Interleukin 10 and Transforming Growth Factor β Cooperate to Induce Anti-CD40–activated Naive Human B Cells to Secrete Immunoglobulin A

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Summary

In the present report, we have investigated the in vitro differentiation of surface(s) sIgD$^+$ and sIgD$^-$ human B cells into Ig-secreting cells in response to various stimuli. sIgD$^+$ B cells homogeneously expressed some of the antigens identifying mantle zone B cells, but lacked expression of germinal center markers, thus confirming that the B cell populations positively selected on the basis of sIgD expression were highly enriched for naive B lymphocytes. Conversely, sIgD$^-$ B cells expressed some of the antigens specifically associated with germinal center B cells. T cell–independent differentiation of sIgD$^+$ and sIgD$^-$ B cells could be achieved by simultaneous crosslinking of sIgs and CD40 in the presence of a mouse Ltk$^-$ cell line stably expressing human CDw32/FcγRII (CDw32 L cells). In this experimental system, sIgD$^+$ B cells were exclusively proned for IgM synthesis, whereas sIgD$^-$ B cells produced IgG, IgM, and IgA. Both the human and viral forms of interleukin 10 (IL-10) strongly increased the Ig secretion by sIgD$^+$ and sIgD$^-$ B cells simultaneously activated through sIgs and CD40. IgM and IgG constituted the predominant Ig isotype produced by sIgD$^+$ and sIgD$^-$ B cells, respectively, in response to IL-10. sIgD$^+$ B cells could be induced for IgA synthesis upon co-culturing with transforming growth factor β (TGF-β) and IL-10, in the presence of an anti-CD40 monoclonal antibody presented by the CDw32 L cells. In contrast, TGF-β suppressed the IL-10–mediated IgG, IgM, and IgA secretions by sIgD$^-$ B cells. sIgD$^+$ B cells could not be induced for IgA synthesis by TGF-β and IL-10 after crosslinking of their sIgs, suggesting that ligation of CD40 was one of the obligatory signals required for commitment of naive B cells to IgA secretion. Limiting dilution experiments indicated that the IgA-potentiating effect of TGF-β was due to its capacity to increase the frequency of IgA-producing cells, most likely as a consequence of class switching. Taken together, our data strongly suggest that TGF-β is involved in the regulation of IgA isotype selection in humans.

The pattern of Ig classes secreted by B cells in response to antigenic stimulation depends upon several factors such as the structure of the antigen and the anatomic site in which the immune response takes place. The production of high affinity antibodies using downstream heavy chain genes, in the course of a secondary immune response, results from a maturation process initiated by the antigen itself, which occurs within the germinal centers of secondary follicles (1, 2). As recently demonstrated in the murine system (3, 4), the molecular mechanism of class switching involves the rearrangement of the genes located on the heavy chain locus and the subsequent deletion of the C_H genes intervening between a variable region exon and the C_H gene to be expressed. Evidence supports the notion that this process is directed and regulated by cytokines and other environmental signals delivered to B cells through interactions with other cell types. In mice, IL-4 has been described to regulate the secretion of IgE and IgG1, whereas IFN-γ and TGF-β have been associated with the induction of IgG2a and IgA synthesis, respectively (5, 6). In humans, it is now recognized that IL-4 is involved in IgE (7, 8) and IgG4 (9, 10) heavy chain switching, but to this date, the cytokine-mediated regulation of IgG1, IgG2, IgG3, and IgA isotypes has not been elucidated. The relative lack of information on that matter in the human system may partly relate to differences in the experimental models used in mice and in humans. Indeed, murine studies have essentially relied on the use of LPS to initiate responsiveness of B cells to switch factors, whereas such a tool was not available for human B cells until recently. Consequently, the study of the cytokine regulation of Ig isotype switching in humans

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has long been hampered by the relatively poor efficiency of the activation systems, precluding the culture of B cells in limiting dilutions. In addition, the maturational state of the B cell populations studied in early experiments was poorly defined and did not allow discrimination between isotype switching and maturation of pre-committed precursors.

However, two novel experimental systems in which highly efficient activation of human B cells could be achieved through triggering of non-Ig–related molecules have permitted us to overcome these technical difficulties. The first one is based on the capacity of syngeneic or allogeneic activated T cells to stimulate entry of B cells into cycle in a non-MHC–restricted fashion via direct cell-to-cell contact (11–14). In the second one, polyclonal activation and sustained proliferation of human B cells is obtained by presentation of an anti-CD40 mAb on irradiated mouse L cells stably expressing CDw32/FcγRII (15). In both models, B cells can be induced for IgE synthesis after IL-4 stimulation (10, 16). The observation that IL-4 is not able to drive purified human B cells to differentiate into IgE-secreting cells unless T cells (10), EBV (17) or an anti-CD40 mAb (16, 18, 19) are provided, is consistent with the hypothesis that Ig isotype switching to IgE requires at least two signals, one delivered by IL-4, the other mediated through CD40 or another B cell surface structure. Therefore, we explored the possibility that the CD40 signaling pathway could also be involved in the control of IgG and IgA isotype selection. We based our experimental approach on the assumption that naive B cells positively selected on the basis of sIgD expression and presumably depleted in post-switch B cells could constitute a suitable target for the detection of switch events. Isolated $sIgD^+$ and $sIgD^-$ B cells were first compared for their capacity to secrete Ig in response to various B cell stimulants. In the absence of exogenous factors, both B cell subsets were inducible for Ig secretion after costimulation with anti-Ig reagents and an anti-CD40 mAb presented on CDw32 L cells. $sIgD^+$ B cells produced IgM exclusively, whereas $sIgD^-$ B cells produced IgM, IgG, and IgA, with a predominance of IgG.

Both human IL-10 and the EBV-encoded protein BCRF-1[1] (or viral IL-10), presenting an extensive homology with the human molecule (20, 21), strongly enhanced the Ig response elicited in both B cell subsets by dual ligation of sIgs and CD40. Furthermore, addition of TGF-β together with IL-10 and crosslinked anti-CD40 antibodies resulted in a selective induction of IgA secretion from $sIgD^+$ B cells, but strongly suppressed the IL-10–mediated production of IgG, IgM, and IgA in the $sIgD^-$ B cell subset. The implications of these findings on the possible role of CD40 and TGF-β in the control of IgA isotype selection are discussed.

Materials and Methods

Reagents. Insolubilized anti-IgM antibodies were purchased from Bio-Rad Laboratories (Richmond, CA) and were used at 5 μg/ml. Formalinized particles of *Staphylococcus aureus* strain Cowan I (SAC) were purchased as Pansorbin from Calbiochem-Behring Corp. (San Diego, CA) and were used at the final concentration of 0.005% (vol/vol). FITC-conjugated streptavidin was purchased from Immunotech (Luminy, France).

Antibodies. The monoclonal and polyclonal antibodies used for phenotypic and functional studies were obtained from the following sources: PE-conjugated anti-CD2 (Leu 5), anti-CD3 (Leu 4), anti-CD20 (Leu 16) mAbs and unconjugated anti-CD10, anti-CD14 (Leu M3), and anti-IgM mAbs (Becton Dickinson & Co., Mountain View, CA); anti-CD38 mAb (Ortho Pharmaceutical, Raritan, NJ); anti-CD2 and anti-CD3 mAbs used for negative selection of B cells with magnetic beads (Aster Laboratories, La Gaude, France); biotinylated, goat anti-human IgD antibodies (Amersham International, Amersham, Bucks, UK); PE-conjugated $F(ab')_2$ fragments of sheep anti-mouse IgG and IgM antibodies used for indirect immunofluorescence stainings (AMD; Eurobio, Paris, France).

The anti-CD23/FcεRII mAb 25 was prepared in the laboratory as previously described (22). The UCL-3D3 mAb specifically recognizing mantle zone B cells (23) and the anti-CD44 mAb NKI-P1 (gp 90, Pgp-1, H-CAM) were kindly provided by Dr. P. Beverley (Courtauld Institute, London, UK) and Dr. S. T. Pals (Free University, Amsterdam, The Netherlands), respectively. The anti-CD40 mAb 89 was produced in the laboratory (24) and was used at a final concentration of 0.5 μg/ml throughout the study.

Factors. Each of the cytokines listed below was tested at various doses chosen above and below the optimal concentration point determined in specific bioassays as described in detail previously (16). For the sake of clarity, only the optimal concentration value is mentioned here. Except where indicated, all cytokines were under the form of purified recombinant material. IL-1α (10^8 U/mg), IL-6 (10^7 U/mg), and TNF-α (2×10^7 U/mg) were purchased from Genzyme Corp. (Cambridge, MA). They were routinely used at 100 U/ml, 5 ng/ml, and 25 ng/ml, respectively. IL-2 (3×10^6 U/mg) and IFN-γ (10^7 U/mg) were purchased from Amgen Biologicals (Thousand Oaks, CA) and were routinely used at 20 U/ml and 500 U/ml. IL-3 (5×10^6 U/mg) and IL-4 (10^7 U/mg) were kindly provided by Drs. S. Tindall and P. Trotta (Schering-Plough Research, Bloomfield, NJ). They were used at 10 ng/ml and 500 U/ml, respectively. IL-5, semi-purified by affinity column chromatography from culture supernatants of Cos 7 cells transfected with the IL-5 cDNA clone, was kindly provided by Dr. R. Coffman (DNAX, Palo Alto, CA). It was usually tested at 15 ng/ml. IL-7 (provided by Dr. F. Lee, DNAX) was used as a 5% dilution (~15 U/ml) of a culture supernatant of Cos 7 cells transfected with the human IL-7 cDNA clone. TGF-β1 (R and D Systems Inc., Minneapolis, MN) was usually tested at 0.6 ng/ml, except where indicated. The EBV-encoded protein BCRF1/viral IL-10 (vIL-10) and human IL-10 (hIL-10) were used as culture supernatants of Cos 7 cells transfected with the vIL-10 or hIL-10 cDNA clones. In some experiments, highly purified *Escherichia coli*-derived rvIL-10 (5×10^5 U/mg) (kindly provided by Dr. R. Kastelein, DNAX) was used at a concentration of 500 ng/ml.

Isolation of $sIgD^+$ and $sIgD^-$ B Cell Populations. Tonsillar mononuclear cells were separated by standard Ficoll/Hypaque gradient method and were next submitted to E rosetting with SRBC. Non-rosetting cells (E^- fraction) were labeled with anti-T cell (anti-CD2 and anti-CD3 mAbs) and antimonocyte (anti-CD14 mAb) mAbs, and subsequently incubated twice with magnetic beads coated with anti-mouse IgG antibodies (Dynal, Oslo, Norway). Residual non-B cells were removed by applying a magnetic field for 10 min. The purity of the B cell populations obtained after this procedure was greater than 95% as estimated by FACScan®

[1] *Abbreviations used in this paper:* BCRF-1, Epstein-Barr virus BamHI C fragment, rightward reading frame 1; SAC, staphylococcus aureus strain Cowan I.

immunofluorescence stainings performed with anti-CD19, CD20, CD2, CD3, and CD14 mAbs. Isolation of $sIgD^+$ and $sIgD^-$ B cell populations was performed using a preparative magnetic cell separation system (MACS®; Becton-Dickinson & Co.) according to the experimental procedure described in detail by Miltenyi et al. (25). Briefly, unfractionated tonsil B cells resuspended in PBS plus 1% BSA plus 0.01% sodium azide plus 5 mM EDTA, were labeled by sequential incubation with biotinylated goat anti-human IgD antibodies (Amersham International) and FITC-conjugated streptavidin (Immunotech, Luminy, France). After washings in PBS plus sodium azide plus EDTA, B cells were resuspended at 2×10^8 cells/ml in the same buffer and incubated with super paramagnetic beads (5 μl of the solution provided by the manufacturers for 10^8 cells) conjugated to biotin. Cells were next deposited on specially designed columns and separated into positive and negative populations using a high-gradient magnetic field. Unlabeled $sIgD^-$ B cells were eluted from the column while the magnetic field was applied and $sIgD^+$ B cells labeled with magnetic beads were next recovered by vigorous washing of the column matrix after its removal from the magnet. Purity of both populations was directly assessed by fluorescence analysis on a FACScan®, since the positive cells were stained by the complex biotin-anti-IgD/FITC-streptavidin/biotin-magnetic beads.

B Cell Cultures. All cultures were performed in Iscove's medium enriched with 50 μg/ml human transferrin, 5 μg/ml bovine insulin, 0.5% BSA, 5×10^{-5} M2-β ME (all from Sigma Chemical Co., St. Louis, MO) and 5% heat-inactivated FCS (Flow Laboratories, Irvine, CA). All B cell cultures were performed in presence of irradiated (7,000 rad) CDw32/FcγRII-transfected Ltk^- cells (CDw32 L cells), kindly provided by Dr. K. Moore (DNAX), according to the experimental procedure described previously (15, 16). In most experiments, B cells were seeded in round-bottomed microtiter trays at a density of 5×10^4 cells per well under a final volume of 200 μl, for a culture period of 10 d. Cytokines, polyclonal B cell activators (SAC, insolubilized anti-IgM antibodies, and the anti-CD40 mAb 89), and irradiated CDw32 L cells (5×10^3/well) were added at the onset of the culture. IgM, IgG, IgA, and IgE levels were measured in culture supernatants by standard ELISA techniques as described elsewhere (26, 27). Each culture point was performed in quadruplicate.

Limiting Dilution Cultures and Analysis. For limiting dilution experiments, $sIgD^+$ B cells were seeded in 96-well V-shaped microtiter trays at decreasing cell concentrations ($0.3–6.7 \times 10^3$ cells/well) under a final volume of 150 μl, in enriched Iscove's medium as described above. For the lowest cell numbers (0.3–243 cells/well), a total of 80 replicate cultures was set up for each cell density. For the highest cell densities ($0.73–6.7 \times 10^3$ cells/well), each culture point was set up as 40 replicates. 5×10^3 irradiated CDw32 L cells were dispensed in each culture well. Four different culture conditions were applied to $sIgD^+$ B cells in limiting dilution experiments: (*a*) SAC plus anti-CD40 mAb, (*b*) SAC plus anti-CD40 mAb plus vIL-10 (2.5%), (*c*) SAC plus anti-CD40 mAb plus TGF-β (0.6 ng/ml), (*d*) SAC plus anti-CD40 mAb plus vIL-10 plus TGF-β. Cultures were individually assayed for their IgA content by ELISA after a culture period of 12 d. Wells in which the OD value was at least four times higher than background levels were considered positive. Calculations to determine the frequency of IgA-secreting cells were carried out according to Taswell (28). The frequency of negative responses at each input number of B cells was used to construct a plot of the $\log_{10}$ of the number of negative cultures versus the number of cultured B cells. The frequency of B cells able to secrete IgA was calculated from the 37% intercept of the regression line, according to the Poisson distribution. The goodness of fit of the regression line was tested using the χ^2 test. In all experiments performed, the results obtained were compatible with the hypothesis of a single hit event, suggesting that we were dealing with a single cell type dilution.

Results

$sIgD^+$ B Cells Display the Phenotypic Features of Mantle Zone B Cells. The phenotype of the $sIgD^+$ and $sIgD^-$ populations recovered after magnetic separation was examined using mAbs directed against surface molecules selectively distributed on mantle zone B cells, such as sIgM (29), CD23 (30), and UCL 3D3 (23), or on germinal center B cells, such as CD10 or CD38 (31). Expression of other antigens, such as CD20 and CD44, which have been reported to display different staining intensities in mantle zone and germinal centers (31, 32), was also assessed on both B cell fractions. As shown in Fig. 1, the fluorescence peak obtained with sIgD in the positive population was monotonal and clearly separated from the control, indicating the virtual absence of $sIgD^-$ B cell contaminants in the positive fraction. Conversely, $sIgD^+$ B cells were consistently undetectable in the negative population even after 48 h of culture (data not shown), therefore excluding underestimation of $sIgD^+$ B cell contaminants possibly due to internalization of sIgD as a consequence of the isolation procedure. All $sIgD^+$ B cells were homogeneously positive for sIgM, CD23, UCL 3D3, and CD44 expression, weakly stained with the anti-CD38 mAb, and lacked CD10. In contrast, $sIgD^-$ B cells did not bind the anti-CD23 and UCL 3D3 antibodies and weakly expressed sIgM as estimated by the low percentages of positive cells (17 ± 8%, mean ± SD of three experiments) and by the low intensity of staining. CD10 and CD38 were distributed on 50 ± 10% and 36 ± 10% (mean ± SD of three experiments) of the $sIgD^-$ B cells, respectively. CD20 was uniformly expressed on all $sIgD^+$ B cells, but the levels of expression of this antigen differentiated three subsets within the $sIgD^-$ compartment: a minor negative population, a dimly stained population, and a brightly stained population. Similarly, three intensities of expression of CD44 were observed on $sIgD^-$ B cells, thus dividing this population in $CD44^{high}$, $CD44^{low}$, and $CD44^-$ subsets. Taken together, these results indicate that B cells positively selected on the basis of sIgD expression are phenotypically related to mantle zone B cells, whereas $sIgD^-$ B cells constitute a heterogeneous population including a subset of cells displaying some of the distinctive phenotypic features of germinal center B cells.

T Cell-independent Differentiation of B cells after Dual Ligation of sIgs and CD40. Functional assays were performed to determine the capacity of $sIgD^+$ and $sIgD^-$ B cells to differentiate in vitro, after ligation of surface Igs, CD40, or both. For this purpose, 5×10^4 purified B cells from each population were seeded in wells of microtiter trays together with 5×10^3 irradiated CDw32 L cells and submitted to the following conditions of stimulation: anti-IgM antibodies, SAC, anti-CD40 mAb 89, anti-IgM antibodies plus mAb 89, and SAC plus mAb 89. IgM, IgG, IgA, and IgE levels

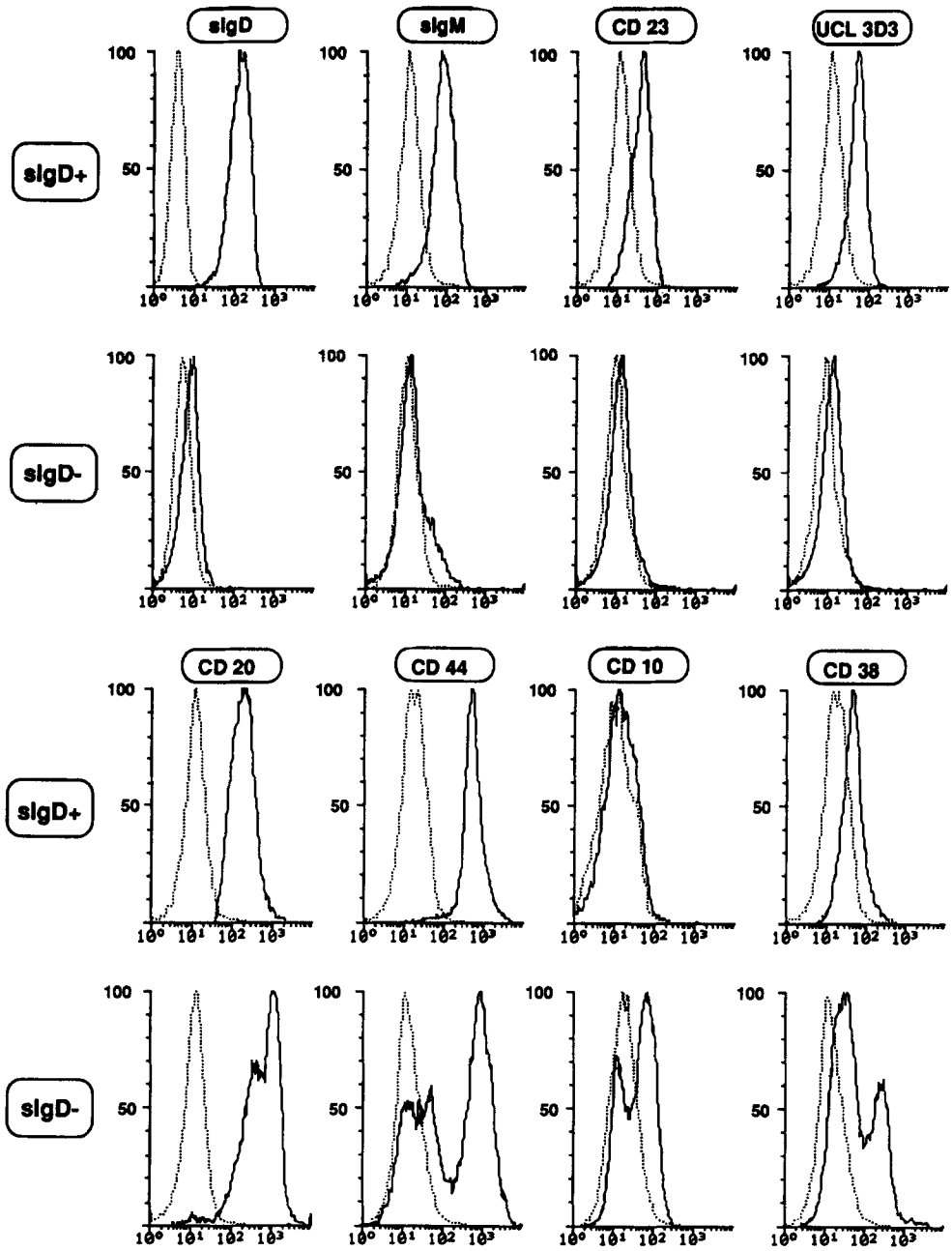

Figure 1. Immunofluorescence analysis of sIgD$^+$ and sIgD$^-$ B cell populations. Horizontal axes illustrate log of fluorescence and vertical axes indicate relative cell numbers. The fluorescence histograms shown are representative of three experiments. Each staining profile (*solid* lines) was superimposed with that of the negative control (*dotted* lines) performed with an isotype-matched unrelated mAb.

were measured in 10-d culture supernatants by ELISA. As illustrated in Table 1, neither anti-Ig reagents nor crosslinked anti-CD40 mAb could stimulate Ig synthesis from sIgD$^+$ B cells. However, when sIgs and CD40 were simultaneously crosslinked (SAC plus anti-CD40 or anti-IgM plus anti-CD40), sIgD$^+$ B cells were induced to secrete high amounts of Igs. The levels of Igs secreted by sIgD$^+$ after costimulation with SAC and crosslinked anti-CD40 antibodies reached, on average, 40 times the amount of Igs produced in response to each of those B cell stimulants used alone (as estimated in 10 separate experiments). The Ig response of sIgD$^+$ B cells under these culture conditions was exclusively restricted to the IgM isotype. However, the levels of IgM secreted by sIgD$^+$ B cells in response to SAC and anti-CD40 were 10–20-fold higher than those induced by the combination of anti-IgM and anti-CD40 antibodies. Similarly, in the absence of exogenous factors, sIgD$^-$ B cells were induced for Ig synthesis when sIgs and CD40 were concomitantly triggered by SAC and anti-CD40 mAb (Table 2). However, the pattern of Ig isotypes produced by sIgD$^-$ B cells in this activation system was strikingly different from that of sIgD$^+$ B cells, since both IgM and IgG were secreted in large amounts. IgG was reproducibly found to be the major isotype induced and constituted on average 76 ± 11% of the overall Ig production (mean ± SD of seven experiments) in these experimental conditions. IgA secretion varied from one tonsil sample to

Table 1. *Stimultaneous Crosslinking of sIgs and CD40 Induces IgM Production from sIgD⁺ B Cells*

Anti CD40 mAb	Anti-Ig reagents	IgM	IgG	IgA	IgE
		μg/ml	*μg/ml*	*μg/ml*	*pg/ml*
–	–	–*	0.04 ± 0.005	0.03 ± 0.007	–‡
–	Anti-IgM	–	0.05 ± 0.004	0.04 ± 0.006	–
–	SAC	–	0.05 ± 0.005	0.03 ± 0.002	–
+	–	0.2 ± 0.09	0.05 ± 0.004	0.03 ± 0.007	–
+	Anti-IgM	1.8 ± 0.2	0.07 ± 0.01	0.04 ± 0.002	–
+	SAC	29 ± 1.6	0.1 ± 0.05	0.07 ± 0.02	–

5×10^4 sIgD⁺ B cells were co-cultured for 10 d with 5×10^3 irradiated CDw32 L cells in complete medium, with anti-Ig reagents (SAC or insolubilized anti-IgM antibodies) with the anti-CD40 mAb 89, or with combinations of anti-Ig reagents and anti-CD40 mAb. Ig levels represent the mean ± SD values of quadruplicate determinations. Representative of seven experiments.
* <0.08 μg/ml.
‡ <150 pg/ml.

Table 2. *Stimulatenous Crosslinking of sIgs and CD40 Induces IgM Production from sIgD⁻ B Cells*

Anti CD40 mAb	Anti-Ig reagent	IgM	IgG	IgA	IgE
		μg/ml	*μg/ml*	*μg/ml*	*pg/ml*
–	–	0.1 ± 0.04	0.1 ± 0.09	0.04 ± 0.003	–‡
–	Anti-IgM	–*	0.1 ± 0.02	0.06 ± 0.009	–
–	SAC	0.1 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	–
+	–	0.3 ± 0.02	0.3 ± 0.08	0.1 ± 0.03	–
+	Anti-IgM	0.2 ± 0.03	0.2 ± 0.04	0.4 ± 0.004	–
+	SAC	4.9 ± 0.1	7.2 ± 0.7	0.09 ± 0.001	–

Same culture conditions as in Table 1. Ig levels represent the mean ± SD of quadruplicate determinations. Representative of seven experiments.
* <0.08 μg/ml.
‡ <150 pg/ml.

another but usually remained a minor component of the Ig response elicited by SAC and anti-CD40 from sIgD⁻ B cells. IgE levels always remained below the threshold of detection whatever the cell population examined. In contrast to sIgD⁺ B cells, sIgD⁻ B cells produced minor amounts of Igs upon costimulation with anti-IgM and anti-CD40 antibodies, a result consistent with the low expression of sIgM on this population.

Activated sIgD⁺ and sIgD⁻ B Cells Produce Large Amounts of Igs in Response to IL-10. We next examined the capacity of various cytokines to modulate the Ig response elicited by SAC and anti-CD40 from sIgD⁺ and sIgD⁻ B cells. Several recombinant factors including IL-1–7, TNF-α, TGF-β and IFN-γ repeatedly failed to enhance Ig synthesis from sIgD⁺ B cells, activated through concomitant triggering of surface Igs and CD40 antigen (data not shown). In contrast, human and viral IL-10 dramatically increased the IgM production elicited by the combination of SAC and mAb 89 from sIgD⁺ B cells (Table 3 and Fig. 2, *top*). IgG and IgA were also produced, but they remained a minor component of the Ig response stimulated by IL-10 from sIgD⁺ B cells since the levels of production of these isotypes constituted, on average, 1.4 ± 0.7% and 0.5 ± 0.2% (mean ± SD of 10 experiments) of the overall Ig synthesis, respectively. IL-10 also synergized with SAC and crosslinked anti-CD40 to stimulate IgM, IgG, and IgA production from sIgD⁻ B cells (Table 3 and Fig. 2, *bottom*). In contrast to sIgD⁺ B cells, IgG was the predominant isotype secreted by sIgD⁻ B cells in response to IL-10 since it accounted, on average, for 68 ± 13% (mean ± SD of 10 experiments) of the overall Ig synthesis, whereas IgM and IgA accounted for 19.5 ± 15% and 12.5 ± 12%, respectively. IgE synthesis was not enhanced above background levels (150 pg/ml) by IL-10 whatever the B cell population considered. The stimulatory effects of IL-10 on IgM, IgG, and IgA synthesis on both populations were confirmed with purified recombinant material (Table 4), indicating that the enhancing effect of IL-10 on Ig synthesis is an innate property of the molecule. IL-10 failed to stimu-

Table 3. *Human IL-10 Enhances Ig Synthesis from Activated sIgD⁺ and sIgD⁻ B Cells*

B cell	Culture	IgM	IgG	IgA
			μg/ml	
	0	-*	0.08 ± 0.03	0:06 ± 0.003
sIgD⁺	SAC + anti-CD40	1.6 ± 0.2	0.1 ± 0.03	0.06 ± 0.01
	SAC + anti-CD40 + hIL-10	172 ± 17	4.2 ± 0.9	0.4 ± 0.1
	0	-	0.1 ± 0.03	0.07 ± 0.01
sIgD⁻	SAC + anti-CD40	0.2 ± 0.07	1.2 ± 0.4	0.1 ± 0.04
	SAC + anti-CD40 + hIL-10	8.2 ± 0.7	26.5 ± 2	10 ± 1.6

5 × 10^4 sIgD⁺ or sIgD⁻ B cells were cultured for 12 d with complete medium or costimulated with SAC and the anti-CD40 mAb 89 presented on CDw32 L cells in absence or presence of Cos-7-derived human IL-10 used at 10%. Ig levels represent the mean ± SD values of quadruplicate determinations.
* <0.08 μg/ml.

late Ig secretion over background levels when added to sIgD⁺ or sIgD⁻ B cells in the absence of stimulatory agents (data not shown).

TGF-β Specifically Enhances IgA Synthesis from sIgD⁺ B Cells while Inhibiting that of sIgD⁻ B Cells. Since TGF-β had been demonstrated to induce LPS-activated mouse B cells to switch to IgA synthesis (3, 33), we tested its ability to influence IgA secretion from sIgD⁺ and sIgD⁻ B cells in the various culture conditions described above. As illustrated in Fig. 3 (*top*), TGF-β strongly potentiated the IgA synthesis induced by vIL-10 from sIgD⁺ B cells, but suppressed both IgM and IgG production elicited by vIL-10 in this cell population. The stimulatory effect of TGF-β on IgA secretion was obtained within a narrow concentration range (0.5–2 ng/ml). Above 2 ng/ml, TGF-β gradually lost its IgA-enhancing effect (data not shown). The IgA levels reached in this particular culture system with optimal doses of TGF-β were generally 10–30 times higher than those obtained with vIL-10 alone.

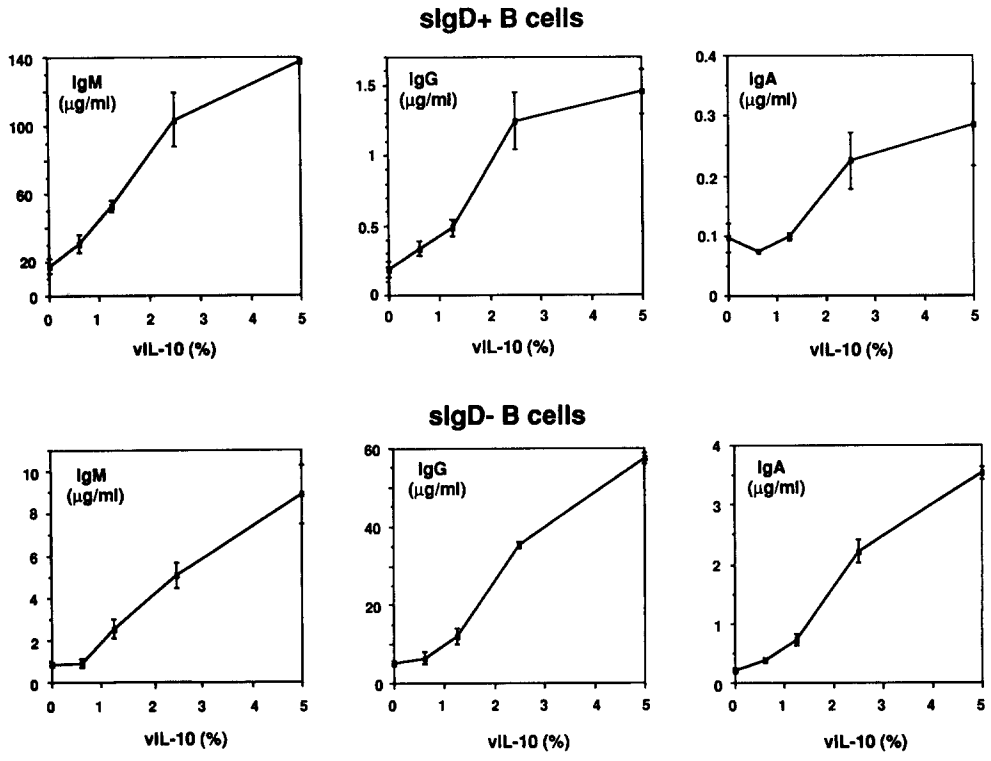

Figure 2. Dose-response curves of the vIL-10 induced IgM, IgG, and IgA synthesis from sIgD⁺ and sIgD⁻ B cells. 5 × 10^4 sIgD⁺ B cells (*top*) or sIgD⁻ B cells (*bottom*) were co-cultured for 10 d in round-bottomed microtiter trays with SAC, the anti-CD40 mAb 89 presented on CDw32 L cells, and serial dilutions of Cos-7-derived vIL-10. Ig levels are expressed in μg/ml and represent the mean ± SD values of quadruplicate determinations. Representative of seven experiments.

Table 4. *Purified vIL-10 Enhances Ig Synthesis from Activated sIgD$^+$ and sIgD$^-$ B Cells*

B cell	Culture	IgM	IgG	IgA
			μg/ml	
	0	0.09 ± 0.02	0.08 ± 0.007	0.04 ± 0.003
sIgD$^+$	SAC + anti-CD40	4.2 ± 0.03	0.1 ± 0.02	0.05 ± 0.01
	SAC + anti-CD40 + vIL-10	235 ± 13	3.7 ± 0.09	0.4 ± 0.08
	0	-*	0.6 ± 0.06	0.07 ± 0.03
sIgD$^-$	SAC + anti-CD40	0.1 ± 0.02	2.5 ± 0.8	0.1 ± 0.02
	SAC + anti-CD40 + vIL-10	7.9 ± 0.5	142 ± 9.6	8.7 ± 0.2

Same culture conditions as in Table 3. Ig levels represent the mean ± SD of quadruplicate determinations. Purified viral IL-10 was used at 500 ng/ml.
* < 0.08 μg/ml.

It is striking that the IgA-enhancing capacity of TGF-β appeared to be strictly restricted to the sIgD$^+$ compartment, since the vIL-10 induced IgM, IgG, and IgA synthesis in sIgD$^-$ B cells (Fig. 3, *bottom*) were inhibited in a dose-dependent manner by TGF-β. Fig. 3 shows that IgA secretion by sIgD$^-$ B cells is blocked by concentrations of TGF-β that are stimulatory for IgA production by sIgD$^+$ B cells. As illustrated by Fig. 4, TGF-β was found to specifically enhance IgA synthesis from sIgD$^+$ B cells costimulated with SAC, anti-CD40, and human IL-10, therefore indicating that the IgA-potentiating activity of TGF-β is equally detected in the presence of the viral or human forms of IL-10. The IgA response of unfractionated B cells to IL-10 was blocked by TGF-β (data not shown).

TGF-β Increases the Frequency of sIgD$^+$ B Cells Driven to IgA Synthesis by vIL-10. Limiting dilution experiments were next performed to determine whether IgA synthesis induced by TGF-β and vIL-10 in sIgD$^+$ B cells resulted from an in-

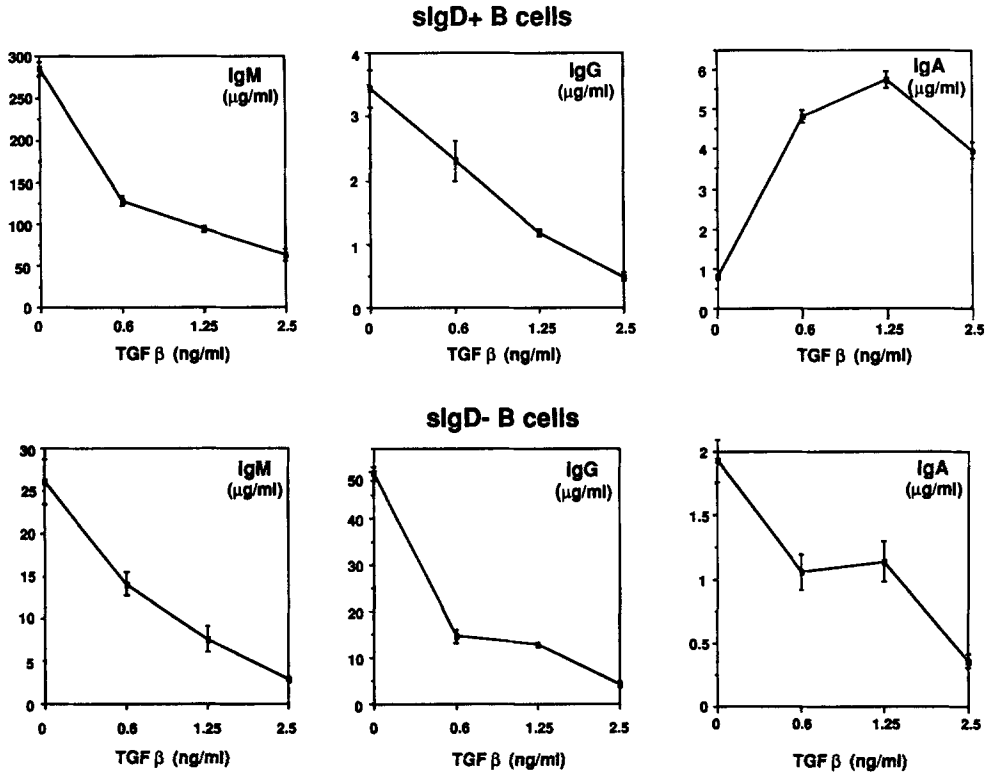

Figure 3. Effect of combinations of vIL-10 and TGF-β on the pattern of Ig isotypes secreted by sIgD$^+$ and sIgD$^-$ B cells. 5 × 10^4 purified sIgD$^+$ B cells (*top*) or sIgD$^-$ B cells (*bottom*), co-cultured with 5 × 10^3 irradiated CDw32 L cells, were stimulated with SAC, anti-CD40, and a fixed dilution of Cos-7–derived vIL-10 (5%), in the absence or presence of three concentrations of TGF-β (0.6, 1.25, and 2.5 ng/ml). IgM, IgG, and IgA synthesis was measured after a culture period of 10 d. Ig levels are expressed in μg/ml and represent the mean ± SD values of quadruplicate determinations. Representative of five experiments.

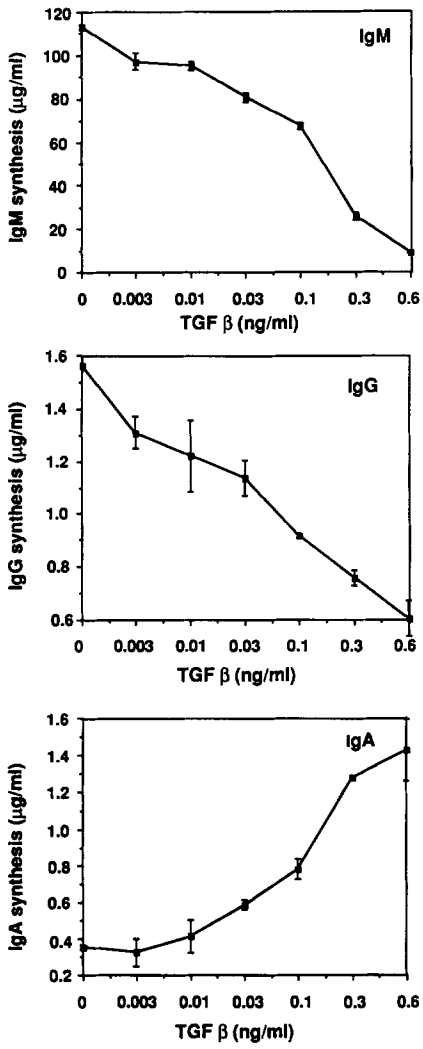

Figure 4. Effect of combinations of hIL-10 and TGF-β on the pattern of Ig isotypes produced by sIgD$^+$ B cells. 5 × 10^4 sIgD$^+$ B cells cocultured with 5 × 10^3 irradiated CDw32 L cells, were stimulated with SAC, anti-CD40, and a fixed concentration of Cos-7-derived human IL-10 (5%) in the absence or presence of serial dilutions of TGF-β. IgG, IgA, and IgM levels were measured after a culture period of 10 d and are expressed in μg/ml. The results represent the mean ± SD values of quadruplicate determinations. Representative of two separate experiments.

creased frequency of IgA-secreting clones or from the expansion and maturation of a few IgA committed precursors contaminating the sIgD$^+$ populations. Therefore, sIgD$^+$ B cells were cultured at various cell densities (0.3–6.7 × 10^3 cells/well) with a fixed number (5 × 10^3) of irradiated CDw32 L cells in V-shaped microtiter trays, for a period of 12 d under different conditions of stimulation. As shown in Fig. 5, virtually no IgA-producing cell could be detected in cultures stimulated by SAC plus anti-CD40 or by SAC plus anti-CD40 plus TGF-β. Costimulation of sIgD$^+$ B cells with SAC, anti-CD40 mAb, and vIL-10 resulted in the emergence of IgA-secreting cells with a frequency of 1/340 for the representative experiment depicted in Fig. 5. Addition of TGF-β to the latter culture condition increased the frequency of sIgD$^+$ B cells recruited to secrete IgA up to 1/75. No IgA-bearing cell could be detected by flow cytometry analysis in the starting sIgD$^+$/sIgM$^+$ populations (data not shown). Therefore, the present results suggest that TGF-β used in combination with vIL-10 enhances IgA synthesis from sIgD$^+$ B cells by increasing the frequency of IgA-producing clones.

Ligation of CD40 is Sufficient to Allow sIgD$^+$ B Cells to Produce IgA in Response to vIL-10 and TGF-β We next attempted to determine the minimal activation signal required for induction of IgA synthesis from sIgD$^+$ B cells. As illustrated by Fig. 6, ligation of sIgs by insolubilized anti-IgM antibodies or SAC did not allow sIgD$^+$ B cells to secrete IgA in response to either vIL-10 or the combination of vIL-10 and TGF-β. This was not due to a lack of responsiveness of B cells to vIL-10 in this assay system, since vIL-10 significantly enhanced IgM synthesis from SAC-activated sIgD$^+$ B cells (3.9 ± 0.6 μg/ml vs. 0.3 ± 0.05 μg/ml in control cultures, for the experiment depicted in Fig. 6). Conversely, sIgD$^+$ B cells were induced for IgA secretion in response to vIL-10 and vIL-10 + TGF-β after crosslinking of CD40 by mAb 89, presented on CDw32 L cells. The stimulatory effect of TGF-β on IgA synthesis was augmented when sIgD$^+$ B cells were simultaneously activated by anti-Ig reagents and crosslinked anti-CD40 antibodies. Taken together, these results indicate that induction of IgA synthesis from sIgD$^+$ B cells in response to vIL-10 and vIL-10 plus TGF-β requires an activation signal delivered by ligation of CD40, but not by crosslinking of sIgs.

Discussion

Naive B cells that coexpress sIgM and sIgD migrate from the bone marrow to the periphery and form the primary follicles in secondary lymphoid organs. After antigen stimulation, primary follicles develop into secondary follicles schematically composed of two major microanatomical structures: the mantle zone in which sIgM$^+$/sIgD$^+$ naive B cells are located, and the germinal center in which the antigen-dependent maturation process occurs (34). Here, we have attempted to isolate naive B cells by the means of anti-IgD antibodies to build up a reliable experimental model that could be used to study the cytokine regulation of isotype switching to IgG and IgA. This technical approach was supported by the fact that IgG- and IgA-committed precursors reside within the sIgD$^-$ B cell subset (35, 36). The results of the phenotypic and functional analysis of sIgD$^+$ B cells both confirmed that our separation criteria allowed isolation of naive B cells. First, sIgD$^+$ B cells homogeneously expressed a panel of markers identifying mantle zone B cells and virtually lacked expression of CD10 and CD38, which are distributed on certain germinal center B cells. Second, sIgD$^+$ B cells costimulated with SAC and an anti-CD40 mAb presented on CDw32 L cells displayed a pattern of isotype secretion exclusively restricted to IgM. The heterogeneity of the sIgD$^-$ B cell subset, suggested by the complex distribution of CD10, CD20, and CD44, could reflect the diver-

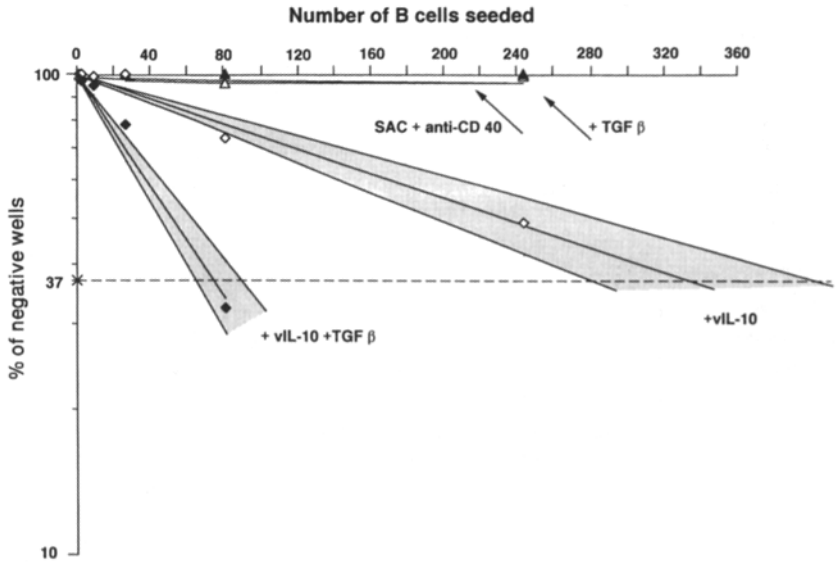

Figure 5. Frequency of sIgD$^+$ B cells recruited to produce IgA in response to vIL-10 or vIL-10 + TGF-β as determined by limiting dilutions experiments. Decreasing concentrations of sIgD$^+$ B cells were cultured for 12 d in the presence of 5 × 10^3 irradiated CDw32 L cells and stimulated with: SAC + anti-CD40, SAC + anti-CD40 + TGF-β (0.6 ng/ml), SAC + anti-CD40 + vIL-10 (2.5%), or SAC + anti-CD40 + vIL-10 + TGF-β. The results are expressed by plotting the percentage of IgA-negative wells against the number of B cells seeded in the culture. *Shaded* areas represent the 95% confidence limits of the regression lines calculated from the experimental points. Representative of two separate experiments.

sity of the stages of B cell maturation (centrocytes versus centroblasts, for instance) found within the germinal center. Yet, considering that a few sIgD$^-$ B cells also lacked CD20 (Fig. 1), expression of which is lost at the plasma cell stage (37), it is probable that a minor population of extra-follicular B cells, such as plasmablasts, is also included within this B cell compartment.

Ligation of sIgs and CD40 can promote B cell proliferation in combination with other stimuli (38, 39), or can directly induce entry of B cells into cycle when the anti-Ig (40) or anti-CD40 antibodies (15) are presented under an immobilized form. However, our data indicate that T cell–independent maturation of human B cells can be achieved, in the absence of exogenous factors, by the combination of Ig-crosslinking agents and immobilized anti-CD40 antibodies. In this respect, this particular mode of activation of human B cells presents some homology with the LPS-induced stimulation of murine B cells. The apparent lack of exogenous factor requirement for Ig synthesis, in this activation system, could be due to the endogenous production of B cell differentiation factors by the irradiated CDw32 L cell transfectants and/or by the B cells themselves. In any case, the absence of secretion of Igs using downstream heavy chain genes by sIgD$^+$ B cells in these culture conditions indicates that simultaneous triggering of CD40 and sIgs is not sufficient to induce Ig isotype switching. Additionally, it is noteworthy that SAC was consistently more potent than anti-IgM antibodies for stimulation of B cell differentiation when combined with the anti-CD40 mAb. In the sIgD$^-$ subset, anti-IgM antibodies failed to cooperate with anti-CD40 for Ig synthesis in spite of the presence of sIgM$^+$ B cells which were inducible for IgM secretion by the combination of SAC and anti-CD40. One plausible explanation for this finding could be that the stimulatory effect of SAC resides in its capacity to trigger both sIgs and MHC class II molecules since Staphylococcal exotoxins have been described to stimulate B cell differentiation through binding to MHC class II antigens (41).

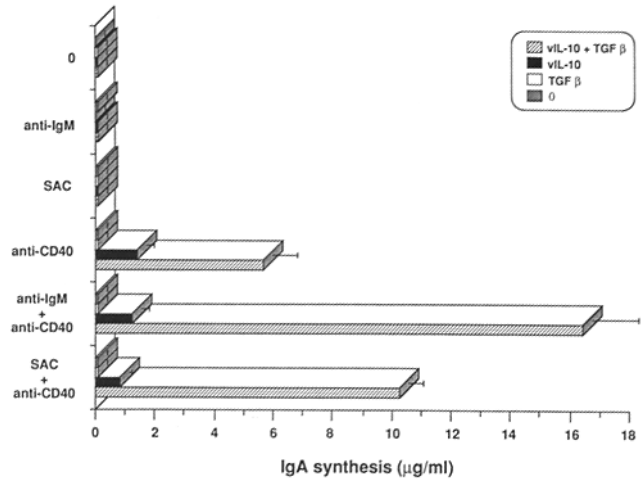

Figure 6. IgA response of sIgD$^+$ B cells to vIL-10 and vIL-10 + TGF-β after activation with Ig-crosslinking agents or with an immobilized anti-CD40 mAb. 5 × 10^4 sIgD$^+$ B cells were stimulated with TGF-β (0.6 ng/ml), vIL-10 (5%), or vIL-10 + TGF-β in the presence of insolubilized anti-IgM antibodies, SAC, anti-CD40, SAC + anti-CD40, or anti-IgM antibodies + anti-CD40. Irradiated CDw32 L cells (5 × 10^3/well) were added to each culture point. IgA levels (μg/ml) were determined after a culture period of 10 d. They are expressed as mean ± SD values of quadruplicate determinations. Representative of three separate experiments.

In our hands, sIgD$^+$ B cells could secrete moderate amounts of IgA upon costimulation with an anti-CD40 mAb presented on CDw32 L cells and IL-10. The frequency of IgA-secreting cells (<0.5%, on average) induced by IL-10 is compatible with a level of contamination of sIgD$^+$ B cells with IgA-committed precursors which would fall beyond the threshold of detection of the flow-cytometry analysis. Therefore, considering that human and viral IL-10 are extremely potent B cell growth and differentiation factors (42), we cannot

rule out the possibility that a minor contaminating population of postswitch B cells could account for the IL-10–mediated IgA synthesis by $sIgD^+$ B cells. However, one of the major points which emerged from the present study was the observation that TGF-β could stimulate IgA secretion from naive human B lymphocytes. Two lines of evidence argue in favor of the hypothesis that IgA isotype switching occurs in response to TGF-β stimulation. First, it is well documented that TGF-β inhibits both proliferation (43) and differentiation (44) of human B lymphocytes. Therefore, it is unlikely that its IgA-potentiating effect could result from outgrowth and maturation of a few $sIgA^+$ contaminants. Second, in accordance with data previously reported in murine models (33), we observed that TGF-β strongly suppressed IgA synthesis in the $sIgD^-$ B cell subset thus excluding the possibility that IgA-committed precursors could constitute the target of the IgA-stimulatory effect of TGF-β observed in the $sIgD^+$ B cell population. In light of these findings, one could reconsider the effects of IL-10 on IgA synthesis by $sIgD^+$ B cells. Indeed, under the extreme conditions of stimulation provided by the combination of SAC, anti-CD40, CDw32 L cells, and IL-10, B cells might be induced for endogenous cytokine release. Since it has been demonstrated that human B cells can produce TGF-β upon SAC stimulation (43), the IL-10-induced IgA secretion observed in $sIgD^+$ B cells might be indirectly mediated by B cell–derived TGF-β.

In conclusion, we have developed an in vitro system, relying on the concomitant ligation of sIgs and CD40, which allows T-independent differentiation of human B cells. This experimental model has permitted us to show that TGF-β can stimulate IgA production from naive B cells, probably as a consequence of class switching. Our data indicate that cross-linking of CD40, but not of sIgs, provides one of the obligatory signals required for induction of IgA synthesis by $sIgD^+$ B cells in response to TGF-β. The TGF-β–dependent regulation of IgA isotype selection in humans appears to differ from the IL-4-dependent regulation of IgE class switching. Indeed, whereas anti-CD40 antibodies and IL-4 are sufficient to induce commitment of human B cells to IgE secretion, combinations of anti-CD40 and TGF-β consistently failed to elicit IgA synthesis from $sIgD^+$ B cells. Therefore, we speculate that (*a*) IgA isotype switching occurs after costimulation of $sIgD^+$ B cells with anti-CD40 and TGF-β; and (*b*) the signal necessary for growth and differentiation of the newly IgA-committed B cells is lacking under these culture conditions, unless IL-10 is also provided. The notion that IL-10 could be involved in the final B cell maturation step is supported by the observation that IL-10 can drive human B lymphocytes to a plasma-like stage of differentiation (F. Rousset et al., unpublished observations). Experiments are presently being carried out to test the validity of such a model.

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