Cellular Immunology **192,** 159–166 (1999)
Article ID cimm.1998.1444, available online at http://www.idealibrary.com on IDEAL®

Neuroendocrine-Induced Synthesis of Bone Marrow-Derived Cytokines with Inflammatory Immunomodulating Properties

Michael Whetsell,* E. Umit Bagriacik,* Gattadahalli S. Seetharamaiah,†
Bellur S. Prabhakar,† and John R. Klein*,[1]

Department of Biological Science and the Mervin Bovaird Center for Studies in Molecular Biology and Biotechnology, University of Tulsa, Tulsa, Oklahoma 74104; and †Department of Microbiology and Immunobiology, University of Illinois College of Medicine, Chicago, Illinois 60612

Received September 8, 1998; accepted December 17, 1998

Although cytokines and other soluble regulators of immunity are known to be involved in hematopoiesis, little is known about the signals that induce the synthesis of those mediators locally. Based on recent studies linking the neuroendocrine hormone thyrotropin [thyroid-stimulating hormone (TSH)] to immune cell function in other tissues, we investigated the capacity of TSH to activate cytokine responses from bone marrow cells. These studies reveal that stimulation of the TSH receptor on bone marrow cells—using highly purified or recombinant TSH or by direct stimulation with anti-TSH receptor antibodies—rapidly induces the synthesis of cytokines from bone marrow cells that are classically used in the regulation of inflammatory responses. Of 13 cytokines screened for activity by ELISA or by RNase protection assays for gene expression, IL-6, IFN-β, TNFα, TNFβ, TGFβ2, and lymphotoxin-β responses were reproducibly induced by TSH within 2–3 h of stimulation. Intracellularly, TSH stimulation of bone marrow cells caused rapid increases in cAMP levels and induced the phosphorylation of the Jak2 protein kinase, thereby defining a novel G-protein-coupled receptor/cytokine synthesis pathway. These findings demonstrate that TSH can serve as a primary inductive signal of cytokine production by bone marrow cells.

INTRODUCTION

Understanding of the events that influence and control the inflammatory response is essential for elucidating mechanisms of immunity at many levels, including host responses to infection, immune senescence during aging, and diseases of the lymphohematopoietic system. Moreover, because the bone marrow in mammals is the source of all immune cell lineages, factors that influence bone marrow hematopoiesis can have profound effects on the overall expression of immunity within the organism. Although cytokines such as IL-6, members of the TNF family, and others have been shown to participate in the regulation of hematopoiesis (1–5), remarkably little is known about the inductive signals that activate bone marrow cytokine responses. While it is possible that foreign antigens or molecules with mitogenic activity such as bacterial polysaccharides function as cytokine activational events, it is highly unlikely that such stimuli find their way into the bone marrow during the natural course of events or that the organism would rely on these mechanisms as inductive signals for immune modulation of hematopoiesis. Similarly, although it is possible that some cells of the immune system may function as "sentinels" during the course of cell trafficking, at present there are no good candidates for this. Therefore, other approaches employing established information systems within the animal may come into play. In that vein, signals received by the immune system via hormones, neuropeptides, or neuroendocrines or possibly even by direct neuronal communication (6, 7) may serve as activational signals in the inductive phase of the immune response, although the extent to which this occurs has yet to be fully explored.

Recently, studies from our laboratory demonstrated that hormones of the hypothalamus–pituitary–thyroid axis, driven principally by thyroid-stimulating hormone (TSH),[2] can substantially influence the composition and distribution of lymphoid cells within the intestinal mucosa (8), a site of lymphopoietic activity in mice (9). In subsequent studies we have shown that TSH is produced locally in the intestine, suggesting that regulatory processes controlled by these immune–endocrine interactions can operate within regional

[1] To whom correspondence should be addressed: Department of Biological Science, University Tulsa, 600 S. College Avenue, Tulsa, OK 74104. Fax: (918) 631-5005. E-mail: john-klein@utulsa.edu.

[2] Abbreviations used: IFN, interferon; IL, interleukin; LT, lymphotoxin; MIF, migration inhibition factor; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone (thyrotropin); TGF, transforming growth factor; TNF, tumor necrosis factor.

0008-8749/99 $30.00

sites (10). Based on those empirical observations, we reasoned that TSH might be involved in the regulation of hematopoiesis at the level of the bone marrow by altering cytokine activities known to have immunomodulating properties. In the present report, using a series of cellular, molecular, and biochemical techniques, we demonstrate that murine bone marrow cells express the TSH-R, that ligation of that receptor leads to cytokine gene activation within 2 h of stimulation and to cytokine secretion from bone marrow cells within as early as 3 h of stimulation, and that intracellular signal transduction occurs via a cAMP/tyrosine kinase pathway leading to cytokine synthesis. The significance of these findings in the overall context of immunity is discussed.

MATERIALS AND METHODS

Mice, cell isolation, and flow cytometric analyses. Animals used in this study were C57BL/6, BALB/c, C3H/HeJ, and CB6F1, derived from breeding stocks obtained from The Jackson Laboratories (Bar Harbor, ME). In comparative experiments, no differences were observed in findings based on animal strain. C.RF-*hyt* mice were raised at the University of Tulsa from breeding pairs obtained from The Jackson Laboratories; homozygous C.RF-*hyt* mutant mice were confirmed by PCR analyses of DNA across the TSH receptor mutation region. Bone marrow cells and thymocytes were isolated by dispersing cell tissues in unsupplemented DMEM. Flow cytometric cell sorting was done by direct cell staining using PE-labeled antibody to CD45 (PharMingen, La Jolla, CA); cells were sorted with a Coulter 751 cell sorter (Coulter Electronics, Hialeah, FL) using a flow cytometric cell-sorting software program (Cytomation, Fort Collins, CO).

In vitro assays. Highly purified human TSH that is nonimmunoreactive for follicle-stimulating hormone, luteinizing hormone, growth hormone, and prolactin, obtained courtesy of Dr. A. F. Parlow (NIDDK Pituitary Hormone and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA), and recombinant human TSH (Sigma Chemical Co., St. Louis, MO; Catalog No. T-4533) were used in these experiments. Antisera to the TSH receptor (TSH-R) was made by immunization of rabbits with a recombinant protein corresponding to the extracellular domain of human TSH-R (11). Rabbit antisera to bovine and rat TSHβ were obtained from the Pituitary Hormone and Antisera Center. Procedures for TSH binding assays were done as previously described (10), using 10×10^6 cells in 1 ml of PBS cultured for 1 h at 37°C with 10 μl of 10 μCi/ml purified ^{125}I-labeled TSH (New England Nuclear, Boston, MA; Catalog No. NEX-107). Inhibition of binding was done by incubation of 10 μl of radiolabeled TSH with 10 μl of 1:10 anti-TSHβ antisera for 1 h at 4°C prior to culture with bone marrow cells or by incubation of 10×10^6 bone marrow cells in 1 ml of PBS with 10 μl of neat anti-TSH-R antisera or normal rabbit control sera for 1 h at 4°C prior to culture with radiolabeled TSH.

For cytokine assays, bone marrow cells were cultured at 5×10^5 cells/ml in 1 ml of DMEM supplemented with 2% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin–streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 5 mM Hepes buffer (Sigma, all reagents) at 37°C in a 10% CO_2 environment. ELISA cytokine capture assays for measuring murine cytokine activities were done with commercial assay kits according to the manufacturer's protocol (Genzyme Diagnostics, Cambridge, MA). Enzyme activity was measured at 450 nm with an automated ELISA reader (Fisher Scientific, St. Louis, MO) and was quantified using standards provided with the ELISA assay kit. For cAMP studies, freshly isolated bone marrow cells were cultured at 3×10^6 cells/ml in supplemented DMEM in the absence of TSH, with 10^{-7} M TSH, or with 10^{-7} M forskolin (Sigma Chemical Co.) for 30 min in the presence of 1 mM 3-isobutyl-1-methylxanthine (Sigma), a phosphodiesterase inhibitor. cAMP activity was assayed with a competitive ELISA assay (R&D Systems, Minneapolis, MN) using the manufacturer's reagents and protocols.

RT-PCR analyses and RNase protection assays. Procedures for extraction of RNA, cDNA construction, and RT-PCR analyses were done as described (10). The downstream primer within exon 2 was 5′-CATGTAAGGGTTGTCTGTGATTTC-3′, the upstream primer within exon 2 was 5′-GACTCATCTGAAGACCATACCCAGTCTTGCA-3′, the upstream primer within exon 1 was 5′-GCGTCTCCACCCTGTGAGTGTCACC-3′. RNase protection assays were done using a commercially prepared assay kit (PharMingen). Bone marrow cells were cultured at 3×10^6 cells/ml in 1 ml of supplemented DMEM for 2 h in the absence of TSH or with 10^{-6} to 10^{-9} M TSH. Cells were collected and lysed with Trizol (BRL, Gaithersburg, MD), and RNAs were precipitated with isopropanol. Total RNAs were resuspended in the manufacturer's hybridization buffer, added to [^{32}P]UTP McK-3b riboprobes, and incubated for 3 min at 90°C and overnight at 56°C. RNA hybrids were treated with RNases for 45 min at 30°C followed by treatment with proteinase K for 15 min at 37°C; hybrids were precipitated with ethanol and electrophoresed in a 6% polyacrylamide denaturing gel. Gels were exposed to radiographic film to identify protected probes.

Immunoprecipitation and Western blotting. For TSH-R immunoprecipitation, 25×10^6 freshly isolated bone marrow cells, and 5×10^6 FRTL-5 cells (American Type Culture Collection, Rockville, MD) from an 80% confluent T-25 tissue culture flask, were washed ×2 in PBS. Cells were lysed in detergent buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1

μg/ml of aprotinin, leupeptin, and pepstatin, 1% NP-40, and 0.25% deoxycholate (Sigma, all reagents). Precleared lysates were mixed overnight with 10 μl of monoclonal anti-TSH-R antibody (clone 28) (12) at 4°C, followed by 40 μl of protein A–agarose (Sigma) for 2 h. Precipitates were collected by centrifugation, washed, boiled in 2× reducing SDS sample buffer, and electrophoresed through a 10% polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were transferred electrophoretically to Immuno-Blot PVDF membranes (Bio-Rad), blocked with 3% nonfat dry milk in PBS, and reacted with anti-TSH-R monoclonal antibody (clone 49) (12) overnight at 4°C, followed by the addition of biotinylated anti-mouse immunoglobulin (PharMingen) for 2 h and streptavidin horseradish peroxidase (Amersham; Buckinhamshire, UK) for 30 min. Enhanced chemiluminescence (Amersham) was used for autoradiographic identification of proteins.

For protein kinase analyses, freshly isolated bone marrow cells were collected, washed, and cultured at 25×10^6 cells/ml in 1 ml of unsupplemented DMEM with or without purified or recombinant TSH in a 37°C water bath. Stimulation by anti-TSH-R antibody was done by culturing 25×10^6 bone marrow cells with 100 μl of anti-TSH-R mAb (clone 28) tissue culture supernatant in 900 μl DMEM at 37°C for 10 min. Inhibition of TSH-induced phosphorylation by anti-TSHβ antisera was done by incubating 100 μl of 10^{-7} M human recombinant TSH with 10 μl of 1:10 anti-rat TSHβ antisera for 30 min at 4°C. This was then added to 890 μl DMEM containing 25×10^6 bone marrow cells for 5 min at 37°C. Cells were pelleted in a microfuge, lysed in the above lysis buffer, and reacted with agarose-conjugated anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Precipitates were collected by centrifugation, washed, boiled in 2× reducing SDS sample buffer, and electrophoresed through 7.5% polyacrylamide gels (Bio-Rad). Proteins were transferred to PVDF membranes, blocked, and reacted with rabbit or goat anti-Jak1, anti-Jak2, or anti-Jak3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-labeled anti-rabbit or anti-goat antibody (Santa Cruz Biotechnology). Membranes were developed for chemiluminescence. Stock solutions of RG-490 (tyrphostin B42) (Calbiochem, La Jolla, CA) were prepared at 10^{-1} M concentration in dimethylsulfoxide (Sigma). Bone marrow cells were suspended in 1 ml of medium containing 10^{-5} M RG-490, or control medium, and incubated for 30 min in a 37°C waterbath prior to the addition of TSH for immunoprecipitation/Western blot analyses. For cytokine assays, RG-490 was diluted to 10^{-5} M in supplemented DMEM. Bone marrow cells (5×10^5) were plated in 1 ml of RG-490 or control medium and incubated for 30 min at 37°C in 5% CO_2, after which 10^{-8} M TSH was added to appropriate wells. Plates were incubated for 20 h and supernatants were assayed for cytokine activity. Control medium for both assays contained appropriately diluted DMSO without RG-490.

RESULTS

Bone Marrow Hematopoietic Cells Express the TSH Receptor

Freshly isolated bone marrow hematopoietic cells were purified by fluorescence-activated cell sorting using antibody to CD45 (leukocyte-common antigen), a marker of nucleated hematopoietic cells. This yielded a population of cells with >98% purity (Fig. 1A). RT-PCR analyses of TSH-R gene expression was done using two sets of primers—one of which spanned a region within TSH-R exon 2 for which we have obtained a cloned gene segment for use as a positive control, the other spanned the intron between exons 1 and 2. The latter was used to confirm that the PCR product was derived from cDNA and not from genomic DNA. PCR products were obtained for both amplified regions from bone marrow cDNA (Fig. 1B, lanes 1 and 2). Moreover, the product obtained in lane 2 (Fig. 1B) was identical in size to the positive TSH-R control (Fig. 1B, lane 3) and was confirmed to be derived from the TSH-R gene by sequence analyses (data not shown) as previously reported (10).

Two approaches were used to assay for surface expression of TSH-R on bone marrow cells. First, immunoprecipitation/Western blotting analyses were done using anti-TSH-R monoclonal antibodies. This revealed a 95- to 100-kDa molecular component typical of the mature TSH-R, plus 55- to 65-kDa bands which are frequently also seen (13) in cell lysates from bone marrow cells and the TSH-dependent FRTL-5 thyroid cell line (Fig. 1C). Thymocytes, which do not express detectable levels of TSH-R (Bagriacik and Klein, unpublished results), served as negative controls for these experiments. To confirm the presence of the TSH-R on the surface of bone marrow cells, binding assays were done using highly purified ^{125}I-labeled TSH according to published protocols (10). Shown in Fig. 2A, there was a high level of TSH binding to CD45$^+$ bone marrow cells, though binding was low for murine thymocytes (Fig. 2A). [It should be noted that TSH binding was unaffected by treatment of bone marrow with antibody to CD3 for depletion of mature T cells (data not shown), indicating that TSH-R expression is associated with a resident cell component of the bone marrow.] The specificity of TSH binding to the TSH-R in this system was confirmed by the ability of TSHβ-specific antisera, or antisera to the TSH-R, to block TSH binding to bone marrow cells (Fig. 2A) and by the capacity of unlabeled recombinant human TSH to compete for binding of ^{125}I-labeled TSH in a dose-dependent fashion (Fig. 2B). These findings thus indicate that hematopoietic cells of the bone marrow express the TSH-R gene and that they bear membrane TSH-R capable of binding to TSH.

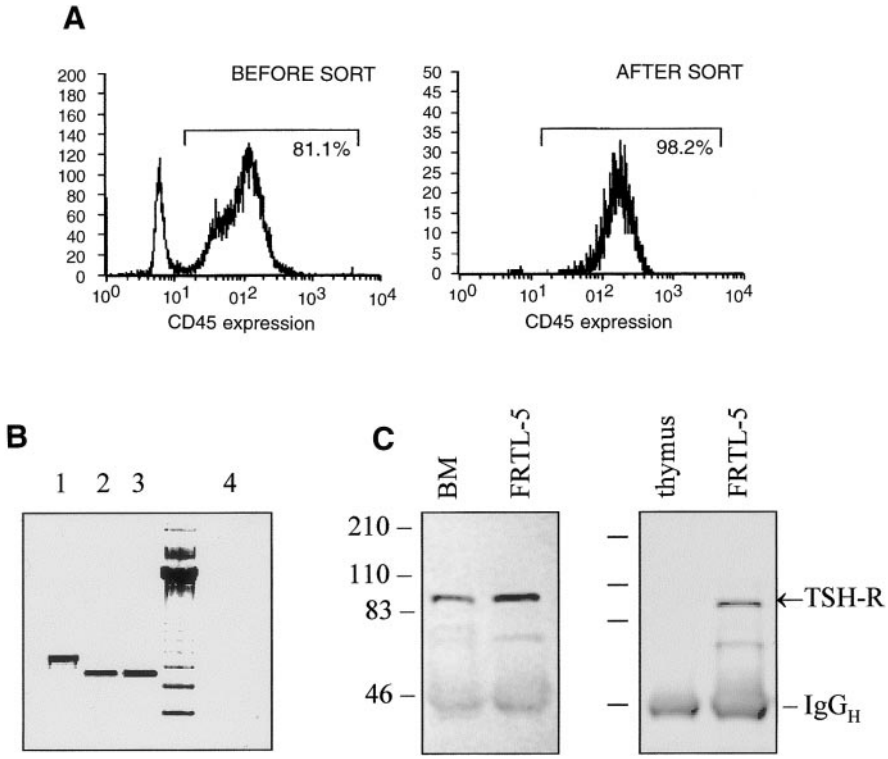

FIG. 1. Bone marrow hematopoietic cells express the receptor for TSH. (A) Enrichment of CD45$^+$ bone marrow cells by flow cytometric cell sorting. (B) RT-PCR analyses for TSH-R gene expression using purified bone marrow cells, demonstrating a PCR product of size predicted for processed RNA across the intron between exons 1 and 2 (lane 1) and within an exon coding region (lane 2) identical in size to a PCR product (lane 3) cloned from TSH-R cDNA with primer sets used in lane 2. Amplification with primers in the absence of cDNA (lane 4). (C) Immunoprecipitation/Western blotting analyses of the TSH-R in cell lysates from bone marrow cells, the FRTL-5 thyroid cell line, and adult thymocytes. Note the presence of the characteristic 95- to 100-kDa form of the mature TSH-R on bone marrow cells and FRTL-5 cells. IgG_H, IgG heavy chain of the anti-TSH-R antibody (clone 28) used for precipitation. Numbers to the left of figure indicate positions of protein size markers (kDa).

TSH Stimulation Leads to Bone Marrow Cytokine Secretion Following Gene Activation

To investigate the functional involvement of TSH on immune-associated activities of the bone marrow, freshly isolated cells were cultured with human TSH, a highly purified reagent with undetectable levels of follicle-stimulating hormone, luteinizing hormone, growth hormone, or prolactin (see Materials and Methods). Cell culture supernatants were collected at 18–24 h and assayed by ELISA for six cytokines (IL-1β, IL-2, IL-6, IL-12, IFNγ, and TNFα) known to be involved in acute or chronic inflammatory responses, hematopoietic cell development, or lymphocyte regulation (14–16). No cytokine activity was detected for IL-1β, IL-2, IL-12, or IFNγ in supernatants from TSH stimulated cultures. However, IL-6 and TNFα were reproducibly present in supernatants from cells stimulated with 10^{-7} to 10^{-9} M TSH (Fig. 3). The ability of TSH to induce cytokine responses was further confirmed for IL-6 in kinetic studies that demonstrated a dose–response effect of TSH on IL-6 secretion (Fig. 4A) and by the fact that cytokine activity was detectable within 3 h of stimulation, with maximal accumulation of IL-6 in *in vitro* cultures occurring at 18 h after stimulation (Fig. 4B). That the induction of cytokine responses were the consequence of engagement of the TSH-R was demonstrated by the ability of TSHβ-specific antisera to block TSH-induced IL-6 secretion, by the ability of antisera to the TSH-R to stimulate IL-6 secretion in the absence of TSH stimulation, by the lack of IL-6 activity from TSH-stimulated bone marrow cells from homozygous C.RF-*hyt* mice that are incapable of TSH-mediated signaling due to a mutation in the TSH-R gene (17); and by the ability of recombinant human TSH also to induce an IL-6 cytokine response from bone marrow cells (Fig. 4C).

To obtain a broader profile of the effects of TSH on the synthesis of other mediators of hematopoietic regulation, particularly those for which bioassays are not readily available, RNase protection assays were done for a panel of 10 cytokines. Bone marrow cells were cultured with or without TSH and were assayed for the presence of cytokine-specific RNAs after 2 h, i.e., just prior to the time when cytokines are detectable in culture supernatants (Fig. 4B). Shown in Fig. 5, transcription was undetectable or minimal for cytokine genes in the absence of TSH (Fig. 5, lane 1), with the exception of some endogenous activity, e.g., migration-

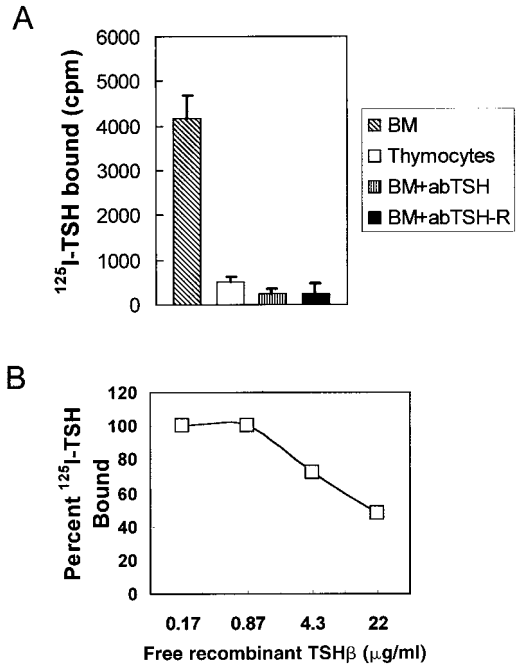

FIG. 2. (A) Binding levels of ^{125}I-labeled TSH to sorted bone marrow cells and TSH-R-negative thymocytes. Inhibition of TSH binding by antiserum to TSHβ incubated with TSH prior to culture with bone marrow cells and by antiserum to the TSH-R incubated with bone marrow cells prior to culture with TSH. Mean values ± range of data from two to five experiments. BM, bone marrow. (B) Unlabeled recombinant TSH inhibits binding of ^{125}I-labeled TSH in a dose-dependent fashion.

inhibition factor (MIF). However, culture of cells with 10^{-6} to 10^{-9} M TSH (Fig. 5, lanes 2–5, respectively) resulted in increases in the transcription of genes for TNFα and β, lymphotoxin (LT)-β, IL-6, IFN-β, and TGFβ2, but had minimal effect or caused no change in levels of transcription of IFNγ, TGFβ1, TGFβ3, or MIF

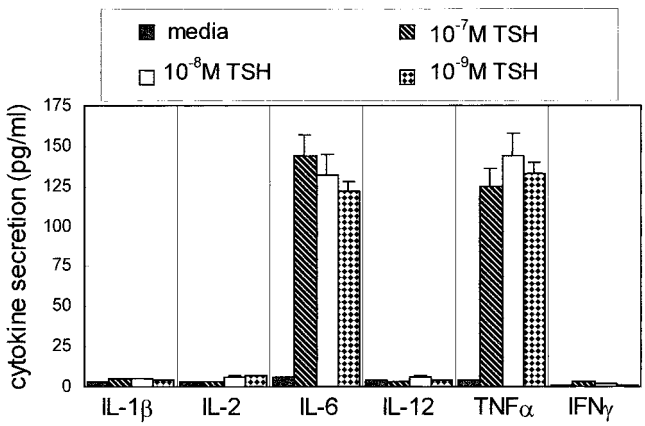

FIG. 3. TSH selectively induces cytokine synthesis from bone marrow stem cells. TSH-induced cytokine activity in bone marrow cell supernatants assayed by ELISA after 18 h of culture with 10^{-7} to 10^{-9} M TSH; mean values ± range of data from two to four experiments.

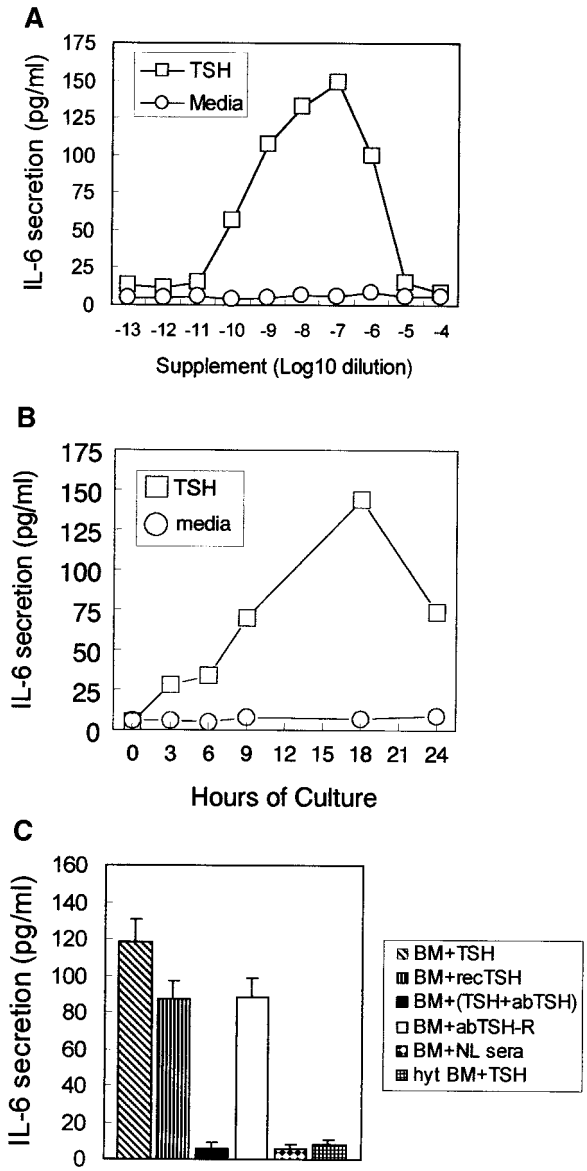

FIG. 4. (A) Dose-dependent TSH induction of IL-6 cytokine activity of bone marrow cells cultured for 18 h with $\log_{10}$ dilutions of TSH, or with supplemented medium in the absence of TSH. (B) Kinetic development of IL-6 cytokine activity of bone marrow cells during a 24-h culture period in the presence of 10^{-8} M TSH or in supplemented medium in the absence of TSH. (C) IL-6 secretion is induced by purified and recombinant human TSH and is blocked by antisera to TSHβ; in the absence of stimulation by TSHβ, IL-6 secretion is induced by rabbit antisera to the TSH-R, but not by normal rabbit sera without TSH; bone marrow cells of homozygous TSH-R mutant C.RF-*hyt* mice fail to produce IL-6 when stimulated with TSH. Mean values ± range of data from two or three experiments. BM, bone marrow.

genes. These findings are of particular interest since they indicate that TSH did not have a mitogenic effect on bone marrow cells since, if that were to occur, this would have led to the activation of all cytokine genes rather than the selective activation of specific cytokine genes as observed.

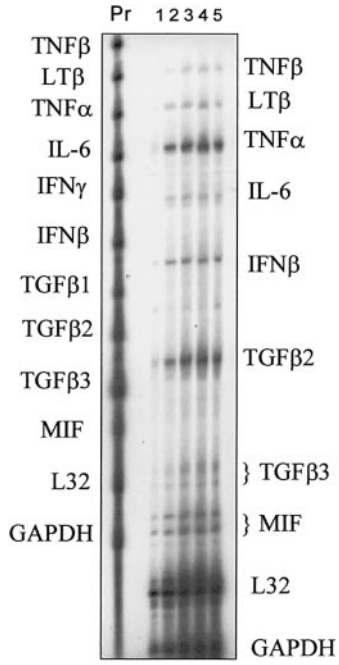

FIG. 5. TSH stimulation leads to selective cytokine gene activation in bone marrow cells. RNase protection assays for 10 cytokine genes in bone marrow cells after 2 h of culture in supplemented medium without TSH (lane 1) or cultured in the presence of 10^{-6} M TSH (lane 2), 10^{-7} M TSH (lane 3), 10^{-8} M TSH (lane 4), or 10^{-9} M TSH (lane 5). Findings similar to these were observed in two additional experiments. L32 and GAPDH controls indicate that equivalent levels of RNAs were analyzed per lane. The locations of protected cytokine probes in lanes 1–5 were determined from the manufacturer's templates.

Intracellular Signaling Following TSH Stimulation Causes Rapid Increases in cAMP and Activation of the Jak2 Protein Kinase

The TSH-R is a G-protein-coupled receptor that when stimulated results in intracellular signaling via cAMP and the activation of various protein kinases. To explore the signaling mechanisms used by bone marrow cells in the course of TSH activation, cells were cultured in the presence or absence of TSH, and cAMP levels were measured at intervals poststimulation using a competitive ELISA assay. Shown in Fig. 6, TSH stimulation of bone marrow cells resulted in rapid increases in intracellular cAMP that were detectable by 10 min of culture with TSH. This activity was approximately one-third to one-half of that induced by forskolin. Those differences may be indicative of variable expression of the TSH-R within the bone marrow versus cAMP production induced by forskolin, a potent activator of cAMP (18).

To assay for protein kinase activity, bone marrow cells were harvested at intervals post-TSH stimulation and solubilized in detergent, and cell lysates were reacted with anti-phosphotyrosine antibody. Precipitated products were electrophoresed, transferred to nitrocellulose, and immunoblotted with antibody to Jak1, Jak2, or Jak3. These were selected because Jak1 has been linked to some cytokine mediated signals (19), Jak2 is expressed in the bone marrow (20) and is utilized by other hormones (21), although a relationship between Jak2 and TSH has not been established; Jak3 is involved in lymphocyte development (22). No differences were observed in Jak1 or Jak3 phosphorylation following TSH stimulation (data not shown). In contrast, phosphorylation of Jak2 occurred in bone marrow cells after 5–15 min of culture with TSH (Fig. 7A). This was a TSH concentration-dependent event with maximal phosphorylation occurring at 10^{-8} M TSH (Fig. 7B). Moreover, preincubation of TSH with anti-TSHβ antisera blocked the ability of recombinant TSH to induce Jak2 phosphorylation, whereas stimulation of bone marrow cells with mAb to the TSH-R resulted in Jak2 phosphorylation similar to that induced by recombinant TSH (Fig. 7C). Finally, incubation of bone marrow cells with tyrphostin (RG-490), a Jak2-specific phosphorylation inhibitor (23, 24), blocked Jak2 phosphorylation (Fig. 7C, top) and reduced IL-6 levels by ~80% (Fig. 8). Note that, similar to reports by others (23), RG-490 had no adverse effects on intracellular Jak2 levels (Fig. 7C, bottom) or on bone marrow cell viability during culture (Fig. 8). These findings, therefore, define a direct functional relationship between TSH stimulation, Jak2 activation, and cytokine secretion by bone marrow cells.

DISCUSSION

The findings reported here suggest that TSH can serve as an inductive signal used in the regulation of immune cytokines from bone marrow cells. Moreover, of particular interest is the fact that the cytokines influenced by TSH are noted as having wide-ranging effects on the immune system involving both positive and negative effects. For example, TNFα is a potent regulator of inflammatory protein synthesis and neutrophil mobilization during acute phase responses (1, 25), and within the bone marrow its effects may be

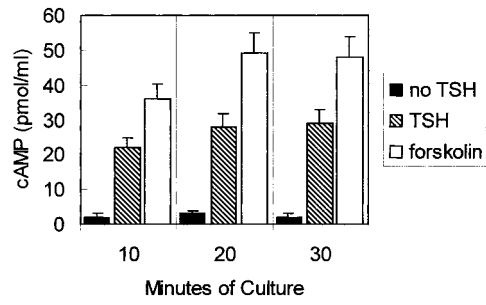

FIG. 6. TSH stimulation of bone marrow cells results in elevated levels of intracellular cAMP. Intracellular cAMP levels from bone marrow cells cultured in supplemented medium in the absence of TSH, with 10^{-7} M TSH, or with 10^{-7} M forskolin, assayed after 10, 20, and 30 min of culture. Mean values ± range of data from three experiments.

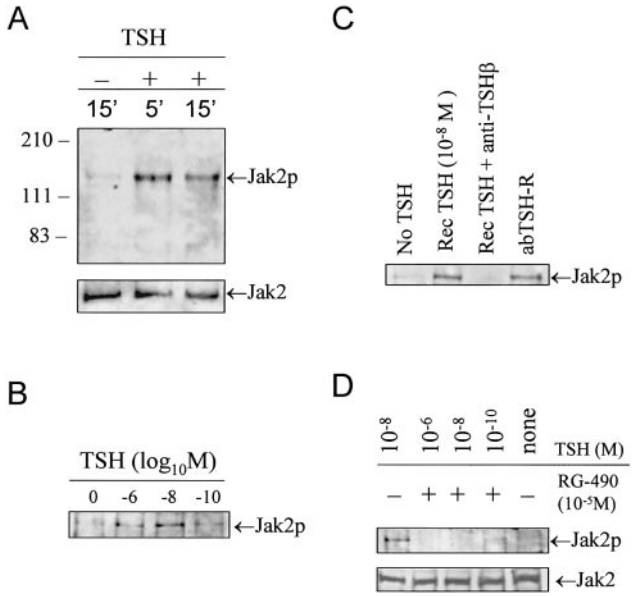

FIG. 7. TSH stimulation of bone marrow cells induces the phosphorylation of Jak2 protein kinase. (A) Anti-phosphotyrosine immunoprecipitation and Jak2 Western blot analyses of bone marrow lysates from cells cultured in the presence or absence of TSH (top) for the times indicated; levels of nonphosphorylated Jak2 were equivalent in cell lysates as demonstrated by direct Western blotting with anti-Jak2 antibody (bottom). Numbers to the left of figure indicate position of protein size markers (kDa). (B) Concentration-dependent phosphorylation of Jak2 in bone marrow cells cultured for 10 min with no TSH or with 10^{-6}, 10^{-8}, or 10^{-10} M TSH. (C) Jak2 phosphorylation occurs following stimulation with 10^{-8} M recombinant TSH, as well as by direct stimulation with anti-TSH-R mAb in the absence of TSH; preincubation of recombinant TSH with anti-TSHβ antisera inhibits Jak2 phosphorylation. (D) Inhibition of TSH-induced Jak2 phosphorylation in bone marrow cells cultured for 30 min with 10^{-5} M tyrphostin (RG-490) prior to 10 min stimulation with or without TSH (top). Anti-Jak2 western blot of cell lysates from bone marrow cells indicates that intracellular Jak2 levels are not affected by RG-490 (bottom). Jak2p, phosphorylated Jak2.

targeted to early hematopoietic cell precursors (26). LTβ and TNFβ moieties have been linked to tumoricidal and proinflammatory responses (27). IL-6 has been shown to have both proinflammatory and anti-inflammatory properties, to suppress TNF responses under appropriate conditions, and to modulate glucocorticoid and other endocrine responses (reviewed in Ref. 28). Similarly, TGFβ2 can influence TNFα activity (29) and may have suppressive effects associated with specific immune responses such as following allograft transplantation (30). IFN-β is noted for its broad anti-inflammatory activities (31, 32). Those cytokine properties collectively suggest that the immune–endocrine interactions described here are part of a complex system of hematopoietic regulation within the bone marrow. Moreover, given that cytokine-mediated control of immunity is a balance between synergistic and antagonistic activities, the full extent of which has yet to be completely understood, it is reasonable to assume that the cohort of TSH-mediated cytokines identified in the present study may likewise achieve their intended results through a series of positive and negative cytokine signals that are selectively targeted to specific bone marrow subsets.

The direct functional link between the immune system and the endocrine system delineated here raises a variety of new questions regarding the collaborative cellular and physiological interactions of those systems in the maintenance of immunity at the organismic level. For example, a long-standing unresolved issue pertaining to the process of immune regulation has to do with the mechanism(s) by which information is transferred to the bone marrow upon immunological challenge in distant locations, so as to bring about the necessary shift in hematopoiesis needed by the host. Some insight into this may be found in studies which indicate that immunologically relevant signals can be delivered via the autonomic nervous system, with its elaborate network of sympathetic noradrenergic nerves that are disseminated into and throughout essentially all primary and secondary lymphoid tissues including the bone marrow (6, 33)—a concept that is continuing to gain support (34). Thus, it is possible that activational signals acquired in that manner might induce the local release of neuroendocrines, such as TSH, that function in a paracrine manner on TSH-R^+ bone marrow cells. Recent findings of *in situ* TSH production in the murine small intestine (10), plus evidence for bone marrow-derived synthesis of other hormones (35, 36), raise the question of whether similar networks also exist elsewhere within the organism. Although the nature of the TSH responsive cell(s) in the bone marrow has not been determined and will require considerably more work to evaluate, the extreme heterogeneity of the bone marrow compartment makes it unlikely that the TSH-R is expressed on all bone marrow cells, but rather that it is restricted to one or a few bone marrow subpopulations. Likewise, it will be of interest to determine the extent to which other hormones associated with the hypothalamus–pituit-

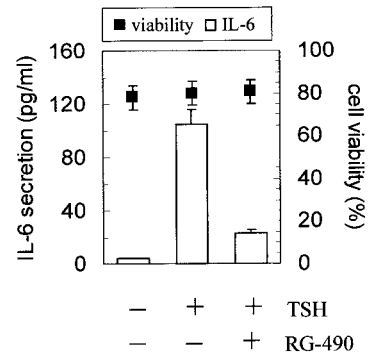

FIG. 8. IL-6 activity (□) and cell viability (■) of bone marrow cells cultured for 20 h in the presence or absence of 10^{-5} M RG-490 with or without 10^{-8} M TSH. Cell viability was determined by dye exclusion for bone marrow cells after culture. Mean values ± range of data from three experiments.

ary–thyroid axis, e.g., thyrotropin-releasing hormone (TRH), are produced by hematopoietic cells of the bone marrow, or possibly by bone marrow stromal cells, and thus may serve as functional inductive signals within a local TSH-mediated immune-endocrine cascade. In that context, the TRH neuropeptide and its precursors have been shown to be made by cells of the immune system, as well as by a variety of other nonneural cells (37).

Finally, two additional important findings emerge from these studies. The first is that cytokine activities can be induced by triggering membrane molecules not generally associated with immune function, in this case the TSH-R, thereby prompting the question of whether TSH also serves as a functional signal to elicit cytokine responses from TSH-R^+ hematopoietic cells in immune compartments outside the bone marrow. In that context also, a second novel finding pertains to the involvement of the Jak2 kinase in TSH-mediated signaling by bone marrow cells. Although Jak2 is used in cell signaling by hormones such as prolactin (38) and growth hormone (39), both of which employ enzyme-linked cytokine-like receptors, and by angiotensin (40), a G-protein-coupled receptor, the present study is the first to link Jak2 to cytokine synthesis following stimulation of a G-protein receptor. Further analyses of these systems may prove useful for understanding the mechanisms by which immune–endocrine interactions converge in the regulation of hematopoiesis and immunity.

ACKNOWLEDGMENTS

The authors thank Drs. Judith A. Kapp and Kenton S. Miller for comments and critical review of the manuscript. This work was supported by NIH Grant DK35566 and by Grant H97-011 from the Oklahoma Center for the Advancement of Science and Technology (OCAST).

REFERENCES

1. Akira, S., Taga, T., and Kishimoto, T., *Adv. Immunol.* **54,** 1, 1993.
2. Hausser, S. P., Kajkenova, O., and Lipschitz, D. A., *Stem Cells* **15,** 125, 1997.
3. Ogawa, M., Yonemura, Y., and Ku, H., *Stem Cells* **15,** 7, 1997.
4. Karkanistsa, L. V., Komarovskaya, M. E., and Krivenko, S. I., *Stem Cells* **15,** 95, 1997.
5. Johnson, R. W., Arkins, S., Dantzer, R., and Kelley, K. W., *Comp. Biochem. Physiol.* **116,** 83, 1997.
6. Felten, D. L., *Neuropsychobiology* **28,** 110, 1993.
7. Maestroni, G. J., *Histol. Histopathol.* **13,** 271, 1998.
8. Wang, J., and Klein, J. R., *Science* **265,** 1860, 1994.
9. Kiyono, H., and McGhee, J. R., (Eds). Mucosal immunology: Intraepithelial lymphocytes. *Adv. Host Def. Mech.* **9,** 1, 1994.
10. Wang, J., Whetsell, M., and Klein, J. R., *Science* **275,** 1937, 1997.
11. Seetharamaiah, G. S., Kurosky, A. K., Desai, R. K., Dallas, J. S., and Prabhakar, B. S., *Endocrinology* **134,** 549, 1994.
12. Seetharamaiah, G. S., Wagle, N. M., Morris, J. C., and Prabhakar, B. S., *Endocrinology* **136,** 2817, 1995.
13. Harfst, E., Ross, M. S., Nussey, S. S., and Johnstone, A. P., *Mol. Cell. Endocrinol.* **102,** 77, 1994.
14. Mohr, A. M., Upperman, J. S., Taneja, R., Wang, M. T., Rameshwar, P., and Livingston, D. H., *Shock* **7,** 324, 1997.
15. Garbe, A., Spyridonidis, A., Mobest, D., Schmoor, C., Mertelsman, R., and Henschler, R., *Brit. J. Haemotol.* **99,** 951, 1997.
16. Scott, P., and Trinchieri, G., *Semin. Immunol.* **9,** 285, 1997.
17. Biesiada, E., Adams, P. M., Shanklin, D. R., Bloom, G. S., and Stein, S. A., *Adv. Neuroimmunol.* **6,** 309, 1996.
18. Miller, M. J., Prigent, S., Kupperman, E., *et al., J. Biol. Chem.* **272,** 5600, 1997.
19. Keegan, A. D., Johnston, J. A., Tortolani, P. J., *et al., Proc. Natl. Acad. Sci. USA* **92,** 7681, 1995.
20. Yoshimura, A., Ichihara, M., Kinjyo, I., *et al., EMBO* **15,** 1055, 1996.
21. Pezet, A., Ferrag, A., Kelley, P. A., and Edery, M., *J. Biol. Chem.* **272,** 25043, 1997.
22. Thomis, D. C., Gurniak, C. B., Tivol, E., Sharpe, A. H., and Berg, L. J., *Science* **270,** 794, 1995.
23. Meydan, N., Grunberger, T., Dadi, H., *et al., Nature* **379,** 645, 1996.
24. Hiraguri, M., Miike, S., Sano, H., Kurasawa, K., Saito, Y., and Iwamoto, I., *J. Allerg. Clin. Immunol.* **100,** S45, 1997.
25. Schrier, D. J., Schimmer, R. C., Flory, C. M., Tung, D. K., and Ward, P. A., *J. Leukocyte Biol.* **63,** 359, 1998.
26. Snoeck, H. W., Weekx, S., Moulijn, A., *et al., J. Exp. Med.* **183,** 705, 1996.
27. Hochman, P. S., Majeau, G. R., Mackay, F., and Browning, J. L., *J. Inflamm.* **46,** 220, 1995.
28. Tilg, H., Dinarello, C. A., and Weir, J. W., *Immunol. Today* **18,** 428, 1997.
29. Pfeilschifter J., Pignat, W., Leighton, J., Marki, F., Vosbeck, K., and Alkan, S., *Biochem. J.* **270,** 269, 1990.
30. Thompson, D. A., Spies, A., Stephan, R. N., Brooks, S. P., Grande, C. C., and Tomasi, T. B., *Transplan. Proc.* **28,** 1948, 1996.
31. Coclet-Ninin, J., *Eur. Cytokine Netw.* **8,** 345, 1997.
32. Lin, Q., Dong, C., and Cooper, M. D., *J. Exp. Med.* **187,** 79, 1998.
33. Felten, D. L., Felten, S. Y., Carlson, S. L., Olschowka, J. A., and Livnat, S., *J. Immunol.* **135,** 755, 1985.
34. Straub, R. H., Westermann, J., Scholmerich, J., and Falk, W., *Immunol. Today* **19,** 409, 1998.
35. Kooijman, R., Malur, A., Van Buul-Offers, S. C., and Hooghe-Peters, E. L., *Endocrinology* **138,** 3949, 1997.
36. Bellone, G., Astarita, P., Artusio, E., *et al., Blood* **90,** 21, 1997.
37. Fuse, Y., Polk, D. H., Lam, R. W., and Fisher, D. A., *Endocrinology* **127,** 2501, 1990.
38. Canbay, E., Normal, M., Kilic, E., Goffin, V., and Zachary, I., *Biochem. J.* **324,** 231, 1997.
39. Postel-Vinay, M. C., and Finidori, J., *Eur. J. Endocrinol.* **133,** 654, 1995.
40. Marrero, M. B., Schleffer, B., Paxton, W. G., *et al., Nature* **375,** 247, 1995.