

β TRANSFORMING GROWTH FACTORS ARE POTENTIAL REGULATORS OF B LYMPHOPOIESIS

BY GRACE LEE,* LARRY R. ELLINGSWORTH,[‡] STEVEN GILLIS,[§]
RANDOLPH WALL,^{||} AND PAUL W. KINCADE*

*From the *Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; the [‡]Connective Tissue Research Laboratories, Collagen Corp., Palo Alto, California 94303; the [§]Immunex Corp., Seattle, Washington 98101; and the ^{||}Molecular Biology Institute, University of California, Los Angeles, Center for Health Sciences, Los Angeles, California 90024*

B lymphocytes and other blood cells are formed within bone marrow under the influence of regulatory glycoproteins that may exist in soluble or insolubilized forms (1–3). Thus far, a number of factors have been found to augment the formation and maturation of B lineage precursors in culture. These include (IL-1, IFN- γ , substances isolated from the serum of young autoimmune mice, factors associated with cyclic neutropenia, one or more factors made by narrow stromal cells, IL-4, and TNF (1, 4, and Lee, G., unpublished observations). A pattern is emerging whereby these substances selectively stimulate particular maturation events and stages in B cell differentiation (1). However, little is known of antagonists which might function to limit the progression of these events.

Transforming growth factor β (TGF- β)¹ was originally described by its ability to confer anchorage independent growth on nonmalignant fibroblasts (5, 6), and is synthesized and secreted by a variety of cells (7–11). It has been found to have stimulatory or inhibitory activities depending on target cell type and presence of other growth factors (5, 8, 12). Recent reports describe several functionally and structurally closely related forms of TGF- β (13–16), and the identities of TGF- β 1 (15) to cartilage inducing factor A (CIF-A) (16) and of TGF- β 2 (15) to cartilage inducing factor B (CIF-B) (14). Receptors for TGF- β are ubiquitous (7–11, 17), and exist in three distinct forms that differentially bind TGF- β -related molecules (15). Known effects on the immune system include inhibition of lymphocyte proliferation, antibody secretion, and NK cell function (10, 11, 17). We now report that TGF- β 1/CIF-A and TGF- β 2/CIF-B are potent inhibitors of the transition of pre-B cells to mature, functional B cells.

Materials and Methods

Cells. The murine pre-B cell line, 70Z/3, and the IgM κ ⁺ B cell lines, WEHI 231 and WEHI 279, were maintained in passage in RPMI 1640 supplemented with 10% FCS, 2-ME, sodium pyruvate, sodium bicarbonate, amino acids, and antibiotics. Single cell

This work was supported by National Institutes of Health grants AI-20069 and CA-12800. Address correspondence to G. Lee, Oklahoma Medical Research Foundation, 825 N.E. 13th St., Oklahoma City, OK 73104.

¹*Abbreviations used in this paper:* CIF, cartilage inducing factor; NZB-F, NZB mouse serum-derived factors; TGF- β , transforming growth factor β .

suspensions of adult B₆D₂F₁ (The Jackson Laboratory, Bar Harbor, ME) spleen or bone marrow were made as described (18). B cells were enriched from the spleens of adult mice by depletion of adherent cells on Sephadex G-10 columns (18), followed by passage through nylon wool (19). B cells retained in the column were shaken loose from the nylon wool and found to be 92% surface κ light chain-positive. Bone marrow B cell precursors were enriched by depletion of B cells on anti-Ig-coated plates (20) and of adherent cells on a G-10 column as above. B cell-depleted preparations contained <1% κ^+ cells. For some experiments, highly enriched B cell precursors were isolated by positive selection on anti-Ly-5(220) (14.8) coated plates after removal of surface Ig⁺ (sIg⁺) cells (20).

Assays. Bone marrow or spleen cells were assayed for clonable B cells in semi-solid agar medium containing 25 μ g/ml LPS as described (18). 70Z/3 and bone marrow cells were analyzed for surface κ or class II molecules before and after incubation with soluble mediators. Cells were stained for 20 min with FITC-anti- κ (Southern Biotechnology Associates, Inc., Birmingham, AL) or unlabeled anti-Ia^d (Becton Dickinson & Co., Mountain View, CA) followed by FITC-anti-IgG2a (Pandex Laboratories, Inc., Mundelein, IL) for an additional 20 min after two washes. Propidium iodide (P-5264; Sigma Chemical Co., St. Louis, MO) was added during the last 5 min of labeling to detect dead cells. Labeled cells were analyzed on an EPICS V (Coulter Electronics Inc., Hialeah, FL) using Omega 590 SP, BP 527, and BP 630 filters. Dead cells and erythrocytes were gated out on the basis of propidium iodide uptake and forward angle light scatter. The presence of TGF- β 1 was detected in fixed cells using an anti-TGF- β 1 antiserum (21) with an immunoperoxidase kit (Zymed Laboratories, San Francisco, CA), or on viable cells by immunofluorescence analysis with FITC-goat anti-rabbit Ig (F-0511; Sigma Chemical Co.) as second antibody. This antiserum does not crossreact with TGF- β 2. Cytoplasmic μ^+ pre-B cells were enumerated as described (20). The phenotypic and functional maturation of B lineage progenitors in short-term culture was monitored as in our previous reports (18).

RNA Analysis. Total RNA was prepared from treated cells, slot-blotted, and probed for κ and μ chain RNA as described (22). Relative amounts of RNA bands were determined by densitometric readings.

Soluble Mediators. Bovine TGF- β 1 (CIF-A) and TGF- β 2 (CIF-B) (13), factors from the serum of young NZB mice (NZB-F) (23), and recombinant IL-4 (formerly called BSF-1) (24) were prepared as described. An additional preparation of TGF- β 1, purified from human platelets, and human IL-1 were purchased from R&D Systems, Inc. (Minneapolis, MN) and Genzyme, Inc. (Boston, MA), respectively. LPS, derived from *Staphylococcus typhosa* 0901, was purchased from Difco Laboratories, Inc., Detroit, MI. Recombinant mouse IFN- γ was obtained courtesy of Dr. H. M. Shepard (Genentech, San Francisco, CA).

Results

Inhibitory Effects of TGF- β on Cloned Pre-B Cells. The pre-B lymphoma cell line, 70Z/3, produces μ heavy chains, has one rearranged κ light chain allele, and can be stimulated to express κ by LPS (25–28). A small percentage, 1–7%, of 70Z/3 cells maintained in culture medium have detectable surface κ , and typically, incubation with LPS for 48 h resulted in an increase of κ^+ cells to 77–90% as determined by channel-by-channel subtraction of cells nonspecifically stained by FITC-goat-Ig from cells labeled with FITC-goat anti- κ . This induction of κ acquisition by LPS was identically inhibited by TGF- β 1 and TGF- β 2 in a dose-dependent fashion (Fig. 1). Viability of cells in all cultures after 48 h incubation was >90%. Cultures containing LPS alone showed some growth inhibition and cell recoveries, determined by counting cells with trypan blue under a light microscope, were 78–89% of medium controls. Little effect on cell recoveries of 70Z/3 was noted in cultures containing TGF- β 1 or TGF- β 2 alone (91–105% of medium controls), or together with LPS (96–112% of LPS con-

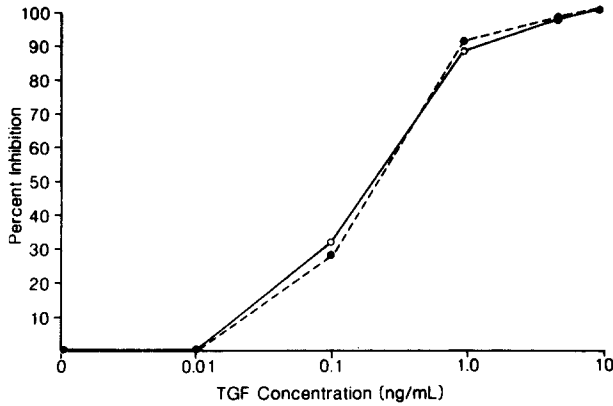


FIGURE 1. Inhibition of LPS induction of cell surface κ expression. 70Z/3 cells were cultured for 48 h at 0.1×10^6 cells/ml with LPS ($10 \mu\text{g/ml}$) and various concentrations of TGF- β 1 (○) or TGF- β 2 (●). Cells were then labeled with FITC-goat anti- κ or FITC-goat Ig (background control) and analyzed by flow cytometry. After subtracting background staining, 81% of LPS-induced cells and 4% of untreated cells were κ^+ . Percent inhibition = [(percent κ^+ with LPS) - (percent κ^+ with LPS + TGF) - (percent κ^+ with medium)] / [(percent κ^+ with LPS) - (percent κ^+ with medium)].

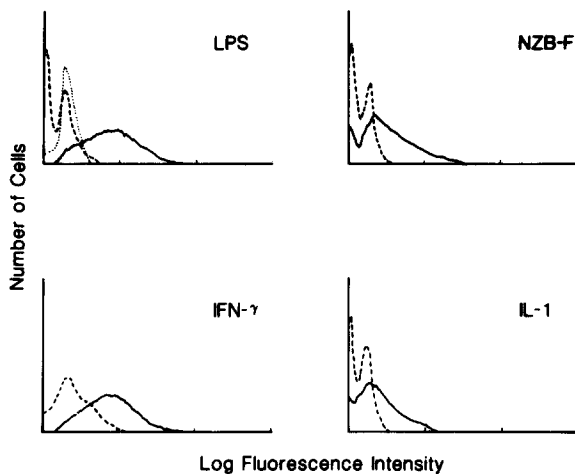


FIGURE 2. Inhibition of induction of LPS, NZB-F, IFN- γ , and IL-1 of cell surface κ . 70Z/3 cells were analyzed for κ expression after culturing as described in Fig. 1 with no additive (....); with LPS ($10 \mu\text{g/ml}$), NZB-F (100 U/ml), IFN- γ (50 U/ml), or IL-1 (25 U/ml) alone (—), or together with TGF- β 1 (10 ng/ml) (---). Similar results were obtained with TGF- β 2 and human platelet-derived TGF- β 1 (not shown).

controls). There was no consistent correlation between cell recovery and the concentrations of TGF- β used.

Light chain expression in response to three other stimuli, IFN- γ , IL-1, and young NZB mouse serum-derived factors (NZB-F), was also inhibitable by TGF- β (Fig. 2). However, while κ induction by IL-1, NZB-F, or LPS was completely abolished, that elicited by IFN- γ was not as sensitive to inhibition by either form of TGF- β . This was also the case when higher concentrations of inhibitors were combined with lower doses of IFN- γ (data not shown).

Like LPS, IFN- γ did not affect cell viability but was somewhat inhibitory to cell growth and in several different experiments, cell recoveries after culture were 80–88% of medium controls. Cell recoveries for TGF- β 1 or - β 2 plus IFN- γ cultures were similar to those for cultures containing only IFN- γ (92–98% of IFN- γ controls). IL-1 and NZB serum factors alone or in combination with TGF- β did not affect cell growth or viability.

κ RNA Levels in TGF- β -treated Cells. Hybridization with a κ gene probe to slot blots of total RNA isolated from 70Z/3 cells suggests that inhibition of κ

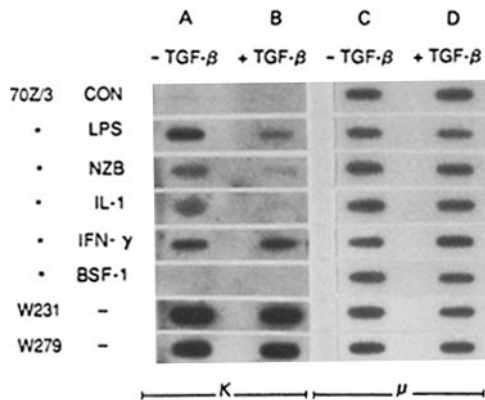


FIGURE 3. Inhibition of κ RNA levels. 70Z/3 cells were incubated overnight with medium alone (control), LPS (10 $\mu\text{g}/\text{ml}$), NZB-F (100 U/ml), IL-1 (25 U/ml), IFN- γ (50 U/ml), or IL-4 (labeled as BSF-1 in figure, 400 U/ml) with or without TGF- β 1 (10 ng/ml). W279 and W231 cells were similarly cultured overnight with or without TGF- β 1 (10 ng/ml). Total RNA was extracted, slot-blotted, and probed for κ RNA (A and B) or μ RNA (C and D). TGF- β 2 was also tested and gave identical results (not shown).

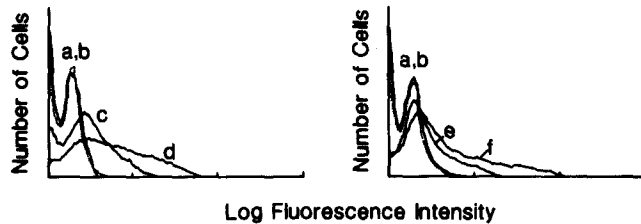


FIGURE 4. Ia induction is not inhibited by TGF β . Class II antigen expression on 70Z/3 cells was detected using anti-Ia mAbs plus FITC-rat anti-mouse IgG2a after 48 h incubation with no additive (a); TGF- β 1 (10 ng/ml) (b); IL-4 (400 U/ml) (c); IL-4 + TGF- β 1 (d); IFN- γ (50 U/ml) (e); or IFN- γ + TGF- β 1 (f). Cells labeled with an irrelevant monoclonal mouse IgG2a antibody plus FITC-labeled second antibody served as background controls. Ia was undetectable on uninduced 70Z/3 cells.

expression by TGF- β may occur by prevention of κ RNA transcription or accumulation (Fig. 3). Induction of κ RNA in response to LPS, IL-1, and NZB-F was inhibited by 82, 91, and 95%, respectively, while that mediated by IFN- γ was decreased by only 21%. Identical results were obtained using either TGF- β 1 or TGF- β 2. In contrast, equivalent amounts of RNA from the same samples hybridized with a μ heavy chain probe showed no effect of TGF- β on levels of μ RNA (Fig. 3).

Enhancement of Class II Antigen Expression. That TGF- β is selectively inhibitory was further demonstrated by its effects on class II MHC molecules (Ia). Some subclones of the 70Z/3 line are responsive to IL-4 (formerly called BSF-1) (29, 30). Consistent with reported observations (30), transcription of class II MHC molecules was markedly increased while Ig gene expression was unaffected (Figs. 3 and 4). Our recently cloned 70Z/3 line was also inducible for Ia by IFN- γ (Fig. 4) but not by IL-1, NZB serum factors, or LPS (data not shown). Acquisition of Ia was not blocked and, in fact, was slightly enhanced by either form of TGF- β (Fig. 4). Elevation of Ia expression by IL-4 on spleen B cells was similarly not inhibited by TGF- β (data not shown).

Inhibition of Bone Marrow B Lineage Cell Maturation. The inhibitory effects of TGF- β were also demonstrable with normal bone marrow cells (Table I). The

TABLE I
TGF- β Inhibits Maturation of Normal Bone Marrow B Cell Precursors

| Cells | Preculture | 48-h Liquid culture | | | |
|--|-----------------|--|-----------------|--------------------|-----------------------------------|
| | B cell colonies | Total cell recovery (percent of control) | B cell colonies | κ^+ B cells | κ^- , $c\mu^+$ Pre-B cells |
| Unfractionated bone marrow | | | | | |
| Control | 953 \pm 20 | | 902 \pm 80 | 21,315 | 5,145 |
| TGF- β 1 | | 98.2 | 630 \pm 10 | 13,725 | 2,928 |
| B cell-depleted bone marrow | | | | | |
| Control | 8 \pm 2 | | 213 \pm 16 | 7,722 | 5,610 |
| TGF- β 1 | | 94.6 | 18 \pm 2 | 3,086 | 2,178 |
| B & adherent cell-depleted bone marrow | | | | | |
| Control | 3 \pm 1 | | 12 \pm 1 | 1,246 | 2,836 |
| TGF- β 1 | | 105.9 | 6 \pm 1 | 1,446 | 980 |
| NZB-F | | 107.7 | 119 \pm 5 | 10,028 | 5,504 |
| NZB-F + TGF- β 1 | | 102.4 | 19 \pm 1 | 1,112 | 1,112 |

Unfractionated, B cell-depleted, and B cell-adherent cell-depleted bone marrow cells from 8-wk-old mice were cultured at 2×10^6 cells/ml for 48 h in medium alone (control), TGF- β 1 (10 ng/ml), NZB-F (100 U/ml), or NZB-F + TGF- β 1 as indicated in the table. Cells were assayed before and after 48-h liquid culture for clonable B cells in semi-solid agar culture with LPS (25 μ g/ml), surface κ , and cytoplasmic μ expression. B cell colonies were scored after 6 d incubation. Pre-B cells were enumerated by surface labeling with FITC-goat anti- κ , followed by cytopinning and fixing of cells on glass slides and labeling with tetramethylrhodamine isothiocyanate-goat anti- μ . Cells that were κ^-/μ^+ were scored as pre-B cells. Data are expressed as colonies, B cells, or pre-B cells per 10^5 initial (preculture) or recovered (after 48-h culture) cells and are representative of similar results from three other experiments using TGF- β 1 and TGF- β 2.

spontaneous emergence in short-term liquid cultures of mitogen responsive, surface κ^+ B cells was stimulated by adherent accessory cells in bone marrow, or by inclusion of NZB serum factors to adherent cell-depleted bone marrow (18, 23). This was strikingly diminished by TGF- β 1 or TGF- β 2 (data not shown for TGF- β 2). In addition, numbers of surface Ig $^-$, cytoplasmic μ^+ cells were significantly reduced, indicating that TGF- β may also inhibit generation of pre-B cells. TGF- β did not significantly affect total cell recoveries after culture as compared with the controls in each group.

Effects of TGF- β on Mature B Cells. As might be anticipated from a recent report (11), TGF- β 1 and TGF- β 2 completely inhibited mitogen-stimulated proliferation of mature B cells when cultured with the B cells for 6 d in the presence of LPS in a semi-solid agar cloning assay (Table II). Short-term (1 or 2 h) exposure of spleen or bone marrow cells to the factors, followed by a wash, had no effect (data not shown). In addition, mature B cells in unseparated marrow suspensions that had been exposed to TGF- β 1 or TGF- β 2 for 48 h and washed still retained functional capability (Table I).

In contrast to the effects on pre-B cells, no reduction in the density of surface Ig or κ RNA was observed after incubating mature splenic B cells or B lymphoma cells with these factors (Figs. 3 and 5). The average increase in κ that follows LPS stimulation was inhibited (Fig. 5).

Presence of TGF- β on B Lineage Cells. TGF- β was easily demonstrable by

TABLE II
Inhibition of Clonable B Cells in Spleen and Bone Marrow

| Inhibitor | Amount used ng/ml | CFU-B/10 ⁵ Cells | |
|-----------|----------------------|-----------------------------|-------------|
| | | Spleen | Bone marrow |
| Control | | 5,570 ± 30 | 895 ± 4 |
| TGF-β1 | 10 | 0 | 0 |
| | 1 | 0 | 6 ± 3 |
| | 0.1 | 394 ± 37 | 0 |
| | 0.01 | 1,320 ± 131 | 100 ± 21 |
| TGF-β2 | 10 | 6 ± 3 | 0 |
| | 1 | 127 ± 32 | 6 ± 3 |
| | 0.1 | 1,443 ± 43 | 257 ± 15 |
| | 0.01 | 3,363 ± 30 | 497 ± 44 |

Single cell suspensions of spleen or bone marrow from 8-wk-old mice were cultured in semi-solid agar medium containing LPS (25 μg/ml) with or without (control) various concentrations of TGF-B1 or TGF-B2. B cell colonies (CFU-B) were enumerated after incubation for 6 d.

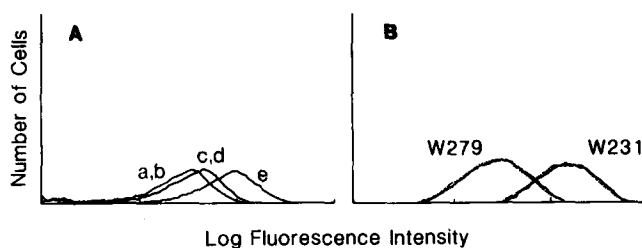


FIGURE 5. Increase of surface κ after stimulation, but not ongoing expression is inhibited in mature B cells. (A) B cells isolated from spleen were incubated for 48 h with LPS (10 μg/ml), TGF-β1 (10 ng/ml), or LPS + TGF-β1. Surface κ was determined before and after culture as described in Fig. 1. Pre-culture cells (a); after 48 h culture with TGF-β1 (b); medium alone (c); LPS + TGF-β1 (d); or LPS (e). (B) κ expression on WEHI 279 or WEHI 231 B lymphoma cells after 48 h culture with (.....) or without (—) TGF-β1 (10 ng/ml). TGF-β2 gave similar results (not shown).

immunofluorescence, using an antibody recognizing the TGF-β1 form, on the surface of 70Z/3, WEHI 231 and WEHI 279 cells, and a majority of spleen and bone marrow nucleated cells. An analysis of adult bone marrow in four separate determinations revealed that 59–71% of nucleated cells were positive and this included populations of B lymphocyte lineage cells. Thus, 70–76% of cells that express Ly-5(220) but not surface Ig (total B lineage precursors) and at least 75% of pre-B cells were positive. TGF-β was also demonstrable by immunoperoxidase staining on lymphocytes and macrophages but not stromal cells in long-term bone marrow cultures established according to the Whitlock-Witte procedure, and TGF-β activity was detectable in these culture supernatants (data not shown).

Discussion

This study demonstrates that members of the TGF-β family are potent inhibitors of an important step during pre-B cell maturation, acquisition of Ig light chains, and prevent emergence of surface κ^+ , mitogen-responsive B lymphocytes after in vitro culture of precursor cells. The factors are also antiproliferative agents for mature B lymphocytes and inhibited the average increase in κ displayed

by splenic B cells that followed LPS stimulation. However, in contrast to the inhibition observed in stimulated pre-B and B cells, no reduction in the density of surface Ig or κ RNA was detected after incubating unstimulated, mature splenic B cells or B lymphoma cells with either TGF- β 1 or TGF- β 2. In addition, levels of μ heavy chain RNA were not reduced in pre-B or B cell lines already constitutively producing μ chains. Therefore, initiation of light chain gene transcription in pre-B cells, and its increase after activation of mature B cells, but not ongoing Ig RNA synthesis and translation, appear to be TGF- β sensitive. Our findings suggest that members of the TGF- β family may selectively influence gene expression at particular stages of B lineage differentiation.

Under conditions that promote the formation of pre-B and B cells in vitro, TGF- β also decreased the numbers of surface κ^- , cytoplasmic μ^+ pre-B cells after short-term culture of bone marrow cells enriched for B lineage precursors. However, no inhibition was observed in levels of μ RNA in pre-B or B cell lines. These observations suggest the possibility that TGF- β might inhibit an earlier stage of B cell differentiation, initiation of heavy chain gene expression. Experiments are planned to determine if TGF- β s have a role in regulation of Ig gene rearrangement, and/or activation of heavy chain genes.

It will be interesting to investigate the mechanism through which TGF- β s prevent the accumulation of κ mRNA in pre-B cells. While destabilization or increased degradation of the message is possible, there are several reasons for suspecting that this is unlikely. There were significant amounts of κ RNA in pre-B cells induced by IFN- γ in the presence of TGF- β , and levels of message in established B cell lines were unchanged by these factors. Thus, a very selective process would have to be invoked, such that message lifespan is dramatically reduced, but only in cells that receive a particular induction stimulus. Changes in chromatin state accompanying κ transcription have been documented with our models (28) and TGF- β could be inhibitory at that level. Similarly, activation of proteins that bind to the κ enhancer (22, 31, 32) might be aborted.

The selective inhibitory activity of TGF- β was further demonstrated by its inability to block class II antigen expression. Neither form of TGF- β induced Ia, but they synergized with IL-4 or IFN- γ to enhance class II expression on 70Z/3 pre-B cells. IL-4 did not induce κ on 70Z/3 cells (Fig. 3) or enhance acquisition of κ by bone marrow B cell precursors (Lee, G., unpublished observations), while LPS, IL-1, and NZB serum factors induced κ but not Ia, and IFN- γ enhanced expression of both molecules on 70Z/3 cells. A recent report (30) indicates that negative regulation of class II expression by maturing pre-B cells may be achieved with prostaglandins. Taken together with our results, this suggests that the acquisition of these two functionally significant B cell surface markers, Ig and Ia, is independently controlled by particular agonists and antagonists. It is also noteworthy that κ induced by immune interferon was more refractory to down-regulation by TGF- β than that induced by other stimuli. Variants of the 70Z/3 cell line have been isolated that are inducible for κ by IFN- γ but not LPS (33). Therefore, activation of the same gene may result from different transmembrane signaling pathways.

An autocrine regulatory function has been proposed for TGF- β (34). Activated B and T cells have increased message and receptors for TGF- β , and the differ-

entiation and/or proliferation of these cells can be inhibited by TGF- β (10, 11). Most of the TGF- β produced by various cell types is in a latent, and presumably precursor form, which becomes functionally potent after acidification (7). Latent TGF- β 1 had no effect on κ or Ia induction in our assays (Lee, G., unpublished observations) and it is not known how the conversion to an active form is achieved in vivo. TGF- β was readily detectable by immunofluorescence on the surface of 70Z/3, WEHI 231, and WEHI 279 cells, a majority of spleen cells, bone marrow cells, and B cell precursors in bone marrow. Interestingly, TGF- β activity was also found in supernatants from long-term bone marrow cultures established according to the Whitlock-Witte procedure (35, and Ellingsworth, L. R., unpublished observations). The material seemed to be most closely associated with macrophages and lymphocytes and it may be significant that pre-B cells in that situation are refractory to maturation-inducing agents (4, 36, and Lee, G., unpublished observations). While we did not distinguish passively acquired from endogenously produced TGF- β , these findings suggest that maturing precursors may auto-regulate their maturation in some circumstances. Certainly, the factors are intimately associated with cells that are potential targets for their action.

Summary

Members of the transforming growth factor β (TGF- β) family of polypeptides were found to be potent in vitro inhibitors of κ light chain expression on normal bone marrow-derived and transformed cloned pre-B cells, and of the maturation of these cells to mitogen responsiveness. The inhibition by TGF- β was selective in that Ia expression was not blocked. Together with the observations that LPS, IL-1, NZB serum factors, IL-4, and IFN- γ preferentially induced either κ or Ia, or both, on a pre-B cell line, these results further suggest that acquisition of Ig and class II molecules is independently controlled by different antagonists as well as agonists. In addition, κ chain induction by IFN- γ does not appear to be as sensitive to TGF- β downregulation as that stimulated by other factors tested, and this raises the possibility that activation of the same gene may result from different transmembrane signaling pathways. In contrast to the inhibitory effects of TGF- β on κ acquisition by pre-B cells and on κ increase after exposure of mature B cells to LPS, as measured by κ RNA levels and/or surface fluorescence, no inhibition was observed on unstimulated spleen B cells or on two cloned B cell lines that constitutively produce κ . Thus, TGF- β may function during specific stages of B cell differentiation by inhibiting initiation of, or increased transcription of Ig genes, and therefore, may be an important negative regulator of B lymphopoiesis. It is the first natural substance found to have this effect.

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