Maintenance of Human Germinal Center B Cells In Vitro

By John D. Pound and John Gordon

The ability to maintain germinal center (GC) B cells in culture should facilitate studies on the molecular and cellular events which accompany affinity maturation and the generation of memory in T-dependent responses. We have investigated the ability of cytokines to maintain human tonsillar GC B cells (IgD+/CD39+/CD38+/CD77+) in the "CD40 culture system." In the absence of added cytokines, CD40 monoclonal antibody held on CD32-transfected L cells effectively sustained DNA synthesis in GC B cells for a maximum 3 to 4 days. Of the following cytokines (interleukin-1β [IL-1β], IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, and stem cell factor), only IL-2 and IL-4 provided a significant enhancement to DNA synthesis in the CD40 culture system; this was modest and short-term. Following a study on the cooperative activity between pairs of cytokines, triple combinations were identified that could maintain high levels of GC B-cell stimulation for at least 10 days. IL-10 was a common component of these synergistic cytokine cocktails, which were IL-10 + IL-4 + IL-7; IL-10 + IL-3 + IL-7; IL-10 + IL-1α + IL-2; IL-10 + IL-1β + IL-3, and IL-10 + IL-3 + IL-6. Culture of GC B cells with these cytokine combinations resulted in a net increase in viable cell numbers increased up to fourfold. Cells recovered from these cultures retained a GC B-cell phenotype with a significant proportion being CD38+/CD44−, features characteristic of centroblasts. Studies with metabolically inactive CD32-L cells supported a role for stromal cell-derived soluble factors in maintaining GC B cells in vitro.

© 1997 by The American Society of Hematology.

From the Department of Immunology, The Medical School, University of Birmingham, Birmingham, UK.

Submitted March 1, 1996; accepted September 18, 1996.

Supported by a Biotechnology Program Grant from the European Community (Contract No. BIO-2CT92-0269) and a Program Grant from the UK Medical Research Council.

Address reprint requests to John D. Pound, PhD, Department of Immunology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1997 by The American Society of Hematology.

From www.bloodjournal.org by guest on May 3, 2017. For personal use only.
MATERIALS AND METHODS

Reagents. Cytokines were human recombinant proteins purchased from R&D Systems Ltd (Oxford, UK) with the exception of IL-4, which was a gift from Dr Steven Gillis (Immunex Research and Development Corp, Seattle, WA). Cytokines were initially assessed at three concentrations; for IL-1β, stem cell factor (SCF), IL-3, and IL-6, these concentrations were the nominal ED50 in reference target cell systems as stated by the manufacturer, together with a 10-fold higher and a 10-fold lower concentration. For IL-10, concentrations were chosen on the basis of effective ranges reported by Rousset et al12; for IL-7, as reported by Saeland et al10 and for IL-2 and IL-4, as reported by Holder et al.11 In experiments to investigate gate synergy, IL-2 was employed at 20 ng/mL and other cytokines at the intermediate concentration from the range shown in Fig 2.

MoAbs G28-5 (CD40; ref 13), BU52 (CD44), and AC2 (CD39; ref 14) were produced from the hybridomas in the Department of Immunology, University of Birmingham (Birmingham, UK) and were purified by ion exchange chromatography on DE52 (Whatman Ltd, Maidstone, UK). The G28-5 clone was obtained from the American Type Culture Collection repository (Rockville, MD) and the BU52 clone from D. Hardie (Department of Immunology, University of Birmingham). Ascitic fluid containing rat IgM CD77 MoAb, 3B-13 was a gift from Dr J. Wielts (Institut Gustave-Roussy, Villejuif, France) and was conjugated to FITC by standard procedures. FITC conjugated CD19 and anti-IgD MoAbs and phycoerythrin conjugated CD3 and CD38 MoAbs were purchased from Dako Ltd (High Wycombe, UK). Phorbol myristate acetate (PMA) was obtained from Sigma Chemical Co (St Louis, MO) and ionomycin from Calbiochem-Novabiochem (Nottingham, UK).

Flow cytometry. Flow cytometry was performed on a Becton Dickinson FACScan and data analysis performed using LYSIS software (Becton Dickinson, Mountain View, CA). Gates for viable lymphocytes and CD32-L cells were set on forward and side scatter. Isotype matched conjugates of irrelevant specificity (Dako Ltd) were employed as controls.

Tonsillar B cells. Human tonsils were obtained from patients undergoing routine tonsillectomy. All procedures were performed at room temperature except where stated. GC B cell1 and resting B-cell2 fractions were isolated as described previously. Briefly, cells were extracted by dissection and dispersal of tonsil tissue in RPMI 1640 (GIBCO Ltd, Paisley, Scotland) and removal of large tissue fragments at 1 g. Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and T cells were depleted by two rounds of rosette formation with 1/10 volume of 0.2% disodium EDTA in phosphate-buffered saline (PBS) pH 7.0 and resuspended in CM. They were γ-irradiated with a dose of 20,000 rads before addition to B-cell cultures at a ratio of (B cells:L cells) 10:1.

Measurement of DNA synthesis. [3H]thymidine ([Amersham International, Amersham, UK] 10 μCi/mL in culture medium, 50 μL per well) was added after the specified interval and cells were procured after a further 16 to 18 hours in culture on a Skatron cell harvester. Assays were performed in triplicate.

Measurement of cell proliferation. Cells recovered from triplicate microplate wells were pooled and incubated for 10 minutes with 1/10 volume of 0.2% disodium EDTA in PBS to disperse aggregates then counted in a Neubauer hemocytometer. Viability was assessed by trypan blue exclusion.

Measurement of Ig. Concentrations of human IgA, IgM, IgE, and IgG subclasses in culture supernatants were measured by enzyme-linked immunosorbent assay using commercially available kits (The Binding Site Ltd).

Fixation of CD32-L cells with carbodiimide. CD32-L cells were fixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI; Calbiochem-Novabiochem, Nottingham, UK) as described by Anderson et al.5 Briefly, CD32-L cells were resuspended in ECDI in saline and incubated on ice for 1 hour, resuspending the cells every 15 minutes to prevent agglutination. They were then washed in serum free RPMI. A concentration of 375 mmol/L ECDI was required to inhibit [3H]thymidine uptake by the CD32-L cells by >95%.

Statistical analysis. The criterion used to define synergy between cytokines in stimulation of B-cell DNA synthesis was that the mean of triplicate counts for [3H]thymidine incorporation for cells cultured with two cytokines less the standard error of the mean (SEM) was greater than the sum of the mean counts for cells cultured with each cytokine alone plus the mean of the SEMs for the responses to the individual cytokines. Statistical significance of positive interactions in data from three experiments was estimated by two-way analysis of variance.

RESULTS

Culture with CD40 MoAb and CD32 transfected L cells can stimulate short-term high-rate DNA synthesis by GC B cells in the absence of exogenous cytokines. High rates of spontaneous DNA synthesis in freshly isolated GC B cells fall rapidly in culture such that [3H]thymidine incorporation cannot be detected after 2 days (Fig 1A), reflecting the progress of these cells to apoptosis in the absence of rescue signals. Neither CD40 MoAb (Fig 1A) nor CD32-L cells (Fig 1B) influenced the decline in DNA synthesis when added to cultures alone; when used together they stimulated a large increase in [3H]thymidine uptake that reached a maximum after 2 days (Fig 1B). The magnitude of this response approached that induced by a combination of PMA (1 nmol/L) and ionomycin (0.8 μg/mL) (Fig 1B), which is an optimal
GROWTH OF GERMINAL CENTER B CELLS IN VITRO

High-density B cells with CD40 MoAb in the presence of CD32-L cells promoted a slow rise in [3H]thymidine incorporation from basal levels over 4 days to a level that was equivalent to only about 30% of the response induced by PMA and ionomycin but was then sustained at this level for at least 10 days (Fig 1C).

Ability of individual cytokines to maintain CD40-dependent DNA synthesis in GC B cells. As an initial screen for growth activity, GC B cells were cultured with various cytokines in the presence and absence of CD40 MoAb and CD32-L cells and [3H]thymidine incorporation was measured after 5 days, a time at which basal CD40-dependent stimulation approached a minimum (Fig 1B).

We confirmed the reported ability of IL-2 to stimulate GC B-cell DNA synthesis in the absence of other factors (Fig 2A) and found that this was the only cytokine to show CD40-independent activity consistently in this regard. IL-7 typically promoted a modest CD40-independent response (Fig 2A) although cells from some tonsils gave responses of a similar magnitude to those stimulated by IL-2 and these were enhanced by both CD40 MoAb and the CD32 transfectant (not shown). IL-4 stimulated dose-related CD40-dependent responses whereas IL-3 and IL-10 each stimulated small CD40-dependent responses that were enhanced in the presence of CD32-L cells (Fig 2A and B). SCF was essentially inactive at the concentrations used.

Cytokines can synergize to stimulate CD40-dependent DNA synthesis in GC B cells. Cytokines from the panel described above (Fig 2) were investigated for potential synergistic interactions in promoting DNA synthesis in 5-day cultures of GC B cells. Four of the 28 possible pairs of cytokines showed synergy in the presence of CD40 MoAb and CD32-L cells. IL-4 synergized with 3 cytokines (Fig 3): IL-1β (P<.019), IL-7 (P<.021), and IL-10 (P<.053) whereas IL-7 synergized with IL-3 (P<.008). We found no evidence of synergy between cytokines in cultures containing either CD40 MoAb or CD32-L cells alone, nor in unsupplemented cultures.

IL-10 is an important costimulatory factor for CD40-dependent DNA synthesis in GC B cells. Having established that synergistic interactions operate between certain pairs of cytokines in stimulating GC B cells we next investigated combinations of three cytokines for cooperative growth stimulation in the presence of both CD32-L cells and CD40 MoAb. Five sets of 3 cytokines showed synergy in 6-day cultures and all of these included IL-10: IL-10+IL-1β+IL-2 (P<.0002), IL-10+IL-1β+IL-3 (P<.0063), IL-10+IL-3+IL-6 (P<.035), IL-10+IL-7+IL-3 (P<.087), and IL-10+IL-7+IL-4 (P<.048). Importantly, three of these combinations exclusively comprised cytokines which stimulated only very small increases in DNA synthesis when used alone at the same concentrations (Fig 2B). Kinetic studies identified a significant CD40-independent component in the activity of the combination of IL-10+IL-1β+IL-2 (see later and Fig 5B). Of the four combinations that were confirmed to show CD40-dependent activity, IL-10 with IL-4 and IL-7 consistently promoted the greatest response (Fig 4A). Resting B cells from the same tonsils did not show enhanced DNA synthesis in 6-day cultures in response to these combi-

**Fig 1.** Kinetics of DNA synthesis by GC and resting B cells. B cells were cultured for 10 days in the presence of CD40 MoAb, G28-5 (●), PMA with ionomycin (△), or CM alone (○). [H]thymidine uptake was measured after the intervals shown. (A) GC B cells; (B) GC B cells in the presence of CD32-L cells; (C) resting B cells in the presence of CD32-L cells. Data are mean cpm for cells purified from the same tonsil and representative of five experiments. Values for [3H]thymidine uptake by CD32-L cells alone (~4,000 to 10,000 cpm) have been subtracted in (B) and (C).
Fig 2. DNA synthesis by GC B cells cultured with cytokines. GC B cells were cultured for 5 days with cytokines in the presence (■) or absence (□) of CD40 MoAb, G28-5, and then \(^{[3}H\)thymidine uptake was measured. The values in brackets indicate the concentration of cytokine employed in ng/mL (U/mL for IL-7). (A) GC B cells; (B) GC B cells in the presence of CD32-L cells. Data are the mean cpm ± SEM and are representative of three experiments. Values for \(^{[3}H\)thymidine uptake by CD32-L cells alone (∼3,000 to 7,000 cpm) have been subtracted in (B).

Cytokine combinations can stimulate a second phase of CD40-dependent DNA synthesis by GC B cells. Having established that certain cytokine combinations could stimulate GC B cells to DNA synthesis in 6-day cultures (see above) we next investigated their activity over longer periods. By measuring \(^{[3}H\)thymidine uptake at intervals more than 10 days we found that the effective cytokine combinations all stimulated a second phase of CD40-dependent DNA synthesis which reached a maximum after 6 to 7 days and was of similar magnitude to the spontaneous levels observed in freshly isolated GC cells (Fig 5A). Only one combination of cytokines (IL-10 with IL-7 and IL-4) enhanced the earlier phase of CD40-dependent DNA synthesis (from day 1 to day 3) (Fig 5A). One cytokine combination, IL-10+IL-1β+IL-2, was found to stimulate DNA synthesis in the absence of CD40 MoAb (Fig 5B).

The time course of \(^{[3}H\)thymidine uptake by GC B cells in the presence of stimulatory combinations of cytokines contrasted with that of resting B cells under the same conditions (Fig 5C). The initial phase of DNA synthesis in high density B cells was enhanced in the presence of all cytokine combinations except for IL-10 with IL-1β and IL-2. Furthermore, no second phase of DNA synthesis corresponding to that seen in GC B-cell cultures was evident for resting B cells (Fig 5C). It is possible that maintenance of DNA synthesis in resting B cells in 10-day cultures in the presence of IL-10+IL-7+IL-4 is mainly attributable to the effect of IL-4 as this is a strong cofactor for CD40-dependent responses in this cell type, whereas that in the presence of IL-10, IL-1β, and IL-2 reflects the IL-2 induced CD40-independent outgrowth of GC B cells that were a significant contaminant in some of these experiments (Fig 5B, C, and D).

Cytokine combinations can stimulate proliferation of GC B cells. Net increases in viable cell numbers could be detected after 3 to 4 days in GC B cells cultured with triple...
cytokine combinations, which stimulated DNA synthesis, and these increases reached maximum values of 50% to 100% after 7 to 8 days (Fig 6A through E). No net increase in cell numbers was observed in the CD40 culture system in the absence of cytokines (Fig 6F). A steady accumulation of nonviable cells in all of these cultures leads to increases in total cell numbers of threefold to fourfold; however, the proportion of nonviable cells produced was greater in the later stages of cultures containing IL-10+IL-1β+IL-2 and IL-10+IL-7+IL-4 (Fig 6A and E).

The largest increases in viable cell numbers consistently occurred in cultures containing IL-10+IL-1β+IL-2 (Fig 6A). However, the combination IL-10+IL-7+IL-4 induced the greatest rate of increase, with a doubling time of approximately 24 hours from day 2 to 3 and this mirrored the greater preceding burst of [3H]thymidine incorporation (Fig 6E).

**Phenotype of cells cultured with cytokines.** Cells recovered after 8 days from GC B-cell cultures containing CD32-L cells, CD40 MoAb, and cytokine combinations were >95% CD19+ and <1% CD3+ arguing against possible outgrowth of contaminating non-B cells, especially T cells. Most of the viable cells in these cultures formed large aggregates and had the morphological characteristics of large blasts on the basis of Jenner-Giemsa staining (not shown). The great majority of cells recovered (>90%) remained CD38+ and a significant proportion of these also retained expression of CD77 but lacked CD44, features typical of centroblasts (Table 1).

**Ig secretion by GC B cells cultured with cytokines.** GC B cells cultured for 10 days with CD40 MoAb, CD32-L cells, and growth stimulatory cytokine combinations secreted only small amounts of Ig (Fig 7). All such cytokine combinations induced an increase in IgA and IgG3 secretion (Fig 7B and E) and there was a slight enhancement of IgG1 and IgG4 secretion in the presence of certain combinations (Fig 7C and F). These differences may not be significant, however, when it is considered that cell numbers were appreciably higher in cultures containing cytokines (data not detailed).

In the absence of CD40 MoAb, IL-10 together with IL-1β and IL-2 stimulated large increases in secretion of IgM, IgA, and all IgG subclasses (Fig 7) but not IgE (not shown) by GC B cells. This was the only cytokine combination that stimulated IgG2 synthesis. These effects were inhibited by the presence of CD40 MoAb.

**CD32-L cells must be metabolically active to support long-term cytokine-dependent DNA synthesis in GC B cells.** Irradiated CD32-L cells may release soluble factors which themselves contribute to the observed CD40-dependent
Fig 5. Kinetics of DNA synthesis by GC and resting B cells cultured with combinations of cytokines. GC B cells (A and B) or resting B cells (C and D) were cultured for 10 days with CD32-L cells in the presence (A and C) or absence (B and D) of CD40 MoAb, G28-5, and various cytokine combinations or CM (C). "[3H]thymidine uptake was measured after the intervals shown. (h) IL-10 + IL-1β + IL-3; (m) IL-10 + IL-7 + IL-4; (●) IL-10 + IL-3 + IL-6. The combination of IL-10 + IL-7 + IL-3 gave a similar profile to that of IL-10 + IL-3 + IL-6. Data are mean cpm for cells purified from the same tonsil and representative of four experiments.

Fig 6. Proliferation of GC B cells cultured with cytokine combinations. GC B cells were cultured in microtiter plates for 10 days with CD32-L cells, CD40 MoAb, G28-5, and various cytokine combinations (A through E) or culture medium alone (F). Cultures were seeded at 10⁵ per well and cells were recovered from 3 triplicate wells and pooled for counts at the times shown. Viable cell numbers are represented by open symbols and nonviable cells by the closed symbols. Tritiated thymidine incorporation (broken line, second ordinate) was measured in parallel cultures after the intervals shown as described for Fig 1. Data are representative of three experiments. (A) IL-10 + IL-1β + IL-2; (B) IL-10 + IL-1β + IL-3; (C) IL-10 + IL-3 + IL-6; (D) IL-10 + IL-3 + IL-7; (E) IL-10 + IL-4 + IL-7.

growth of GC B cells. We investigated this possibility by fixing the CD32-L cells with ECDI, a reagent that renders cells metabolically inactive yet allows their surface molecules to interact with receptor ligands. The ability of ECDI-fixed and irradiated CD32-L cells to support cultures of GC B cells was then compared.

Although ECDI-fixed CD32-L cells were able to support a similar level of DNA synthesis by GC B cells in the presence of CD40 MoAb to that provided by irradiated transfectants, the enhancement of these responses by IL-10 with IL-4 and IL-7 was not evident when fixed L cells were used (Fig 8A). Thus, the costimulatory effect of the cytokine combination appears to require metabolically active stromal cells, most likely so that they can secrete additional growth factors. ECDI-fixed CD32-L cells showed a much reduced capacity to support resting B-cell cultures in the presence of CD40 MoAb alone than did irradiated transfectants; however, IL-4 and to a lesser extent the combination of IL-10 with IL-4 and IL-7, were able to overcome this (Fig 8B). The similarity of the CD40-dependent IL-4 induced responses in resting B cells supported by irradiated and fixed L cells provides evidence that the ability of the
Table 1. CD77 and CD44 Expression by GC B Cells Maintained in the CD40 Culture System

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>%CD38%/CD77</td>
<td>%CD38%/CD44</td>
</tr>
<tr>
<td>IL-10</td>
<td>72</td>
<td>79</td>
</tr>
<tr>
<td>+ IL-1β + IL-3</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>IL-10 + IL-1β + IL-2</td>
<td>33</td>
<td>30</td>
</tr>
<tr>
<td>IL-10 + IL-3 + IL-7</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>IL-10 + IL-4 + IL-7</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>IL-10 + IL-3 + IL-6</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>IL-10</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>+ IL-4 + IL-7</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>IL-10</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>+ IL-3 + IL-6</td>
<td>33</td>
<td>25</td>
</tr>
</tbody>
</table>

GC B cells were cultured for 8 days with CD40 MoAb, G28-5 in the presence of CD32-L cells and various combinations of 3 cytokines. Cells recovered were >93% CD38+, >95% CD19+, and <1% CD3-.

DISCUSSION

As Ig v-gene hypermutation is believed to be initiated in the centroblasts, the signals that regulate proliferation of GC B cells are likely to be important in the events regulating affinity maturation. Centroblasts in GC dark zones initially arise from antigen-specific primary B-cell blasts, which have colonized a follicle following their activation as resting B cells in T-zones; once established, however, it is possible that this pool may also be supplied by centrocytes which re-enter the dark zone. This possibility was first raised by Holder et al4 by demonstrating that membrane-bound CD40L was capable of maintaining GC cells in active cycle over 3 to 4 days. More recently, Han et al18 have suggested that in order for the population of the GC to remain stable, rescued B cells must divide several times. Although a major role of CD40 is probably to direct selected centrocytes into the memory pathway,2,7 it is not known whether GC B cells that have undergone initial interactions via CD40 are then receptive to additional signals that can drive their re-entry into cell cycle and possibly sustain their cycling status for some time. The present study aimed to identify factors that can maintain CD40-rescued GC B cells in cell cycle and thereby, indirectly, provide a potential first step toward characterizing the external signals underlying affinity maturation.

CD40 MoAb alone has been regarded as a potent rescue signal for GC B cells but a poor stimulator of their proliferation.2 In the presence of CD32-transfected L cells, however, we have now shown that CD40 MoAb can provide a strong, albeit transient, stimulus for GC B cell DNA synthesis. Under these conditions of the “CD40 culture system,” not only is the decline in spontaneous DNA synthesis of GC B cells prevented but the initial rate is enhanced to near maximal levels achievable before subsiding after 2 days. A major contribution to this potent stimulation is likely to be the optimal receptor cross-linking facilitated by the CD32

Fig 7. Ig secretion by GC B cells cultured with cytokine combinations. Ig concentration was measured in the supernatant of GC B cells cultured for 10 days with CD32-L cells and cytokine combinations in the presence (●) or absence (□) of CD40 MoAb, G28-5. (A) IgM; (B) IgA; (C) IgG1; (D) IgG2; (E) IgG3; (F) IgG4.
transfectant as no stimulation occurred in its absence. That metabolically inert ECDI-fixed CD32 transfectants supported similar levels of stimulation to their irradiated counterparts makes it unlikely that soluble factors from the L cells played a major role in enhancing basal CD40-induced responses.

As rescue via CD40 is known to extend the viability of GC B cells in culture about 2 to 3 days the kinetics of DNA synthesis in the presence of CD40 MoAb and CD32-L cells could reflect initial rescue of centrocytes which re-enter cell cycle for 2 days but then succumb to apoptosis. Alternatively, continued cycling of centroblasts could also make a contribution to this profile and our current data do not allow us to distinguish between these possibilities. The GC dark zone is reported to be essentially devoid of T cells and our current data do either the culture medium or any of the stimulatory reagents; cocktails was reflected in increased cell numbers in cultures with or without CD40 MoAb, G28-5. Cultures contained CD32-L cells that had been irradiated (as in Figs 1 through 7) or fixed with ECDI. 

![Fig 8. DNA synthesis by GC and resting B cells cultured with irradiated and ECDI-treated CD32-L cells. GC B cells (A) or resting B cells (B) were cultured with CM, IL-4 (20 ng/mL) or a combination of IL-10 + IL-7 + IL-4 with or without CD40 MoAb, G28-5. Cultures contained CD32-L cells alone or fixed with ECDI. (H)Thymidine uptake was measured after 7 days. Data are means ± SEM. Values for CD32-L cells alone (~8,000 cpm) have been subtracted.](https://www.bloodjournal.org/)

CD40mAb
CD32-L cells
- irradiated
+ ECDI

CD40mAb
CD32-L cells
- irradiated
+ ECDI

Our finding that IL-10 was a component of all triple combinations of cytokines that acted synergistically in stimulating CD40-dependent DNA synthesis by GC B cells suggests that this T-cell product may be a particularly important growth factor for GC B cells. Levy and Brouet have recently reported that IL-10 can rescue splenic GC B cells from apoptosis. Therefore, it is possible that the role of IL-10 that we identified in promoting DNA synthesis was related to its ability to improve survival despite our inability to detect a significant direct effect on the rescue of tonsillar GC B cells from programmed death.

The stimulation of GC B-cell DNA synthesis by cytokine cocktails was reflected in increased cell numbers in cultures (Fig 6). In the experiments documented we did not replenish either the culture medium or any of the stimulatory reagents; therefore, the responses observed in longer term cultures may well have been suboptimal. The greater proportion of nonviable cells found in cultures containing IL-10 + IL-7 + IL-4 (Fig 6A and E) as compared with other stimulatory combinations may be a consequence of nutrient depletion resulting from the preceding higher rates of cell division. An alternative possibility is that this reflects the generation of larger numbers of short-lived blasts under these conditions. The high observed rate of proliferation induced by the combination IL-10 + IL-7 + IL-4 (doubling time ~24 hours, Fig 6E) suggests that these culture conditions may be relevant to the cell cycle kinetics of the follicular reaction.

When GC B cells from cultures containing the cytokine combination IL-10 + IL-4 + IL-7 were recovered and then washed and transferred to cultures with fresh cytokines, CD32-L cells, and CD40 MoAb after 7 or 8 days and then at weekly intervals thereafter, high rates of DNA synthesis could be maintained for up to 4 weeks (data not detailed). Therefore, we believe that long-term maintenance of GC B cells will be feasible using the conditions described.

Five of the seven cytokines which were seen to cooperate in stimulating GC B-cell DNA synthesis have been identified in cells found in the GC suggesting that they might be of some physiological relevance. IL-2, IL-4, and IL-10 have each been identified at the mRNA level in GC T cells and IL-1β and IL-7 FDC at both the mRNA and protein levels. Although IL-3 is a product of TH cells, there are as yet no specific reports of IL-3 production by cells in the GC. IL-6...
is an important autocrine B-cell growth and differentiation factor whereas a study reporting that IL-10 can be produced by activated human splenic B cells indicates that GC B cells may produce their own IL-10.

Three lines of evidence show that the cells being maintained in the CD40 system with triple combinations of cytokines were indeed GC B cells. Firstly, when comparing GC B-cell populations with resting B cells, the cytokine effects were selective: for resting B cells, they failed either to enhance CD40-dependent DNA synthesis at day 5 or to promote a second phase of CD40-dependent [3H]thymidine incorporation, each of which was evident for GC B-cell populations. Secondly, and most importantly, cells recovered from 8-day cultures of GC B cells with cytokine combinations that maintained DNA synthesis retained a GC B-cell phenotype; these cells were not only CD38− (strongly expressed by all GC B cells) but a substantial proportion were also CD77+ and/or CD44+. Although CD77 is considered as a general but highly specific marker for B cells of GC origin, it is more strongly expressed by centroblasts than centrocytes; the absence of CD44 is a good marker for centroblasts and distinguishes them from centrocytes that are weak expressors of this receptor. The finding that these cultures contained significant numbers of CD38+CD44− cells and CD38+CD77+ cells (Table 1) is consistent with the notion that proliferating centroblasts are being encouraged under the culture conditions established. Finally, we have seen that withdrawing either the CD40 stimulus or the cytokines results in cessation of DNA synthesis followed by death of the cultured cells within 48 hours (data not detailed); this is in keeping with maintaining a potentially apoptotic GC B-cell population in these cultures. The requirement for continuous stimulation via CD40 may reflect the importance of CD40 in maintaining proliferation of GC B cells in vivo; indeed, studies in mice have shown that antibody to CD40L can abrogate an established GC reaction.

GC B cells maintained in cycle by cytokines under the conditions described secreted only small amounts of Ig (Fig 7) indicating that no significant differentiation to plasma cells occurred. The high levels of IgM secretion by GC B cells cultured with IL-10+IL-1β+IL-2, which stimulated long-term CD40-independent DNA synthesis, probably reflects the action of IL-2, which has been reported to promote the outgrowth of a CD5− subset to IgM secreting plasmablasts. Our finding that IL-10 and IL-1β cooperated with IL-2 to stimulate DNA synthesis suggests that the former two cytokines may be relevant cofactors in that pathway.

Recently, Arpin et al reported that a combination of IL-2 and IL-10 stimulates CD40-dependent proliferation of GC B cells in 3-day cultures. These cells acquire some of the phenotypic characteristics of memory cells (CD38+/CD20−) after a further 4 days but if the CD40 stimulus is withdrawn, differentiation toward plasma cells occurs. We did not identify cooperativity between IL-10 and IL-2 in our studies but this may simply reflect kinetic considerations; thus, because our intention was to identify the factors for long-term growth of GC B cells, we specifically screened for interactions among cytokines in stimulation of DNA synthesis in 5-day cultures and later while not investigating earlier time points.

With the one exception (IL-10+IL-1β+IL-2), cooperation among cytokines in stimulating DNA synthesis of GC B cells was identified only in cultures containing both CD40 MoAb and CD32-L cells. This suggests that the majority of cytokine-mediated effects observed may depend on extensive cross-linking of CD40 and/or the presence of stroma. In the absence of exogenous cytokines, ECDI-fixed CD32 transfectants that are metabolically inert supported the basal CD40-dependent DNA synthesis in GC B cells, almost as effectively as did their irradiated counterparts arguing against a major role for feeder cell-derived soluble factors. However, the combination of exogenous IL-4 with IL-7 and IL-10 which enhanced CD40-dependent DNA synthesis in the presence of irradiated CD32-L cells had no effect when ECDI-fixed transfectants were employed. Therefore, soluble factors from the transfectant may act in concert with exogenous cytokines and this would support a role for stromal derived factors in long-term CD40-dependent GC B-cell growth. Thus, this study provides further evidence that growth of GC B cells is likely to be regulated by stroma.

The description of a culture system capable of maintaining B cells of GC origin for at least 10 days, and potentially for several weeks, should aid studies aimed at elucidating mechanisms underlying somatic hypermutation on Ig v-region genes and possibly other events associated with the GC response. The results presented here also provide candidate factors that may be physiologically relevant to the process of sustaining centroblast proliferation.

ACKNOWLEDGMENT

We are grateful to Michelle Holder for Ig measurements.

REFERENCES


Maintenance of Human Germinal Center B Cells In Vitro

John D. Pound and John Gordon

Updated information and services can be found at:
http://www.bloodjournal.org/content/89/3/919.full.html

Articles on similar topics can be found in the following Blood collections
  Immunobiology (5474 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml