IgG subclass isotypes. Second, splenic B cells were used in our studies, while previous investigations largely used peripheral blood or tonsil B cells. Intrinsic differences between B cells isolated from different tissues cannot be excluded without detailed comparisons of the responses of these different B cell populations. Third, and most importantly, different B cell isolation and activation systems were employed in some of the previous studies mentioned above, including purification of naïve B cells on the basis of IgD positivity or addition to cultures of CD4+ T cell clones as a source of T cell-derived factors (11). Such methods may provide activation signals in addition to CD40L. Indeed, coactivation of CD40-stimulated human B cells via surface Ig can induce very high levels of secreted Ig (14, 17, 22, 41), and membrane TNF-α expressed by activated CD4+ T cells can provide a costimulatory...
signal for Ig production by human B cells (9, 53). This may explain our finding that the amounts of IgG secreted by CD40L-activated naive B cells were 10- to 100-fold less than those previously reported from total B cell populations (Table II) (31, 39–41). Lastly, the negligible isotype switching to IgG4 observed may not correlate with Ig secretion. It has been found that anti-IgG-induced murine B cell blasts stimulated with LPS and IL-4 could express IgG1, but failed to secrete detectable levels of IgG (55). More recently, a similar reciprocal relationship was reported for Ig secretion and isotype switching by activated human B cells (56). Consequently, the lack of expression of IgG4 by human B cells activated with CD40L and IL-4 does not mean that the cells are incapable of secreting IgG4. Similarly, CD40L-activated B cell stimulated with IL-4 may express IgG1, IgG2, and IgG3, but may not secrete these isotypes. In support of this idea is the observation that although IL-4 induced the transcription of germline mRNA by CD40-stimulated human B cells, two-thirds of these B cells failed to produce IgE (57). Moreover, according to recent evidence, dendritic cells (DC) and follicular DC can influence B cell activation by modulating survival, proliferation, differentiation, isotype switching, and Ig secretion (56, 58–67). Based on these observations, a model can be proposed whereby initial interactions with T cell-derived signals such as CD40L and IL-4 induce the expression of switched isotypes on the surface of activated B cells, but further signals delivered via follicular DC or DC are required for Ig secretion. The validity of such a model is currently being explored.

Ig secretion by activated human B cells can be modulated by other cytokines and PGs. For example, IL-5 (68), IL-6 (69), IL-7 (70), IL-9 (71), and TNF-α (57) enhance Ig production, while IFN-γ, IFN-α (51), TGF-β (17, 57), IL-12 (72), and PGE3 (51, 73) inhibit it. The ability to describe Ig isotype switching with reference to division history now makes it possible to delineate whether this array of factors modulates Ig secretion by affecting cell division. The division-based rate of isotype switching, the amount of IgG secreted by CD40L-activated human B cells, and the overall regulation of the humoral immune response in health and in conditions such as allergy.

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