Cytokine mRNA Expression during an In Vitro Response of Human B Lymphocytes: Kinetics of B Cell Tumor Necrosis Factor α, Interleukin (IL)6, IL-10, and Transforming Growth Factor β_1 mRNAs

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Summary

Expression of mRNA for eight cytokines was analyzed in an in vitro response—proliferation and Ig-secretion—of normal human B lymphocytes. This was made possible by the use of murine thymoma cells as helper cells in conjunction with human T cell supernatant, and the design of human DNA sequence–specific primers for RT-polymerase chain reaction. mRNAs for interleukin (IL)2 and IL-4, but also for IL-1α and IL-1β remained undetectable during the whole culture period in highly purified B cells prepared by a three-step purification protocol. However, tumor necrosis factor α and IL-6 mRNAs peaked during days 1–3 after culture start and became undetectable after 5–6 d, shortly before bulk B cell proliferation started to decline. In contrast, transforming growth factor β_1 mRNA, after a progressive increase during the first few days, and IL-10 mRNA, after a peak on days 1–3, remained detectable in immunoglobulin (Ig)-secreting cultures throughout the observation period of 22 d. Clonal analysis on 8-d cultures that had been seeded with single B cells by autocloning with the cell sorter, revealed that 85% of 77 B cell clones studied, expressed TGF-β_1 mRNA, and only 19% IL-10 mRNA. These findings show a differentiation stage–related cytokine program during a B cell response, whereby (a) B cells can become activated without IL-1α or IL-1β expression; (b) mRNA for positive (IL-10) and negative (TGF-β_1) autoregulatory factors coexists in cell populations during the later phase of the response, although not necessarily in all B cell clones; and (c) normal Ig-secreting cells cease IL-6 expression in contrast to their malignant counterparts, myeloma cells.

Until cytokine production was first demonstrated in EBV-transformed B cell lines (1), B lymphocytes were considered to be just effector cells of the immune system, producing Ig after activation by T lymphocytes. More recent studies showed the production of many cytokines in EBV-transformed and leukemic B cell lines (2–6), including IL-2 (7). IL-1 and -6 have been reported to be autocrine growth factors for human myeloma cell lines (8, 9). A physiological and possible immunoregulatory role for B cell–produced cytokines was suggested, when it was found that cytokines, such as IL-1, -6, and -10, TNF-α and TGF-β could be expressed by normal murine (10) and human B cells (4, 11–15).

In the present study, we reinvestigated the expression of mRNA for various cytokines (IL-1α, -1β, -2, -4, -6, and IL-10, TNF-α and TGF-β_1) during the different stages of an in vitro response of normal human B lymphocytes, from activation of resting B cells to the differentiation into Ig-secreting cells. For this purpose, we used an in vitro culture system in which murine mutant EL-4 thymoma cells stimu-

late B lymphocyte proliferation and differentiation into high-rate Ig-secreting cells (16, 17), and analyzed cytokine mRNAs by means of RT-PCR.

The mRNA of only four of the studied cytokines, but notably not that of IL-1α or -1β, was detected in highly purified B cells. TNF-α and IL-6 mRNA expression occurred transiently during the first few days of culture, whereas IL-10 and TGFβ-1 mRNA expression persisted in Ig-secreting cell cultures. In single cell–derived 8-d B cell clones, TGFβ-1 mRNA was expressed by most of the clones, whereas IL-10 mRNA was only detected in a minor subset. The results show a differentiation stage–dependent B lymphocyte cytokine program.

Materials and Methods

Cell Separations: Two Different B Cell Populations Were Obtained

B Cell Population 1. Mononuclear cells from the peripheral blood

of healthy donors were prepared by centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). Adherent cells (monocytes) were depleted by 1-h incubation in petri dishes at 37°C in normal culture medium—10% FCS and T cells (E⁺ fraction) were removed through rosetting with neuraminidase-treated sheep erythrocytes for 1 h at 4°C. After staining with FITC-conjugated Leu12 mouse IgG1 (anti-CD19) mAb (Becton Dickinson & Co., Mountain View, CA), B lymphocytes were positively selected with a cell sorter (Epics V; Coulter Electronics, Hialeah, FL), as described (18). B cell populations thus obtained were typically >95% surface Ig⁺ when analyzed with PE-conjugated goat anti–human total Ig (Becton Dickinson & Co.).

B Cell Population 2. Adherent cell depletion as above was performed in the presence of 0.1% NaN₃, followed by T cell depletion as previously described. CD19⁺ B cells were then positively selected with magnetic beads (PAN-B Dynabeads M-450; Dynal, Oslo, Norway) at 4°C, detached using Detacha-beads (Dynal) for 1 h at room temperature in the presence of 0.1% NaN₃ to reduce B cell activation, and finally, after staining with FITC-conjugated goat F(ab')₂ anti–human μ chain (Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC-conjugated F(ab')₂ anti–human Ig (Cappel Laboratories, Cochranville, PA), B lymphocytes were positively selected with the cell sorter.

CD5⁺ and CD5⁻ B Cell Subpopulations. These were obtained with the cell sorter by positive or negative selection with Leu1 antibody (Becton Dickinson & Co.) of CD19⁺ cells (18), purified by magnetic beads.

CD3⁺ T Cells. CD⁺ T cells were sorted from the E⁺ fraction with FITC-conjugated anti-CD3 mAb (Dakopatts, Glostrup, Denmark) and cultured with 10 ng PMA/ml and 5 μg PHA/ml (10⁵ cells/200 μl) for 4, 8, and 24 h, in order to prepare RT-PCR controls for IL-2, -4, and -10, respectively.

Adherent Cells. Monocytes were recovered after separation from mononuclear cells by 1-h incubation on plastic dishes at 37°C in normal medium—10% FCS. Such cells were then cultured in the EL-4 system (1,000 cells/well). Cells from the tumor cell line U937 were used as positive RT-PCR control for lysozyme.

B Cell Cultures

300 B cells or single B cells (CD19⁺ B cells, distributed with the Autoclone apparatus of the cell sorter [19]) were added to 200-μl cultures (96-well, flat-bottomed plates) together with 5 × 10⁴ irradiated (50 Gy), mutagenized, murine EL-4 thymoma cells (clone 6.1.5.5.) and 5% T cell supernatant (from 10⁶ E⁺ cells/ml, incubated for 36 h in the presence of 10 ng/ml PMA and 5 μg/ml PHA), as described (17). All cultures were performed at 37°C, in 5% CO₂ and in RPMI 1640 medium (Gibco, Basel, Switzerland), supplemented with 2 mM L-glutamine, 10 mM Hepes buffer, 5 × 10⁻⁵ M β₂-ME, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Gibco), and 10% heat-inactivated FCS (Seromed, Berlin, Germany). To extend such EL-4 cultures, secondary cultures were set up by pooling, after 8 d, the B cells from a series of primary cultures, separating them from dead EL-4 cells and cell degradation products on a Ficoll gradient, and reculturing them (10⁴ cells/well, 200 μl) by adding fresh EL-4 cells and T cell supernatant as above.

Detection of Cell Proliferation and Ig Secretion

The number of total viable cells after various culture periods was determined by trypan blue exclusion. To determine the number of B cells, cell counts from parallel cultures containing only EL-4 cells were subtracted. Thymidine incorporation was measured according to standard procedure (17). EL-4 cells are thymidine-kinase

deficient and thus do not incorporate thymidine. Ig levels in culture supernatants were quantified with IgM, IgG, and IgA isotype–specific ELISAs as described (19).

RNA Extraction

At different time points, culture wells were pooled, transferred to Eppendorf tubes, and centrifuged. Total RNA was recovered after cell lysis in 100 μl of guanidine-thiocyanate buffer by acid phenol/chloroform extraction, followed by ethanol precipitation (20).

RT-PCR

RNA was retrotranscribed after annealing with 0.1 nM oligo dT primers (10 mer; Böehringer Mannheim, Germany) in the presence of 3 U of AMV RT (Promega Corp., Madison, WI), 25 U of RNAsin (Promega Corp.), 1 mM DTT, 100 μg/ml BSA, and 1 mM of each deoxynucleotide (Böehringer Mannheim) in a total volume of 25 μl for 1 h at 42°C (21). Aliquots of the RT mixture were then amplified in a thermocycler (Techne Corporation, Duxford, Cambridge, UK) in a volume of 50 μl with 25 U/ml of Taq polymerase (Promega Corp.) diluted in the buffer supplied by the manufacturer, 100 μM of each deoxynucleotide and 500 nM of each of the two primers (22). The cycling conditions were 0.8 min at 94°C for denaturation, 0.8 min at temperatures between 58 and 66°C for annealing, depending on the t_M of the pair of primers used (23), and 0.8 min at 72°C for elongation.

The primers, synthesized and purified at the University of Geneva, Department of Microbiology, had the following sequences, 5' and 3' primers, respectively: β₂ microglobulin (β₂m),[1] CCCCC ACTGA AAAAG ATGAG and TCACT CAATC CAAAT GCGGC; lysozyme, CCAAA TGGGA GAGTG GTTAC and TCAGC GATGT TATCT TGCAG; CD 2, GTCTT TGTGG CACTG CTCG and AGGAG GATGT TGGGA AGTTG; IL-1α, GAGGA AGAAA TCATC AAGCC and CATCT TGGGC AGTCA CATAC; IL-1β, CTGCG TGTTG AAAGA TGATA AG and CCACA TTCAG CACAG GACTC; IL-2, GCAAC TCCTG TCTTG CATTG and AATGT GAGCA TCCTG GTGAG; IL-4, CTGTG CTCCG GCAGT TCTAC and ACGTA CTCTG GTTGG CTTCC; IL-6, GCCAG AGCTG TGCAG ATGAG and AGGAA CTCCT TAAAG CTGCG; IL-10, ATGCT TCGAG ATCTC CGAGA and TTTGT AGATG CCTTT CTCTT GG; TGF-β₁, TTTCG CCTTA GCGCC CACTG and TCCAG CCGAG GTCCT TGCGG; and TNF-α, CAGCC CTCTG GCCCA GGCAG and GGTGA GGAGC ACATG GGTGG. Primers for the amplification of Ig C regions were designed on exons 3 and 4 for IgM (ACCTG CCCTC GCCAC TGAAG and GCTGC CCCCT CTGCA TCCAC) and on exons 2 and 3 for IgG (CAAGG AGTAC AAGTG CAAGG and AAGAA GGAGC CGTCG GAGTC), respectively. For the amplification of Ig V regions, the upstream primer was designed to recognize a consensus sequence on the CDR3 of the V region (ACACG GCCGT GTATT ACTGT) (similar to reference 24), and the downstream primer to recognize sequences on the first exon of the respective Ig-isotype C regions: for IgM, GAATT CTCAC AGGAG ACGAG; for IgG, AGGTG TGCAC GCCGC TGGTC; and for IgA, AGACC TTGGG GCTGG TCGGG. The primer pairs used were designed (a) in order to anneal to human and not to murine gene sequences or EL-4–derived sequences; (b) to span over introns to allow the detection of potential contaminating genomic DNA; and (c) to contain restriction sites to confirm PCR specificity (PC/Gene program; IntelliGenetics, Mountain View, CA). Digestion of all amplified cDNA

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[1] *Abbreviation used in this paper:* β₂m, β₂ microglobulin.

fragments with at least two specific restriction enzymes resulted in each case in the expected restriction fragments. RT-PCR with all the above-mentioned primers on EL-4 mRNA led to no detectable bands, thus confirming their species specificity (data not shown).

For "nested PCR", 1/10 of the reaction volume of the primary PCR was added to a fresh PCR mix, containing the following 5' and 3' primers, respectively: IL-10, TTGTT GTTAA AGGAG TCCTT GC and TTGCT CTTGT TTTCA CAGGG A; and TGF-β_1, TCAAC GGGTT CACTA CCGGC and the same 3' primer as above.

An aliquot of PCR product (see below) was then visualized after electrophoresis through 2.0% agarose gels by staining with ethidium bromide (25). To ensure the absence of sample contamination, an RNA extraction control (murine EL-4 cells alone) was run in parallel to each PCR.

Estimation of Relative cDNA Amounts

The RT-PCR signal generated by β_2m mRNA was chosen to estimate the amounts of cDNA obtained from different B cell samples, since β_2m cDNA from equal numbers of B cells and plasma cells gave rise to similar band intensities after PCR (only two to three times higher in activated B and plasma cells than in resting B cells) and since human sequence–specific primers could be designed. Moreover, the absence of β_2m pseudo genes (26) prevents false interpretations of cDNA-PCR signals in case of possible contamination by genomic DNA.

Results

B Cell Response in the EL-4 Culture System and RT-PCR. Coculture of human B lymphocytes with irradiated mouse T cells from a mutant EL-4 thymoma cell line and human T cell supernatant leads to their activation, proliferation, and differentiation into Ig-secreting cells (17–19). In the present study, the number of B cells at culture start typically increased 100-fold after 8 d (Fig. 1). Cells harvested after 8 d of culture and passed over a Ficoll gradient were found to be >95% cytoplasmic Ig^+, morphologically plasmocytic, and surface $CD19^+/CD21^-/CD38$ bright cells. When recultured with fresh EL-4 cells and T cell supernatant, such cells almost stopped proliferating, but continued to secrete Ig (100–200 pg/24 h/cell) for 2 wk (26a).

To detect significant changes in cytokine mRNA expression at different times of the B cell response, we chose the expression of β_2m mRNA as an approximate reference (within an error range of a factor of 2 to 3) for the relative amounts of cDNA obtained at different activation stages and from different numbers of cells per culture (see Materials and Methods). Adjustment of cDNA from the B cell culture samples before PCR according to counted cell numbers resulted in similar β_2m RT-PCR signals throughout the culture period (Fig. 1). IgM and IgG mRNA expression was found to increase in such samples between days 3 and 7. After 8 d, B cell degradation progressively occurred in primary EL-4/B cell cultures (see decrease of Ig mRNA in Fig. 1). Conversely, in secondary cultures, Ig mRNA expression was found to remain constant until the end of the observation period (22 d), in accordance with ongoing Ig secretion.
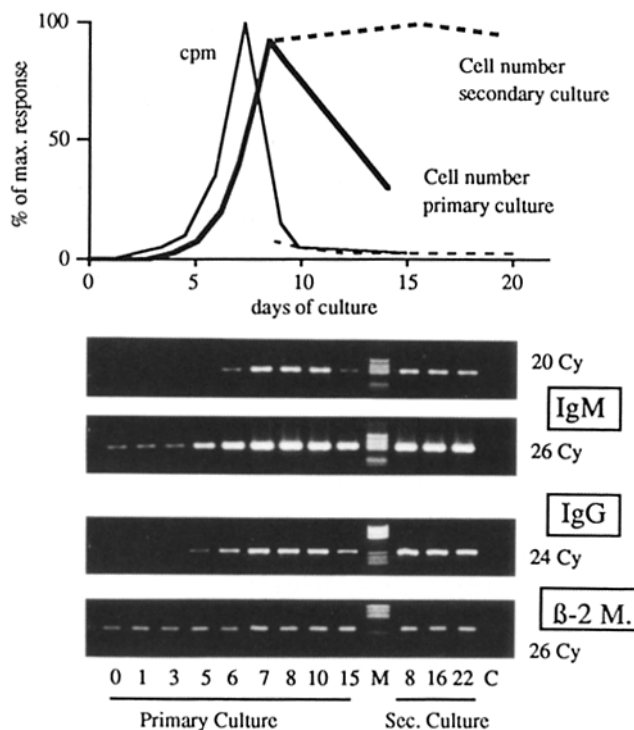

Figure 1. Kinetics of cell proliferation in EL-4/B cell cultures (*graph*) and IgM, IgG, and β_2m mRNA expression in EL-4/B cell samples. The graph shows thymidine incorporation (*thin lines*) and increase of viable cell number (*thick lines*) in primary (*continuous lines*) and secondary cultures (*broken lines*), as percent maximal values (18,000 cpm/culture on day 7 for 300 B cells/culture at primary culture start; 120 times the starting cell number on day 16). Results are expressed as mean values from four different experiments. cDNA was obtained at the indicated days from pooled series of 30 replicate cultures set up as above. PCR with IgM, IgG, and β_2m-specific primers was performed for the shown amplification cycles with cDNA aliquots corresponding to the equivalent of 1,000 viable B cells as determined by cell count (except for days 1–6, where cDNA preparations were adjusted for β_2m expression). Ethidium bromide–stained agarose gels in this and the following experiments were prepared with 1/10 of the volume of the PCR mix. This experiment with B cell population 1 cells is representative of similar experiments performed with either B cells from B cell population 1 (three experiments) or B cell population 2 (three experiments) (see Materials and Methods). (*M*) Molecular weight marker (DNA molecular weight marker V, Böehringer Mannheim); (*C*) RNA extraction control (mouse EL-4 thymoma cells only).

Correlation of IL-2 and -4 mRNA with CD2 mRNA Expression. Expression of mRNA of the T cell cytokines IL-2 and -4 was barely detectable in 1,000 B cells from B cell population 1 (see Materials and Methods) at a detection limit of cDNA from 1 and 10 PMA/PHA–activated T cells for IL-2 and -4, respectively (Fig. 2). A similar weak expression in B cell cultures was found for the T cell marker CD2. IL-2, IL-4, and CD2 cDNA signals were undetectable in B cells from B cell population 2 (data not shown). Thus, IL-2 and -4 mRNA were not expressed by the B cells in this culture system.

Correlation of IL-1α and -1β mRNA with Lysozyme mRNA Expression. EL-4/B cell cultures set up with B cells from B cell population 1 revealed clearly detectable IL-1α and -1β
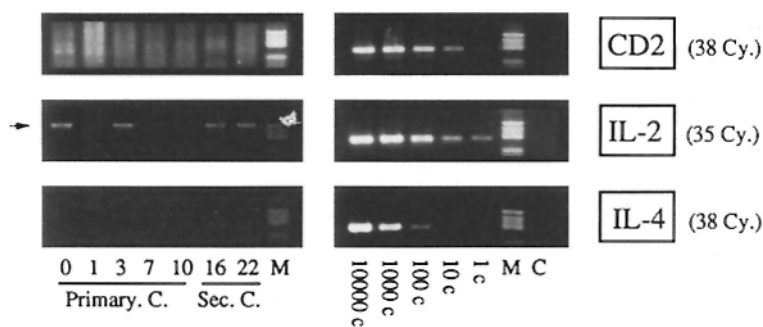
Figure 2. Comparison of T cell cytokine mRNAs (*IL-2* and *IL-4*) in B cell cultures and T cells. PCR was performed with cDNA samples from B cell cultures (*left*; same experiment as in Fig. 1; cDNA from ~1,000 B cell population 1 cells per PCR) and with serial dilutions of cDNA from purified CD3^{+} T cells, that had been stimulated with PMA/PHA (see Materials and Methods; from 1–10[4] T cell equivalents; *right*). (*Arrow*) Amplified fragments from genomic DNA contaminants. These were only detectable in the IL-2 PCR. Amplified cDNA fragments migrate further on the gel (compare with *right*).

mRNAs from the beginning of the culture until day 3. However, analysis of mRNA for the monocyte/macrophage marker lysozyme in these B cell cultures, and comparison with U937 cells, at a detection limit of cDNA from 1 to 10 U937 cells, indicated a monocyte contamination of ~1% (data not shown). When adherent cells were added to the EL-4 culture system, they expressed IL-1α and -1β mRNA with very similar kinetics (Fig. 3). Thus, it was not possible to exclude that the IL-1 mRNAs in EL-4/B cell cultures were derived from contaminating monocytes. To further investigate this point, we performed a more elaborate B cell purification. B cells were obtained by (*a*) depletion of monocytes by adherence and of T cells by SRBC rosetting; (*b*) positive selection with anti-CD19 magnetic beads; and (*c*) FACS® sorting of membrane IgM or total Ig^{+} cells with goat F(ab')₂ reagents (see Materials and Methods). In pooled mRNA, representing 1/10 of the mRNA from 10,000 such B cells (named B cell population 2), we could no longer detect lysozyme mRNA. In addition, IL-1α and -1β mRNAs were also no more detectable (Fig. 3). Thus the IL-1 mRNAs could not be attributed to B cells in this culture system.

Time Pattern of IL-6, TNF-α, IL-10, and TGF-β₁ mRNA Expression in EL-4/B Cell Cultures. We detected mRNAs for four cytokines with two different time patterns in EL-4/B cell cultures set up with highly purified B cells (B cell population 2) (Fig. 4). (*a*) IL-6 and TNF-α mRNAs were

upregulated between days 0 and 1, showed maximal expression during days 1–3, and became undetectable on days 5–6. (*b*) IL-10 mRNA (after a peak on days 1–3) and TGFβ-1 mRNA (after a progressive increase during the first 5 d) remained detectable until the end of the observation period (day 22). Thus, only the latter two cytokine mRNAs were detectable in cultures with Ig-secreting cells throughout the secondary culture.

A comparison with myeloma cells (U266 and RPMI 8226 cell lines), as well as with PMA/PHA–activated T cells, showed similar IL-10 mRNA expression for the same numbers of normal Ig-secreting, myeloma, and T cells. Thus, the requirement for nested PCR for IL-10 mRNA detection may be due to the lower efficiency of the selected human cDNA-specific primers rather than to effective mRNA levels. IL-6 mRNA was detectable in U266 myeloma cells (data not shown).

Contrary to the findings of a study with murine B cells (27), the analysis of EL-4/B cell cultures set up with sorted CD5^{+} and CD5^{−} B cells showed no difference in IL-10 mRNA expression between the two subsets of cells (data not shown).

Analysis of IL-10 and TGFβ-1 mRNA Expression in Individual B Cell Clones. By clonal analysis, we studied whether the same cell clones produce mRNAs for IL-10 and TGFβ-1. EL-4 cultures with single CD19^{+} B cells were set up with
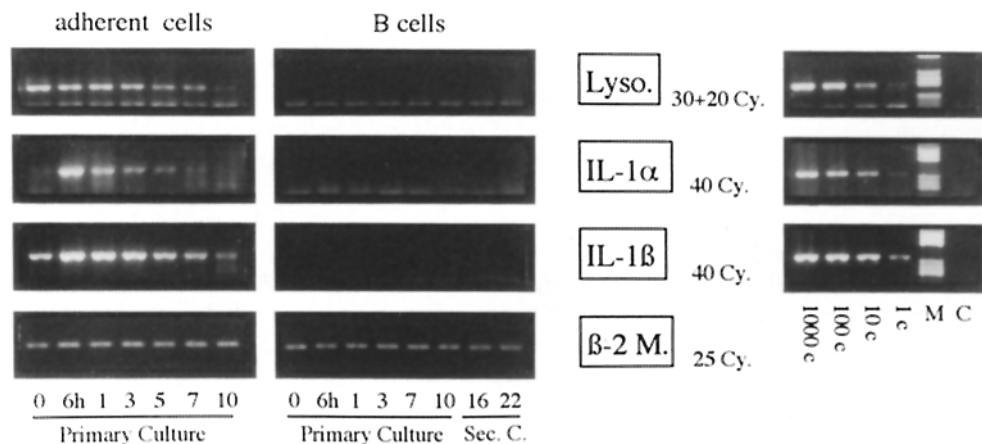

Figure 3. Absence of IL-1α and -1β mRNA in highly purified B cells. (*Left*) PCR for the detection of IL-1α, IL-1β, lysozyme, and β₂m cDNAs was performed with cDNA from adherent cells and from highly purified B cells (B cell population 2) cultured for the indicated time periods in the EL-4 system. cDNA samples corresponded to ~1,000 cells per PCR. Two consecutive rounds of PCR amplification (30 and 20 cycles) were done for the detection of lysozyme cDNA. The cDNA detection limits were established with U937 cells (*right*).
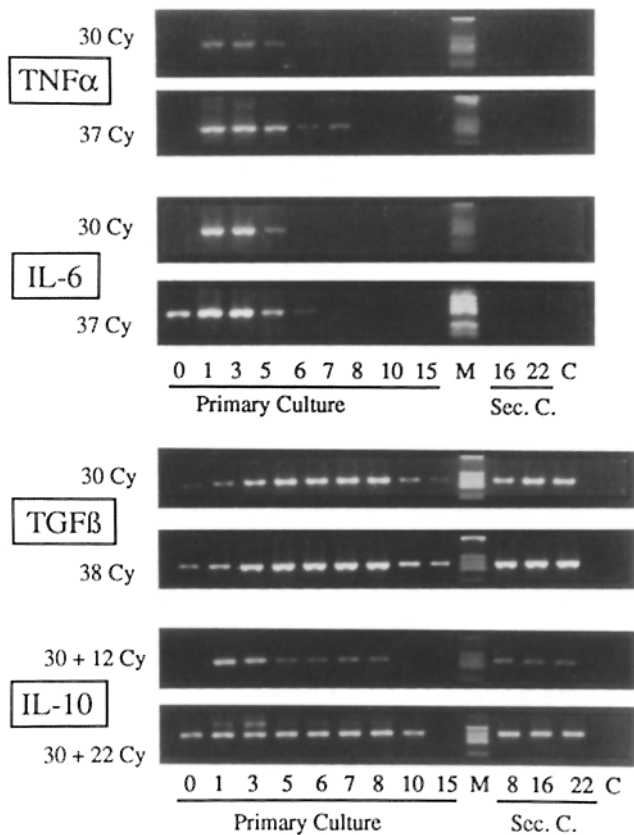
Figure 4. Cytokine mRNA expression in B cells at different times of culture. cDNA samples were adjusted for β_2m signals and corresponded to cDNA from ~1,000 B cells (see Fig. 1). Results obtained after two different numbers of amplification cycles, before complete saturation of bands from all samples, are shown. Nested PCR was performed for the detection of IL-10 mRNA. (Reaction 1) PCR for 30 cycles; (reaction 2) PCR for 12 and 22 cycles, respectively. The results for TNF-α, IL-6, and IL-10 mRNA were obtained with B cell population 2 cells and are representative for two other experiments with total surface Ig$^+$ or surface IgM$^+$ B cell population 2 cells. The results for TGFβ-1 were obtained with B cell population 1 cells, but B cell population 2 cells gave the same band pattern.

Figure 5. Typical RT-PCR results from single B cell clones. Single B cells were seeded in EL-4 cultures with the use of the Autoclone apparatus of the cell sorter, and cultured for 8 d. RNA from culture wells, in which Ig secretion had been detected by ELISA, was analyzed by RT-PCR for the presence of the indicated mRNAs. PCR input corresponded to 1/4 of the total RT volume for IL-10 and TGF-β1, and to 1/10 of the RT volume for β_2m and IgM, IgG, and IgA. Positive control, (B), is cDNA from an EL-4/B cell bulk culture (equivalent of 1,000 B cells/PCR; day 8). Note that Ig-PCRs with the chosen primers results in smeared bands for polyclonal B cells, in contrast to the monoclonal band patterns in autoclone wells. Nested PCR was performed for the detection of IL-10 and TGF-β1 mRNA.

the Autoclone device of the cell sorter. After 8 d, Ig levels were measured by ELISA assays and Ig secretion was detected in 77 of 140 tested cultures (IgM in 5, IgM and IgG in 6, IgM and IgA in 11, IgM, IgG, and IgA in 31, IgG in 7, IgA in 10, and IgG and IgA in 7 cultures). These results show a B cell responder frequency of 55% and frequent IgM to IgG or IgM to IgA isotype switch, in accordance with our previous autocloning results (19). cDNA preparations were made from all Ig$^+$ wells and β_2m mRNA was detected in 95% of them. mRNA or cell degradation before day 8 possibly occurred in the remaining Ig$^+$ cultures. On the other hand, in 10 cDNA samples prepared from Ig$^-$ culture wells, no β_2m signal was detected. RT-PCR with V and C region primer pairs detected clonal IgM, IgG, and/or IgA mRNA bands in 20 Ig ELISA-positive cultures analyzed, as opposed to smears obtained with B cell bulk cultures (examples are
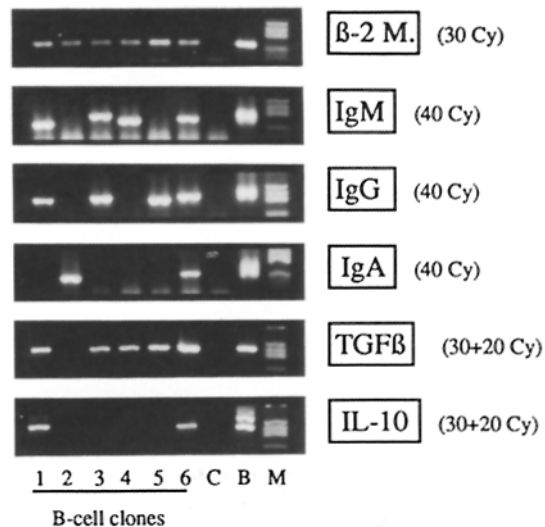
shown in Fig. 5). These results were in accordance with the ELISA results.

TGFβ-1 mRNA was detectable by nested PCR in 85% of Ig ELISA- and β_2m mRNA–positive cultures, whereas IL-10 mRNA was found in only 19% of such cultures (Fig. 6). All cultures containing detectable IL-10 mRNA coexpressed TGFβ-1 mRNA. No correlation between secreted Ig isotypes and the presence of TGFβ-1 and IL-10 mRNA could be established. Also, the presence of IL-10 mRNA was not correlated with strong β_2m mRNA signals or high Ig levels. Thus, absence of IL-10 mRNA seemed not to be related to small B cell clones. The number of clones with detectable IL-10 mRNA did not change over a 10-fold difference of PCR amplification cycles (inset, Fig. 6). The data strongly suggest that, whereas almost all B cell clones express TGFβ-1, additional IL-10 expression occurs at this time point only in a subset of such clones.

Discussion

The analysis of cytokine mRNA expression in normal human B lymphocytes during a T cell–dependent response was made possible by the use of murine thymoma cells as helper cells in conjunction with human T cell supernatant, and the design of human cDNA sequence-specific primers for RT-PCR. Recent experiments showed that our mutant
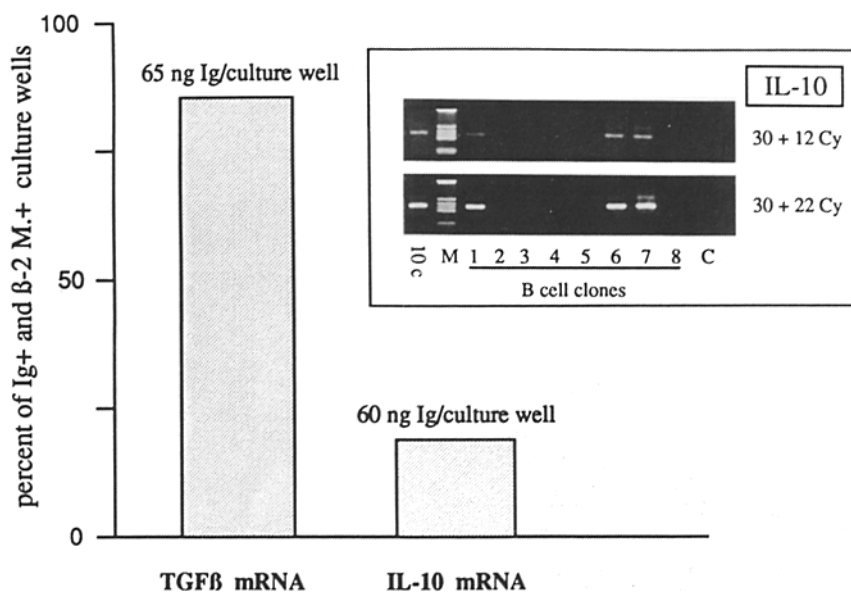
Figure 6. Frequencies of IL-10 and TGF-β1 mRNAs expressing B cell clones. The 77 autoclone culture wells, in which Ig secretion had been detected by ELISA and β2m mRNA by RT-PCR, were tested for IL-10 and TGF-β1 mRNA expression, as shown in Fig. 5. All IL-10 mRNA positive clones also contained TGF-β1 mRNA. The percentage of positive wells is depicted and the mean Ig production per culture well is indicated. (*Inset*) Nested PCR results for the detection of IL-10 mRNA from 8 B cell clones after 30 plus 12 and after 30 plus 22 cycles. Note that the presence or absence of bands did not change over the 10-cycle interval. The mRNA equivalent from 10 EBV-transformed B cells was run as a positive RT-PCR control (*10 c*).

EL-4 thymoma cells express CD40 ligand in a way similar to normal helper T cells, and that their helper activity is totally inhibited by murine or human soluble CD40-Ig Fc constructs (Werner-Favre, C., J. F. Gauchat, G. Mazzei, J. Y. Bonnefoy, and R. H. Zubler, unpublished results). Thus, like another assay for efficient human B cell activation (28), this system involves a CD40-mediated B cell activation signal. However, long-term growth of B cells (28) does not occur in the EL-4 system.

mRNA expression for eight cytokines was studied. IL-2 and IL-4 mRNAs can, most probably, not be attributed to B cells. This contrasts with findings on EBV-immortalized B cells, which were found to produce IL-2 and -4 mRNAs (29), and IL-2 bioactivity (7), but no IL-4 immunoreactive protein (29). More unexpectedly, the presence of IL-1α and -1β mRNAs in EL-4/B cell cultures correlated with that of the monocyte marker lysozyme. B cell population 1 cells, which contained ∼1% of monocytes, expressed IL-1α and -1β mRNAs, whereas triple-isolated B cell population 2 cells without detectable lysozyme mRNA showed no IL-1 expression. This also differs from observations on EBV-transformed B cells (11, 29). In addition, IL-1–like bioactivity (11), immunoreactive IL-1, and IL-1α and -1β mRNAs (by Northern blot) have previously been detected in normal human B cells (12). The latter observations were made with B cells activated with anti-Ig antibody. The absence of IL-1 mRNAs and the presence of the other cytokine mRNAs, as found with the EL-4 system in the presence of total T cell supernatant, were confirmed with a system (28) that uses IL-4 and murine fibroblasts coated with anti-CD40 antibody (our unpublished results). Thus, IL-1 expression is at least not obligatory during a B cell response. It can be asked whether IL-1 expression in normal B cell populations is always due to monocyte contamination. This is not yet clear, but if it were the case, it would be in accord with the reported difference in

in vivo effects on naive, resting T cells of antigen presentation by B cells versus that by macrophages in mice (30, 31).

Four cytokine mRNAs with two different time patterns of expression were detected in B cells. TNF-α and IL-6 mRNAs peaked during days 1–3 and became undetectable after 5–6 d, shortly before the bulk B cell proliferation declined. In contrast, TGFβ-1 mRNA, after a progressive increase during the first few days, and IL-10 mRNA, after a peak on days 1–3, remained detectable throughout the observation period of 22 d. This shows a B cell differentiation stage–related cytokine program. Previous studies have revealed that B cells can effectively synthesize (and also respond to) these cytokines (4, 13–15, 32, 33). It becomes therefore difficult to predict the relative dependence on exogenous (T cell and monocyte-derived) cytokines during an in vivo B cell response. In a recent study on the effects of adding various cytokines and cytokine inhibitors to B cells cocultured with EL-4 thymoma cells in the absence of T cell supernatant, we found that IL-1 was required to induce TNF-α responsiveness of B cells, and that TNF-α was required to induce responsiveness to other cytokines such as IL-2, -4, and -6 (34). This hierarchy of cytokine effects fits with the occurrence of the IL-1 mRNA peak (at 6 h) before the TNF-α mRNA peak (days 1–3) in B cell population 1 cells. However, the results obtained with B cell population 2 cells show that the IL-1 is an exogenous cytokine in this system. It is possible that various activation signals differentially influence cytokine expression by B cells.

Clonal analysis, performed by RT-PCR on 8-d cultures that had been seeded with single B cells by autocloning with the cell sorter, showed that most Ig-secreting B cell clones express TGFβ-1 mRNA (85%), and only 19% IL-10 mRNA. Both TGFβ-1 and IL-10 were found to influence isotype switch to IgA (35), but no correlation between the expression of these cytokine mRNAs and secreted Ig isotypes (IgM, IgG,

and IgA) or amounts of Ig could be established. It is known that adult versus cord blood–derived CD19$^+$ B cells (36), and CD19$^+$ total B cells versus membrane IgM$^+$ B cells (>85% of adult CD19$^+$ B cells) (19) switch to other isotypes with very similar high frequencies in the present culture system and even produce similar proportions of clones producing only IgG or IgA (derived from IgM/G or IgM/A double isotype–positive cells). Thus the present data are only representative for naive B cells. CD5$^+$ but not CD5$^-$ B cells were found to be the main source of IL-10 mRNA in mouse B lymphocytes (27). Our experiments with bulk cultures of sorted CD5$^+$ and CD5$^-$ B cells did not show such a difference. However, in human B cells, the CD5 molecule behaves like an activation marker rather than a marker for a special B cell lineage (18, 37), and most CD5$^-$ B cells become transiently CD5$^+$ during the first few days in the EL-4 culture system (18). IL-10 mRNA is in fact also increased at that time.

TGFβ-1 was shown to inhibit (14) whereas IL-10 was found to stimulate human B cell growth and Ig secretion (38). These opposite effects of TGFβ-1 (34) and IL-10 (our unpublished results) were confirmed in the EL-4 system. Our bulk culture data thus suggest that positive and negative autoregulatory factors coexist during the whole B cell response, as in many other biological systems. But it is not known whether plasmocytic cells or a minor subset of less differentiated cells express TGFβ-1 and/or IL-10 mRNA. Also, it is not clear whether B cell clones only differ with regard to cell differentiation or whether they are heterogeneous regarding cytokine expression like T cell clones (the TGFβ-1 and IL-10 mRNAs remained detectable until day 22).

In agreement with previous studies (8, 39) our data show that normal Ig-secreting cells produce neither IL-1 nor -6, and, therefore, clearly differ from their malignant counterparts, myeloma cells (8, 9). It has recently been reported that detectable serum IL-10 indicates a better, and detectable serum IL-6 a worse, prognosis in multiple myeloma patients (40). According to the differentiation stage–related cytokine program of normal B cells shown above, IL-6–producing myeloma cells should be less differentiated than IL-6–nonproducing myeloma cells.

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