INTERLEUKINS AND IgA SYNTHESIS

Human and Murine Interleukin 6 Induce High Rate IgA Secretion In IgA-committed B Cells

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The antibody response is dependent upon interleukins that induce activation, proliferation, and terminal differentiation of B cells. The latter events, namely the production of plasma cells secreting Ig in high amounts are mainly induced by the cytokine B cell stimulatory factor 2 (BSF-2)[1], now termed IL-6 (1). The interleukins, including IL-6, are all pleiotropic, and rDNA cloning has now shown that previously cloned IFN-β2 (2), 26-kD inducible protein (3), and hybridoma/plasmacytoma growth factor (4) are identical to BSF-2 or IL-6 (5, 6). Although IFN-β2 was originally derived from human fibroblast lines induced with stimuli that result in production of IFN-β, several studies have now shown that IL-6 does not have IFN-β activity (7, 8). Other effects of IL-6 include stimulation of hematopoietic stem cells (9), nerve cells (10), and hepatocytes to produce acute-phase proteins (11). IL-6 is also produced by T cells and monocytes/macrophages, and induces thymocyte growth and differentiation of mature T cells (12–14). The receptor for IL-6 (IL-6R) has now been characterized (15) and cloned (16), and the IL-6R is present on activated B cells, e.g., multiple myelomas and EBV-transformed B cell lines (1, 17).

Models have been proposed to explain interleukin-sensitive stages in B cell differentiation. For example, the sequential steps of B cell activation, division, and terminal differentiation are mainly influenced by IL-4, IL-5, and IL-6, respectively (1). In this regard, small, resting B cells express IL-4R, and IL-4 promotes entry into cell cycle with an associated increase in class II MHC antigen expression (18, 19). LPS-induced B cell blasts have been shown to express receptors for IL-5 (20), which are absent on noncycling cells, clearly suggesting that B cell blasts are the IL-5-responsive subset. Receptors for IL-6 have been demonstrated only on activated and dividing

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[1] *Abbreviations used in this paper:* BSF-2, B cell stimulatory factor 2; hr, human recombinant; m, membrane; mr, murine recombinant; PP, Peyer's patch; TGF, transforming growth factor.

B cells and on B cell and plasma cell tumor populations (15, 16). It has also been shown that cytokines regulate movement of B cells through restriction points in the cell cycle (21). One of these occurs in G_1 where macrophage-derived factors are essential, while a second occurs 2–4 h before division (G_2) and is controlled by IL-2 and IL-5, but not by IL-3 or IL-4 (22).

Our own studies have been directed toward determining the principal cytokines involved in the mucosal IgA response. Antibody of the IgA isotype comprises 60–65% of the total Ig produced daily in higher mammals (23). IgA associated with mucosal surfaces is produced locally by IgA plasma cells distributed in the subepithelial spaces of mucosal membranes and in secretory glands, such as the lacrymal and salivary glands (24). Peyer's patches (PP) are major IgA inductive sites and contain B cells that are the precursors of IgA plasma cells at mucosal effector sites (25, 26). The PP contain both small, resting and blast B cell populations, and B cells that have recently committed to IgA (membrane IgA^+ [$mIgA^+$]) (26). In this study, we have analyzed the effects of recombinant mouse and human IL-6 on various PP B cell subsets to determine the role of this interleukin in regulation of IgA synthesis.

Materials and Methods

Murine IL-6. Murine rIL-6 (mrIL-6) was obtained as a reverse-phase HPLC-purified protein derived from transfected COS-7 cell supernatants (27). Briefly, GY30 bone marrow stromal cells were induced for 7 h with human rIL-1 at ~650 U/ml. Total cellular RNA was extracted using the guanidinium isothiocyanate method (28) and poly(A)$^+$ RNA was selected by oligo(dT)-cellulose chromatography. cDNA libraries were constructed by the method of Okayama and Berg (29) using the pcD-SRα plasmid vector (30). pcD-mIL-6 plasmid DNA was transfected into COS-7 monkeys cells and mrIL-6 was purified by reverse-phase HPLC. mrIL-6 was >95% pure as shown by SDS-PAGE analysis (31). Units of mrIL-6 were determined using the factor-dependent cell line NFS-60. 10 U/ml IL-6 was defined as the concentration of IL-6 inducing half-maximal stimulation of NSF-60 cells as determined by the MTT colorimetric assay (32).

Human IL-6 and Polyclonal Anti-IL-6 Antibody. Human rIL-6 (hrIL-6) was prepared by expressing a cDNA for IL-6 in *Escherichia coli* followed by further purification, the details of which are described elsewhere (6, 33). Briefly, the plasmid pTBCDF-12, which contains the *E. coli trp* promoter for expression, was constructed in order to generate a pT9-11 fusion protein. The translated fusion protein was then digested with kallikrein and aminopeptidase-P to obtain biologically active hrIL-6. This recombinant material was further purified by reverse-phase HPLC. The purity of hrIL-6 was >95%, as shown by SDS-PAGE analysis, and 10^5 U/19.3 μg/ml of hrIL-6 was used as the starting material in this study. To obtain the polyclonal antibody to human IL-6, a goat was immunized with hrIL-6. This antiserum neutralized 1 U/ml of hrIL-6 activity at a dilution of 10^{-5}; however, this antibody did not neutralize human IL-2, IL-4, or IL-5.

Source of Cytokines. mrIL-4 and IL-5 were obtained from HeLa cells transfected with cDNA for IL-4 (34) and IL-5 (35). These interleukins were purified from concentrated culture supernatants as previously described (36). mrIL-1α was generously provided by Drs. A. Stern and P. Lomedico (Hoffman-La Roche Inc., Nutley, NJ). hrIL-2 and mrTNF-α were kind gifts from Biogen Corp. (Cambridge, MA). mrIFN-γ was kindly provided by Genentech (South San Francisco, CA). Purified human platelet-derived transforming growth factor β (TGF-β) was purchased from Collaborative Research Inc. (Bedford, MA). TGF-β was >95% pure, as shown by SDS-PAGE analysis, giving a single band of 25 kD under nonreducing conditions and a single band of 12.5 kD under reducing conditions. TGF-β supported the growth of normal rat kidney cells cultured in soft agar with a half-maximal colony-forming response occurring at 1.35 ng TGF-β per milliliter of culture medium. mrIL-7 was kindly provided by Immunex Corp. (Seattle, WA) (37).

Cytokine Assays. Both hrIL-2 and mrIL-4 were assayed on the HT-2 factor-dependent cell line (38), as previously reported (36). For assessment of mrIL-4 activity, appropriate dilutions were tested in the presence (or absence) of the anti-IL-4 antibody derived from the B cell hybridoma 11B11, generously provided by Dr. W. Paul (National Institutes of Health, Bethesda, MD) (39). The mrIL-1α activity was assessed in the C3H/HeJ thymocyte comitogenic assay (40). The activity of mrIL-5 was measured using the dextran-sulfate B cell costimulation assay as previously reported (36). Finally, mrIFNγ was assessed by inhibition of growth of WEHI 279, an IFN-γ-sensitive cell line (41) kindly provided by Dr. K. Bottomly (Yale University School of Medicine, New Haven, CT).

Mice. C3H/HeN mice obtained from the Frederick Cancer Research Facility (NCI, Frederick, MD) were maintained in horizontal laminar flow cabinets and provided food and water ad libitum. All mice used were between 7 and 12 wk old.

Preparation of PP B Cells. PP were aseptically removed and dissociated using the enzyme Dispase® (Boehringer Mannheim Biochemicals, Indianapolis, IN) in Joklik-modified medium (Gibco Laboratories, Grand Island, NY) (42). Cells were cultured overnight at 2×10^6 cells/ml in a 75-ml flask in complete medium consisting of RPMI 1640 (Gibco Laboratories) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, nonessential and essential amino acids, 5×10^{-5} M 2-ME, gentamycin, and 10% (vol/vol) FCS (Flow Laboratories, Inc., McLean, VA). B cells were prepared by panning on petri plates (15 × 100 mm; Falcon Labware, Oxnard, CA) coated with goat IgG anti-mouse $F(ab')_2$ as previously described (43). The adherent B cell population was then treated with a rat monoclonal anti-T cell cocktail (anti-Thy-1.2, Clone HO-13-4 [44]; anti-Lyt-2, Clone 53.6-72 [44]; and anti-L3T4, Clone GK1.5 [45] for 30 min at 4°C followed by incubation (30 min at 4°C) with monoclonal anti-rat κ chain [Clone MAR 18.5 [46]), then incubated for 30 min at 37°C with a 1/10 dilution of baby rabbit complement (Pel-Freeze Biologicals, Rogers, AR). Cells obtained by this method were >98% mIg^+.

B Cell Fractionation. Purified PP B cells were fractionated on discontinuous Percoll gradients as previously described (36, 43). Briefly, Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was diluted 9/1 with 10× HBSS (Gibco Laboratories). From this 100% stock solution, 50, 60, 65, and 70% Percoll solutions were made by dilution in 1× HBSS. Gradients were prepared by layering 2-ml aliquots of 70, 65, 60, and 50% Percoll in a 15-ml centrifuge tube (Falcon No. 2057; Becton Dickinson & Co., Palo Alto, CA). A suspension of $3\text{-}5 \times 10^7$ purified B cells in 1 ml of incomplete medium (RPMI 1640 supplemented with Hepes buffer, L-glutamine, sodium pyruvate, nonessential amino acids, gentamycin, penicillin, and streptomycin) was carefully layered over the 50% Percoll layer and the gradient was centrifuged at 2,000 rpm for 20 min at 4°C (with the brake off). Fractions were collected from the Percoll interfaces with a Pasteur pipette and washed twice in incomplete medium before use in in vitro assays.

Depletion/Enrichment of $mIgA^+$ B Cells. For the depletion or enrichment of $mIgA^+$ B cells, PP B cells were stained with FITC-conjugated goat anti-mouse α chain (Southern Biotechnology Associates [SBA], Birmingham, AL) for 30 min at 4°C. Cells were then washed twice with incomplete medium and resuspended at 5×10^6 cells/ml for cell sorting. Cell separations were performed by use of the FACStar® (Becton Dickinson & Co., Mountain View, CA) and FITC-anti-α-treated B cell populations were sorted into $mIgA^+$ and $mIgA^-$ cell fractions. According to the flow cytometry profile, brightly stained cells (4–6%) were collected as $mIgA^+$ B cells.

In Vitro Cultures. Purified unsorted PP B cells or mIgA-depleted PP B cells were cultured in complete medium at 10^5 cells in 0.2 ml total volume/well in 96-well flat-bottomed microtiter plates (Falcon Labware), and rmIL-6 was added to give final concentrations of 0.5, 5, 50, and 150 U/ml. $mIgA^+$ B cells were cultured at 5×10^4 cells per well. All cultures were incubated for 7 d at 37°C in a humidified atmosphere of 83% N_2, 7% O_2, and 10% CO_2.

Isotype-specific RIA. Solid-phase absorbed and affinity-purified goat IgG antibodies specific for murine μ, γ, and α H chains were obtained from SBA. Their specificity was confirmed in RIA using purified mAbs and appropriate myeloma proteins as substrates. Radioiodination with carrier-free Na ^{125}I (Amersham Corp., Arlington Heights, IL) was performed using the chloramine T method, modified to reduce oxidative damage to proteins (47).

Immulon Remova well assay strips (Dynatech Laboratories, Chantilly, VA) were coated with goat anti-mouse μ, γ, or α at 1 μg/ml in borate-buffered saline, pH 8.4, overnight at 4°C. Control strips were left uncoated, but all strips were blocked for 2 h at 25°C with 1% (wt/vol) BSA (Sigma Chemical Co.) in borate-buffered saline, which was also used as the diluent for all samples and ^{125}I-labeled reagents. Appropriate dilutions of tissue culture supernatants were added to washed wells in triplicate and incubated for 6 h at 25°C. After washing, 100,000 counts/min of ^{125}I-labeled isotype-specific anti-Ig reagent was added to each well and incubated overnight at 4°C. After removal of the unbound ^{125}I antibodies by washing, the cells were counted in a gamma 5500 spectrometer (Beckman Instruments, Irvine, CA). Calibrations were made using serial twofold dilutions of purified mAb 1F3-C1.5 or 2F.11.15 for IgM and IgA, respectively, or a standard mouse serum (Miles Laboratories, Naperville, IL) for IgG. Calibration curves and interpolation of unknowns were obtained by computer using a "Logit-log" BASIC program (RIA 001; Biomedical Computing Technology Center, Vanderbilt Medical Center, Nashville, TN).

Enumeration of IgA-producing Cells. To quantitate the number of antibody-forming cells, the enzyme-linked immunospot (ELISPOT) assay (48) was used. For this assay, 96-well nitrocellulose bottom plates (Millititer HA; Millipore Corp., Bedford, MA) were coated overnight at 4°C with goat anti-mouse Ig (SBA) diluted to 5 μg/ml in PBS (100 μl/well). Control wells were coated with PBS. All wells were then blocked for 60 min with complete medium. IL-6-stimulated or control PP B cells (5×10^3 to 5×10^4 cells/well) were incubated on coated plates in 100 μl of complete medium for 6–8 h. The plates were then thoroughly washed with PBS containing 0.05% Tween (PBS-T) and then incubated with 100 μl/well biotin-labeled goat anti-mouse IgA (SBA) diluted 1:1,000 in PBS-T containing 1% FCS. After overnight incubation at 4°C, the plates were washed three times with PBS-T then incubated for 2 h with 100 μl/well avidin-peroxidase diluted 1:1,000 in PBS-T. Spots representing single antibody producing cells were developed using 3-amino-9-ethylcarbazole as substrate, and visualized with the aid of a dissecting microscope. The results are presented as the mean ± SD for triplicate determinations.

Results

Cytokine-mediated Support of IgA Synthesis by PP B Cells. Although numerous studies have examined the ability of various purified natural and recombinant cytokines to regulate the level and isotype of Igs secreted by activated B cells, few experimental systems have allowed an examination of defined leukocyte-derived factors that support commitment to, and secretion of, IgA antibodies (26). The levels of IgA secreted by cytokine-supported cultures of LPS-stimulated spleen B cells have been uniformly low, and the possible skewing of the isotype profile by LPS induction cannot be discounted. Our previous studies have shown that high levels of IgA are secreted by cultures of noninduced PP B cells supplemented with mrIL-5 (36). As an extension of this work, we have used cultures of purified PP B cells to systematically examine the ability of interleukins and cytokines to support the later stages of B cell differentiation to the secretion of IgA. Extensive titrations of seven recombinant human and mouse interleukins (mrIL-1α, hrIL-2, mrIL-4, mrIL-5, mrIL-6, hrIL-6, and mrIL-7) and three additional cytokines (mrIFN-γ, mrTNF-α, and platelet-derived TGF-β) revealed that only mrIL-5 and rIL-6 (murine and human) significantly enhanced IgA secretion by PP B cells (Fig. 1). While none of the other factors increased the level of IgA in the culture supernatants by >50%, the IL-5 and IL-6 preparations supported ~400 and 1,000% of the IgA secreted in the control cultures, respectively. Both the murine and human rIL-6 reproducibly induced secretion of approximately twice the amount of IgA compared with IL-5, and therefore the activities of this interleukin were investigated in greater detail.

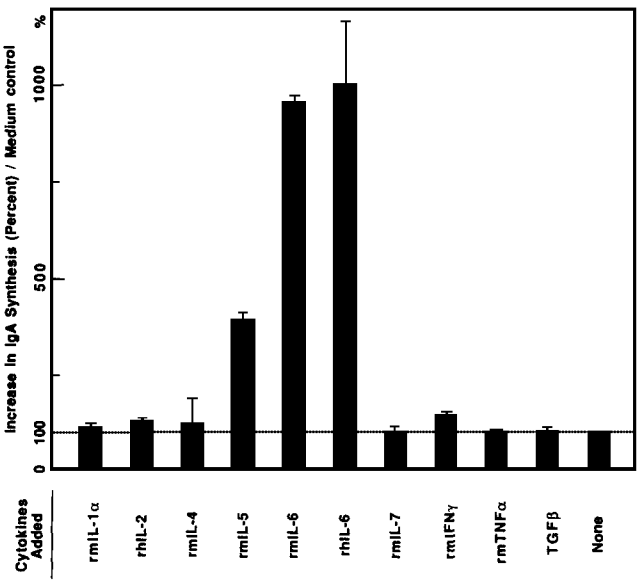

FIGURE 1. The potential effects of cytokines on IgA synthesis in PP B cell cultures. In this study, the cytokines mrIL-1α (100 U/ml), hrIL-2 (100 U/ml), mrIL-4 (10 U/ml), mrIL-5 (50 U/ml), mrIL-6 (50 U/ml), hrIL-6 (50 U/ml), mrIL-7 (50 U/ml), mrIFN-γ (100 U/ml), mrTNF-α (2.5 ng/well) and TGF-β (40 pg/ml) (see details in Materials and Methods) were added to purified PP B cell cultures (10^5 B cells/well) and incubated for 7 d. Each cytokine was titrated in an appropriate bioassay; in these studies multiple doses of each cytokine were added and the results presented are typical responses in these cultures. Levels of IgA in the culture supernatant was determined by RIA. Values are the mean ± SD of triplicate cultures. Mean IgA in medium control cultures (eight experiments) was 1,276 ± 637 ng/ml.

mrIL-6 Selectively Enhances IgA Secretion by PP B Cells. The ability of mrIL-6 to influence the relative proportions of IgM, IgG, and IgA secreted by PP B cells in the absence of any other exogenous stimulus was tested across a range of concentrations to examine whether this lymphokine plays a role in determining the enrichment for IgA production characteristic of the cells from this mucosal tissue (Fig. 2). mrIL-6 exhibited a dose-dependent ability to enhance IgA synthesis, with a peak response 5–20 times that of control cultures. In contrast, IgM and IgG secretion was only marginally increased by this lymphokine, regardless of the dose used. Comparison of the levels of each isotype induced by optimal doses of mrIL-6 and mrIL-5 revealed that mrIL-6 is not as effective as mrIL-5 at supporting IgM or IgG synthesis, despite the fact that it supports a substantially higher level of IgA secretion. These results illustrate that both of these interleukins preferentially support IgA secretion by PP B cells, and that this effect is more pronounced, both in absolute amount and relative to IgM and IgG, with mrIL-6.

mrIL-6 and hrIL-6 Induce Identical Activities in Mouse PP B Cells. hrIL-6 has recently been shown to augment Ig secretion in PWM-stimulated human B cell cultures, and to induce mouse hemopoietic colony formation (1, 9). Direct comparison of the effects of mrIL-6 and hrIL-6 after their addition to cultures of PP B cells demonstrated that they possessed essentially identical abilities to support Ig synthesis (Fig. 3). Both caused a dose-dependent increase in antibody secretion that was almost exclusive for the IgA isotype. The availability of goat antiserum specific to human IL-6 allowed a control experiment to be performed to confirm that the enhancement of IgA synthesis by PP B cells is a property of IL-6. Inclusion of the anti-IL-6 antiserum at a final concentration of 1:200 in the IL-6 supplemented cultures resulted

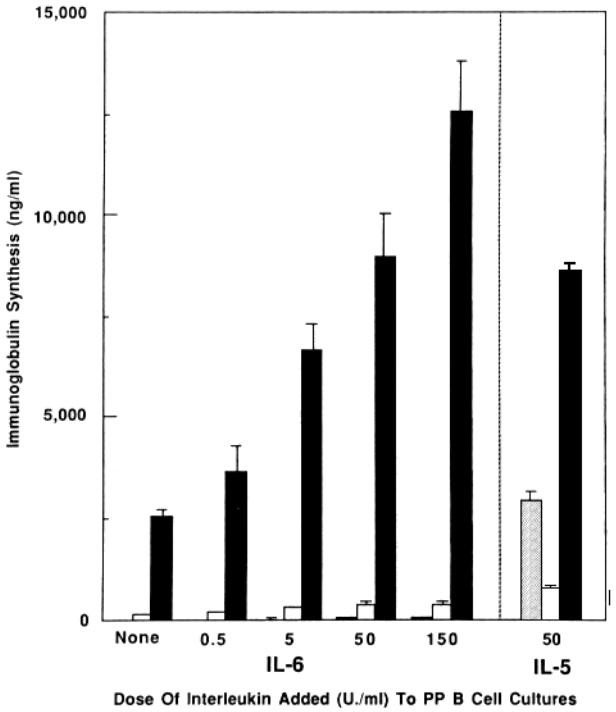

FIGURE 2. mrIL-6 effects on Ig synthesis in PP B cell cultures. Purified PP B cells were cultured at 10^5 cells/culture for 7 d in the presence of increasing amounts of mrIL-6 and mrIL-5 (50 U/ml). Levels of IgM (▨), IgG (□), and IgA (■) in culture supernatants were determined by isotype-specific RIA. Values are representative of the mean ± SD of triplicate cultures for five separate experiments.

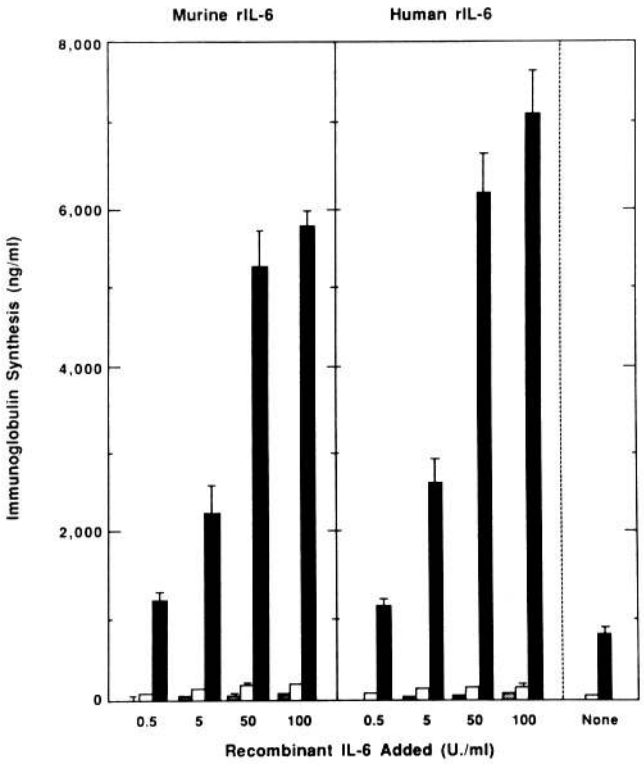

FIGURE 3. hrIL-6 effects on Ig synthesis by PP B cells. PP B cells obtained by panning were cultured at 10^5 cells/well with hrIL-6 and mrIL-6 for 7 d. IgM (▨), IgG (□), and IgA (■) levels in culture supernatants were determined by isotype-specific RIA. Values represent the mean ± SD of triplicate cultures.

in a 98% reduction in IL-6-mediated IgA secretion, while normal goat serum was without effect (Fig. 4).

mrIL-6 Acts Predominantly on Low Density PP B Cells. Human tonsillar B cells have been shown to be refractory to human IL-6 unless they are first activated with mitogens (1), and we have shown that the IL-5-responsive B cells in mouse PP are predominantly in the low density (activated) fraction (36). To determine the activation status of the cells responding to IL-6, PP B cells were cultured in the presence of various concentrations of IL-6 after fractionation into low density (activated) and high density (predominantly resting) populations on discontinuous Percoll gradients. Determination of the levels of IgM, IgG, and IgA in the culture supernatants after 7 d of culture indicated that the cells that responded to IL-6 with increased Ig synthesis were enriched in the low density fraction (Fig. 5). As in the case of unfractionated PP cells, the predominant isotype secreted by both the high and low density cells was IgA. While the enhancement of Ig secretion by mrIL-6, as well as that previously demonstrated with mrIL-5 (36), is pronounced in the low density (activated) cell fraction, there does not appear to be a strict correlation between membrane isotype expression by activated PP B cells and isotype secretion. Although a higher proportion of the PP mIgA$^+$ cells are in cycle compared with mIgA$^-$ cells, the absolute number of cycling mIgM$^+$, mIgA$^-$ cells is several times higher than that of mIgA$^+$ cells (36). Despite the enrichment of the activated mIgM$^+$ cells into the low density fraction, virtually no enhancement of IgM secretion is observed in the presence of IL-6. Thus, these results demonstrate that the PP B cells that respond to IL-6 with increased Ig secretion are in cycle and that those PP B cells destined for IgA secretion are uniquely sensitive to the differentiation-supporting activity of this interleukin.

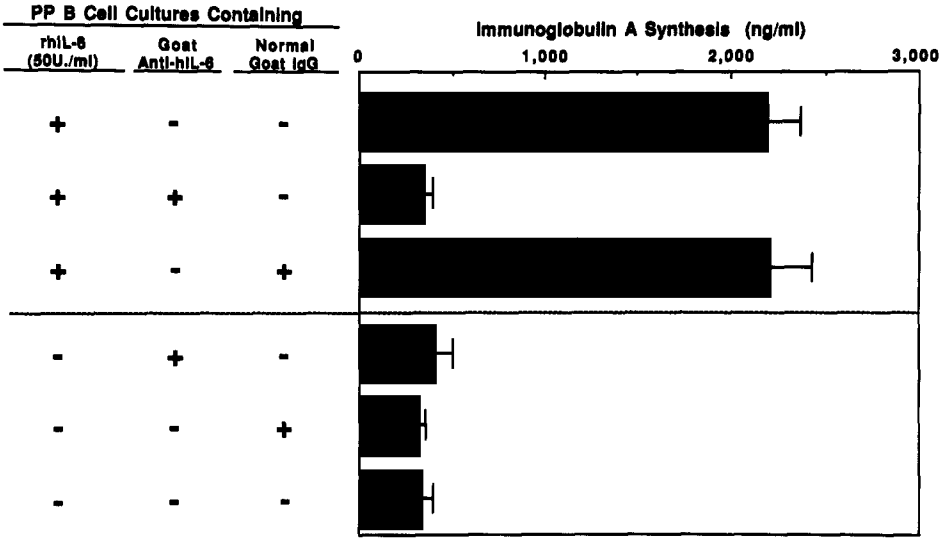

FIGURE 4. Goat anti-human IL-6 inhibits hrIL-6-mediated IgA secretion. Purified PP B cells were cultured for 7 d at 10^5 cells/culture with hrIL-6 at 50 U/ml. Goat anti-human IL-6 (1:200 final dilution) and normal goat serum (1:200) were added to cultures 30 min before addition of B cells. IgA levels in culture supernatants were determined by isotype-specific RIA. Values represent the mean ± SD of triplicate cultures.

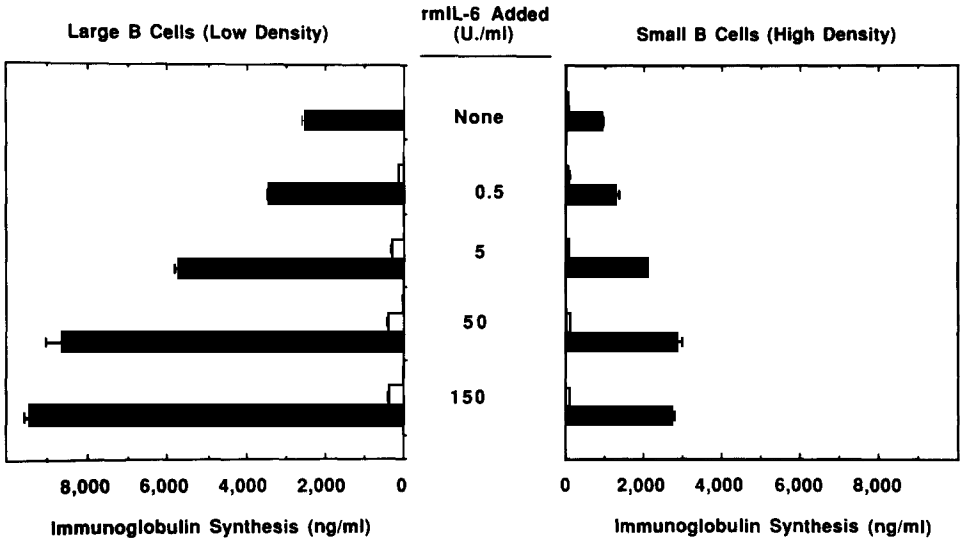

FIGURE 5. mrIL-6 effects on PP B cell subpopulations separated on Percoll density gradients. Purified PP B cells obtained by panning were separated into large B cells (50 and 60% Percoll interfaces) and small B cells (65 and 70% Percoll interfaces) and were cultured at 10^5 cells/culture for 7 d with increasing amounts of mrIL-6. IgM (▨), IgG (□), and IgA (■) levels in culture supernatants were determined by isotype-specific RIA. Values represent the mean ± SD of triplicate cultures.

The PP B Cells that Respond to mrIL-6 with Increased IgA Synthesis Are mIgA$^+$. A central issue pertaining to mucosal immune inductive sites, such as the PP, involves the mechanism(s) through which they are predisposed to the selective induction of IgA class antibody responses. To differentiate between the possibilities that IL-6 directs isotype switching, as opposed to selectively potentiating the differentiation of previously committed B cells, flow cytometric sorting was used to separate PP B cells into mIgA$^+$ and mIgA$^-$ subpopulations. Culture of these separate subpopulations in the presence of various concentrations of IL-6, followed by analysis of the secreted Igs for the levels of IgM, IgG, and IgA, clearly demonstrated that the cells that secrete IgA in response to IL-6 express mIgA at the initiation of the culture (Fig. 6). Removal of the mIgA$^+$ cells abolished both background and IL-6-supported IgA secretion. In contrast, the enriched mIgA$^+$ subpopulation responded in a dose-dependent manner with enhanced IgA secretion. In conjunction with the data obtained with density-separated cells presented above, these results are consistent with a model in which IL-6 acts on activated mIgA$^+$ (IgA-committed) B cells to support their synthesis of increased amounts of IgA.

mrIL-6 Supports an Increase in the Number of IgA-secreting Cells in the Absence of Proliferation. Based on the comparative "background" levels of IgA synthesis in nonsupplemented cultures of low density versus high density and mIgA$^+$ versus mIgA$^-$ PP B cells, it is clear that the PP B cell population represents an enriched source of activated mIgA$^+$ cells that are either secreting IgA at the time of isolation, or that will progress to IgA secretion in the absence of exogenously added lymphokines. The ability

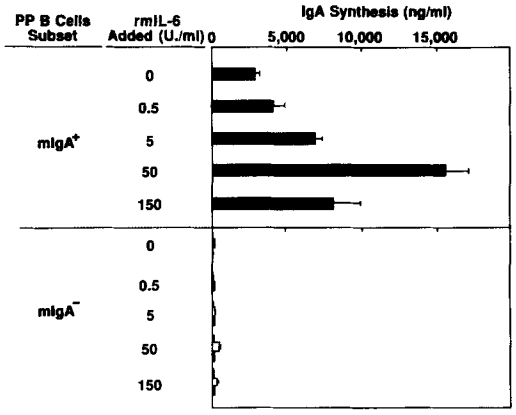

FIGURE 6. mrIL-6 effects on FACS separated PP B cell populations. PP B cells obtained by panning were stained with FITC-labeled anti-α and separated into mIgA$^+$ and mIgA$^-$ populations. Whole PP B cells and mIgA$^-$ PP B cells were cultured at 10^5 cells/culture for 7 d with IL-6. mIgA$^+$ PP B cells were cultured at 5×10^4 cells/culture for 7 d with IL-6. IgM (▨), IgG (□), and IgA (■) levels in culture supernatants were determined by isotype-specific RIA. Values represent the mean ± SD of triplicate cultures.

of IL-6 to increase the absolute amount of secreted IgA could be mediated through proliferation and expansion of these "synthesis committed" cells, by enhancement of the synthetic rate of these committed cells, or through the recruitment and differentiation to IgA secretion of additional cells from the activated mIgA$^+$ pool. To differentiate between these possibilities, we measured the number of IgA secreting cells and the amount of IgA secreted as a function of time. As detected in an ELISPOT assay, the number of cells secreting IgA in the presence of exogenously added IL-6 rose to near a plateau number (greater than eight times background) within the first 24 h of culture (Table I). Additionally, no difference in the number of viable cells was detected over the first 3 d of culture, either in the presence or absence of IL-6. Parallel studies demonstrated that in the presence of IL-6 plateau levels of IgA were secreted into the culture supernatants by day 3 (data not shown). These data demonstrate that the IL-6 enhancement of IgA synthesis measured in these studies is a rapid event and do not indicate that proliferation is involved. Rather, the results suggest that the PP contain a significant fraction of IL-6-sensitive activated mIgA$^+$ B cells that are poised to terminally differentiate to IgA secretion in the presence of this regulatory lymphokine.

TABLE I

IL-6 Enhances the Frequency of IgA-producing Cells in PP B Cell Cultures

	Number of IgA SFC/10^6 B Cells			
	Day 1		Day 3	
Exp.	mrIL-6	None	mrIL-6	None
1	9,440 ± 640	1.160 ± 120	9,760 ± 800	740 ± 120
2	ND	ND	6,560 ± 660	280 ± 34
3	11,920 ± 720	1,400 ± 80	16,001 ± 640	1,180 ± 120

Panning purified PP B cells (10^6 cells/culture) were incubated for 1 or 3 d with mrIL-6 at a final concentration of 50 U/ml. Control cultures were incubated in culture medium alone. At the end of culture, cell viability was determined by trypan blue dye exclusion and IgA spot-forming cells (SFC) responses were examined by ELISPOT assay (see Materials and Methods).

Discussion

The PP are major IgA inductive sites in higher mammals (26); however, we only partially understand the mechanisms involved in regulation of this response. Past studies have shown that isolated T cells from PP can selectively regulate IgA synthesis in vitro (49). Other studies using cloned T cells from this tissue have shown that two broad regulatory mechanisms are operative (50–53). First, cloned T cells from mice (50) and hybridoma cell lines from human appendix (51) could apparently induce B cells to switch from mIgM to mIgA expression. Second, more classical Th cell clones were also derived from PP that preferentially supported IgA responses (52, 53). Taken together, these studies would suggest that PP T cell subsets occur that can produce switch factors, while possibly others may produce cytokines for regulation of IgA synthesis. In this latter respect, it is likely that a unique profile of cytokines will be of importance in the IgA response.

We have taken advantage of two major aspects of IgA inductive sites, e.g., the presence of endogenously triggered B cells that are in cell cycle and an increased frequency of B cells which have committed to IgA. This has allowed us to avoid the use of polyclonal B cell activators such as LPS and PWM, which can also induce certain isotype response patterns and thus possibly skew results and their interpretations. Our past studies and those of others have shown that mrIL-5-enhanced IgA synthesis in PP B cell cultures (36, 54, 55). The use of LPS in these PP B cell cultures yielded somewhat discrepant results. In one study, it was shown that rIL-5 induced IgA synthesis in LPS-triggered $mIgA^+$ but not $mIgA^-$ B cells (54). However, the second study suggested that IL-5 enhanced IgA synthesis in LPS-stimulated, $mIgA^-$ PP B cells (55). We addressed the effects of mrIL-5 by addition to nonstimulated cultures, and showed that this interleukin significantly enhanced IgA synthesis (36). Thus, the PP B cell system offers the advantage of provision of B cells that are cycling and that express receptors for cytokines that regulate the events that lead to production of this isotype.

We have examined in this study all of the currently available interleukins (except IL-3), as well as murine IFN-γ, TNF-α, and TGF-β for potential effects on murine PP B cells. Our results clearly show that rIL-5 and, especially, rIL-6 induce significant increases in IgA synthesis. This does not prove that the other cytokines do not contribute to B cell activation or proliferative responses; however, it does suggest that these other cytokines alone are not involved in terminal events that lead to the production of IgA. It would also suggest that the effect seen with rIL-6 was not due to contamination with one of the other cytokines tested. Since the effect of IL-6 (including both human and mouse rIL-6) is so pronounced for enhancement of IgA synthesis in PP B cell cultures, we have extensively studied this effect..

It has been shown that resting B cells do not express IL-6R (15), and others have shown that human tonsillar B cells, which were refractory to IL-6, could be induced to respond after stimulation with PWM (56). Our studies were directed towards determining whether activated B cells would also be responsive to IL-6. Thus, PP B cells were separated on Percoll gradients into activated (blast, low density) and resting (small, high density) B cell populations. Generally, IL-6 induced two- to threefold higher IgA responses in the low density B cell population than occurred in the high density population; however, IL-6 did induce small B cells to respond and to ultimately secrete IgA in vitro. Separation of B cells on density gradients does not result

in complete separation of activated and resting B cells. The receptor on human B cells has been fully characterized (15) and cloned (16); however, little is known regarding the IL-6R on murine B cells. We are therefore performing studies to identify IL-6R on murine PP B cell subpopulations.

It is clearly possible that IL-6 could induce PP B cells to undergo an isotype switch to IgA and this possibility has been extensively investigated. First, we have previously shown that both high and low density PP B cell populations contain approximately equal numbers of $mIgA^+$ B cells (36). Thus, experiments were undertaken with PP B cells that were depleted of $mIgA^+$ B cells by flow cytometry. When $mIgA^-$ B cells were cultured with mrIL-6, no increase in IgA secretion was observed. However, when cultures containing enriched numbers of $mIgA^+$ PP B cells were examined, optimal levels of mrIL-6 induced up to 13 μg/ml of IgA in B cell cultures containing $\sim 5 \times 10^4$ $mIgA^+$ B cells. In other studies, we have shown that depletion of $mIgA^+$ B cells from both low and high density B cell cultures abrogated the IL-6 effect. Taken together, these results indicate that the major target of IL-6 in the PP B cell population is a cell that has already committed to IgA.

Since mrIL-6 induced some IgA synthesis in small B lymphocyte populations, it was important to determine if this interleukin was inducing both proliferative and differentiative signals. Of course, most studies have indicated that IL-6 acts on dividing B cells that express IL-6R and promotes terminal differentiation to Ig secretion (1). Our studies support this mode of IL-6 effect. When PP B cell cultures were supplemented with mrIL-6, it was shown that between 24 and 72 h of incubation, the cells did not undergo significant rounds of division. No increase in [^{3}H]thymidine incorporation was detected in IL-6-supplemented cultures compared with control cultures at 24, 48, or 72 h (data not shown). However, the numbers of B cells secreting IgA dramatically increased in cultures assessed at 1 and 3 d in that there was an ~8-fold increase on day 1 and a 15–18-fold increase by day 3 of culture. These results clearly indicate that mrIL-6 induces PP B cells to terminally differentiate into IgA-producing cells. It would also appear that most IL-6-responsive cells express mIgA and these cells are subsequently stimulated to produce IgA.

We were aware of the possibility that the mrIL-6 preparation could contain a small amount of another cytokine, which would account for the observed effect. One possibility would be TGF-β, which is known to be produced by COS-7 cells and has a similar molecular mass to IL-6 (25 kD for TGF-β and 26 kD for IL-6). We directly addressed this point by testing several relevant concentrations of TGF-β (8 pg to 1 ng) in PP B cell cultures. We were unable to observe any changes in levels of IgA (IgM or IgG) secreted. More direct evidence for an IL-6 effect was provided by studies with hrIL-6 and anti-IL-6. In this case, we obtained an identical dose-dependent increase in IgA synthesis with human IL-6 and this effect was blocked specifically by the anti-IL-6 antibody. We thus conclude that the dramatic effects of both mouse and human IL-6 on murine IgA synthesis are directly due to these interleukins and not to contaminating proteins.

The results presented thus far suggest that IL-6 plays an important role in the terminal stages of mucosal immune responses. We have preliminary data suggesting that T cells from murine PP secrete IL-6 and these studies are ongoing. Furthermore, IL-6 is known to be produced by a wide variety of cells including T cells (57), macrophages (58, 59), and fibroblasts (3). Others have recently shown that IL-6 mRNA

is transcribed at high levels in human spleen, liver, kidney, and peripheral blood leukocytes (60). Receptors for IL-6 have also been found on resting T cells (16), and IL-6 has been shown to affect the development of hemopoietic stem cells and thymocytes. These studies indicate that this lymphokine plays an important role in the development of many cell types, including B cells. Because of the pleiotropic nature of IL-6 and its widespread production by different tissues, it is likely that events that drive PP B cells toward the IgA isotype occur before these cells become responsive to IL-6, and that the major function of IL-6 in the PP is to induce the terminal differentiation of these cells into IgA-secreting plasma cells.

Summary

Freshly isolated murine PP B cells were cultured with 10 different cytokines, including IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IFN-γ, TNF-α, and TGF-β, to investigate a possible role for these cytokines in induction of Ig synthesis. Of interest was the finding that only IL-5 and both mouse recombinant (mr) and human recombinant (hr) IL-6 enhanced IgA synthesis. The effect was greater with either mrIL-6 or hrIL-6 than with mrIL-5. IL-6 induced cycling mIgA$^+$ PP B cells to secrete high levels of IgA ($\sim$7-fold increase over control). Of importance was the finding that mrIL-6 had little effect on secretion of IgM or IgG by PP B cell cultures. hrIL-6 also increased IgA secretion by PP B cells and this enhancement was abolished by a goat anti-hrIL-6 antiserum. mrIL-6 did not cause B cell proliferation but induced a sharp increase in numbers of B cells secreting IgA. Isotype-switching was not a mechanism for this marked increase in IgA synthesis since mIgA$^-$ PP B cells were not induced to secrete IgA by mrIL-6. From these studies we conclude that IL-6 plays an important role in promoting the terminal differentiation of PP B cells to IgA-secreting plasma cells.

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