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Transforming Growth Factor Beta (TGFβ) Is Produced by and Influences the Proliferative Response of *Xenopus laevis* Lymphocytes

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Both TGFβ2 and 5 have been described in the South African clawed frog *Xenopus laevis* and have been cloned from the tadpole-derived fibroblast cell line, XTC. Because TGFβ has such a profound inhibitory effect on the mammalian immune system, this study was performed to determine whether TGFβ: (a) has any *in vitro* effects on the growth of *Xenopus* lymphoblasts, and (b) is produced by mitogen-activated *Xenopus* lymphocytes.

Following stimulation with mitogen or alloantigen, T lymphocytes from *Xenopus* secrete a T-cell growth factor (TCGF) that is functionally homologous to mammalian interleukin-2 (IL-2). Both recombinant human TGFβ1 and *Xenopus* TGFβ5 inhibit TCGF-induced proliferation of *Xenopus* splenic blasts and this inhibition can be reversed with anti-pan TGFβ antiserum. The *Xenopus* mitogen-induced saturated ammonium sulfate precipitated TCGF-containing supernatant (SAS TCGF SN) also contains latent TGFβ as assayed on mink lung fibroblasts and *Xenopus* splenic blasts, and experiments utilizing anti-TGFβ antiserum showed that only TGFβ5 is present in this supernatant.

KEYWORDS: TGFβ, *Xenopus*, immunity, lymphocyte, amphibian, cytokine.

INTRODUCTION

TGFβ is a pleiotrophic cytokine produced by a number of different cell types including platelets, macrophages, fibroblasts, and T and B lymphocytes. TGFβ is secreted in a 100-kD biologically inactive latent form. This latency peptide can be cleaved by a change in pH or proteolysis to yield a 25-kD disulfide-bonded homodimer (reviewed in Roberts and Sporn, 1990). Five types of TGFβ have been cloned: TGFβ1, 2, and 3 were originally described in humans (Assoian et al., 1983; Wrann et al., 1987; ten Dikje et al., 1988); TGFβ4 has been found only in chickens (Jakowlew et al., 1988); TGFβ5 has been found only in *Xenopus* (Roberts and Sporn, 1990). The amino-acid-sequence identity of the aforementioned processed TGFβs (after cleavage of latency peptide) is between 60% and 80%. TGFβ5 is 76% identical to TGFβ1, 66% identical to TGFβ2, 69% identical to TGFβ3, and 72% identical to TGFβ4. Regions of identity include a highly conserved site and nine conserved cleavage cysteine residues. TGFβ 1 through 5 also show functional conservation in a number of assays, including the inhibition of proliferation of mink lung fibroblasts (MLF) and the stimulation of normal rat kidney (NRK) fibroblast colony formation in the presence of epidermal growth factor (Roberts and Sporn, 1990).

TGFβ plays a role in the control of the formation of extracellular matrices, myogenesis, formation and remodeling of bone, and in embryogenesis. TGFβ also functions in the immune system. It inhibits T- and B-cell proliferation, NK-cell activity, and generation of mixed lymphocyte responses and cytotoxic T cells (Massague, 1990). Kehrl et al. (1986) reported that TGFβ is secreted and TGFβ receptors are expressed by mitogen-activated T cells. These and other investigators believe that TGFβ acts to limit T- and B-cell clonal expression and to stimulate fibroblast proliferation to regulate inflammation and promote healing.

TGFβ2 and 5 have been cloned from the *Xenopus* tadpole-derived fibroblast cell line, XTC,

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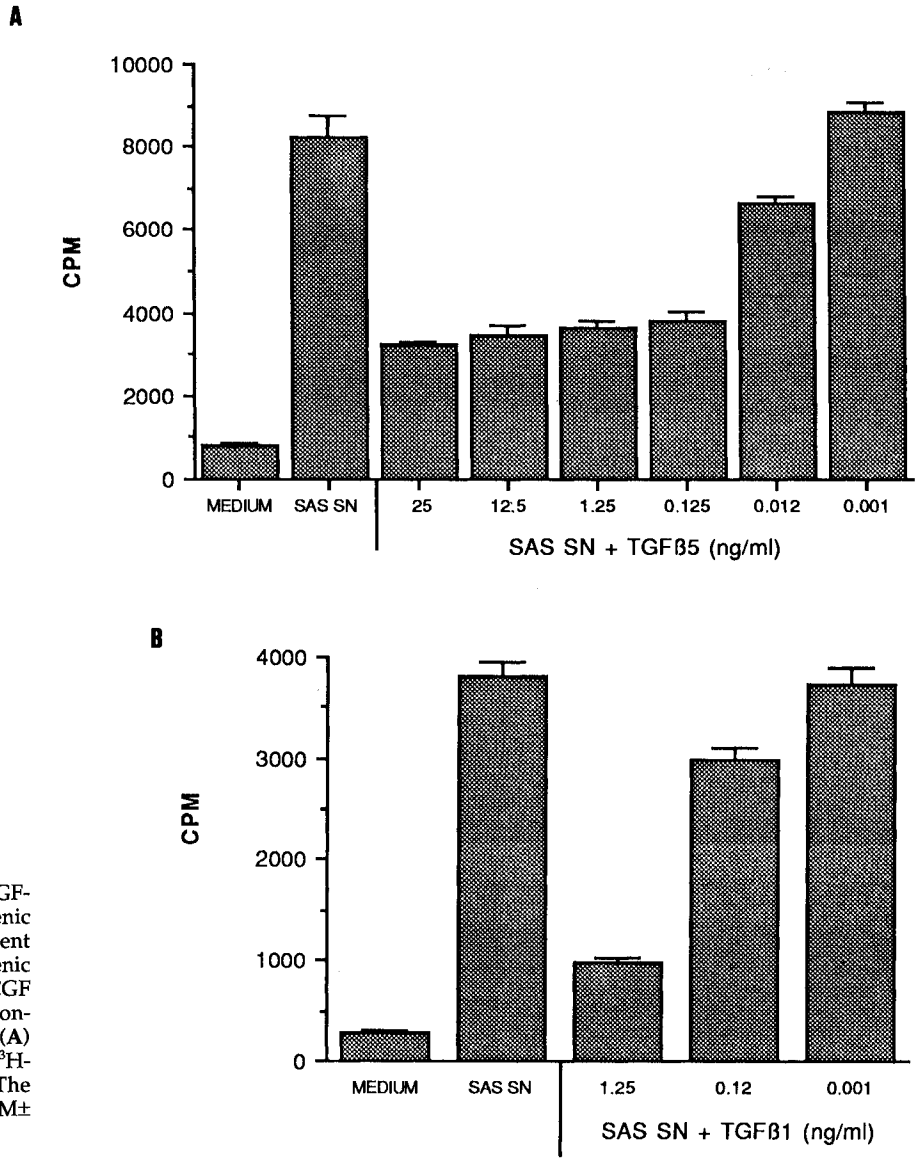

FIGURE 1. TGFβ inhibits TCGF-induced proliferation of splenic blasts. Representative experiment showing 3-day-old *Xenopus* splenic blasts assayed with 25% SAS TCGF SN alone or with the indicated concentrations of *Xenopus* rTGFβ5 (**A**) or human rTGFβ1 (**B**) in a 3-day ^{3}H-thymidine incorporation assay. The results are expressed as mean CPM± SE.

and they have mesoderm-inducing activity on explants of amphibian ectoderm (Roberts and Sporn, 1990). Because TGFβ has such a profound effect on the mammalian immune system, this study was performed to determine whether TGFβ has any *in vitro* effects on the growth of *Xenopus* lymphoblasts and if it is produced by mitogen-activated *Xenopus* lymphocytes.

RESULTS

Xenopus SAS TCGF SN contains a TCGF that is functionally homologous to mammalian interleukin-2 (IL-2) (Watkins and Cohen, 1987; Haynes and Cohen, 1993). One of its main *in vitro* activities is the induction of proliferation of activated, but not resting, splenocytes. Recombinant *Xenopus* TGFβ5 (rTGFβ5) inhibited the SAS TCGF SN-induced proliferation of 3-day-old *Xenopus* splenic blasts (Fig. 1A). Maximum inhibition was seen with 0.125 ng/ml and this inhibition could be titrated until no inhibition was seen with 0.001 ng/ml. Similar results were obtained with recombinant human TGFβ1 (Fig. 1B).

To determine whether the inhibition of lymphoblast proliferation associated with rTGFβ5 resulted from TGFβ5 or from some contaminant

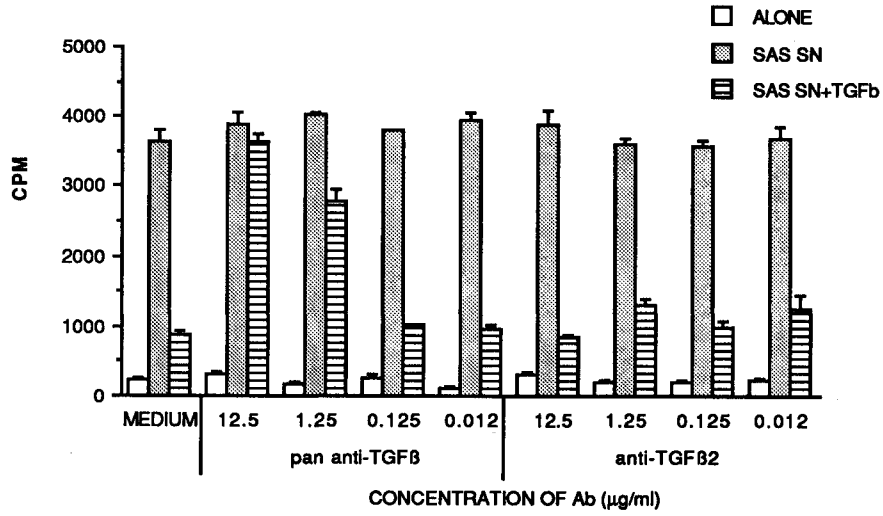

FIGURE 2. Anti-pan TGFβ antiserum reverses TGFβ-induced inhibition of TCGF-induced proliferation. Three-day-old *Xenopus* splenic blasts were cultured alone or with 25% SAS TCGF SN and 1.25 ng/ml rTGFβ5 with or without the indicated concentrations of rabbit anti-pan TGFβ or rabbit anti-TGFβ2 antiserum, as described in Materials and Methods, in a 3-day ^{3}H-thymidine incorporation assay. Results are expressed as mean CPM ±SE.

in the TGFβ5 preparation, TGFβ5 was preincubated with anti-pan TGFβ antiserum and then assayed (Fig. 2). Whereas the anti-pan TGFβ antiserum reversed the inhibition of proliferation, the control anti-TGFβ2 antiserum (which does not recognize TGFβ5) did not. The observed inhibition of proliferation, therefore, was specifically due to TGFβ5. Neither antibody was mitogenic for *Xenopus* blasts (Fig. 2, open bars).

The next set of experiments tested for the presence of TGFβ in the SAS TCGF SN by assaying acid-treated (to activate latent TGFβ) SAS TCGF SN for biological activity on mink lung fibroblasts and *Xenopus* splenic blasts. Figure 3A demonstrates that rTGFβ5 inhibited the proliferation of MLF in a dose-dependent manner; Fig. 3B reveals that the SAS TCGF SN exhibited similar inhibitory activity, but only upon acid activation. Thus, PHA-stimulated *Xenopus* splenocytes secrete both TCGF and a latent form of TGFβ. Indeed, the undiluted SAS TCGF SN exhibits activity equivalent to 125 pg/ml of rTGFβ5.

Figure 4 shows that acid-activated SAS TCGF SN also induced reduced proliferation of *Xenopus* splenic blasts when compared to untreated SN. This reduction resulted from TGFβ activity and not from denaturation of the TCGF molecule during the acid treatment, because the full stimulatory activity of the SAS TCGF SN could be restored when it was first treated with the anti-pan TGFβ antiserum (Fig. 5).

Finally, because both TGFβ2 and 5 have been described in *Xenopus* (Roberts and Sporn, 1990), antiserum specific for each of these proteins was used in an attempt to neutralize the TGFβ biological activity found in the acid-treated SAS TGCF SN. Figure 6 shows that the anti-TGFβ2 has no effect, whereas the anti-TGFβ5 reverses the inhibitory activity found in the acid-treated supernatant.

DISCUSSION

In mammals, TGFβ is produced by mitogen-activated T cells, inhibits IL-2-dependent T-cell proliferation, and downregulates the immune response (Kehrl et al., 1986). Our study shows that these observations also are applicable to the *Xenopus* immune system. *Xenopus* splenic blasts proliferate in response to TCGF(s) present in mitogen-induced SAS TCGF SN and this proliferation can be inhibited by either rTGFβ (1 or 5) or by activating the latent TGFβ that is present in the supernatant. In blocking experiments with an anti-pan TGFβ antiserum, this inhibition was shown to result from TGFβ. Results of an additional two experiments with antisera specific for either TGFβ2 or TGFβ5 are consistent with the proposition that the inhibitory effects of the acid-activated TCGF-rich supernatant are due solely to TGFβ5. That is, there was no reversal of inhibition with anti-TGFβ2; the extents to which the anti-pan TGFβ antiserum and the anti-TGFβ 5-specific antiserum reversed inhibition were similar, and the lymphoblast growth-promoting activity of the acid-activated supernatant was completely restored with the anti-TGFβ5-specific

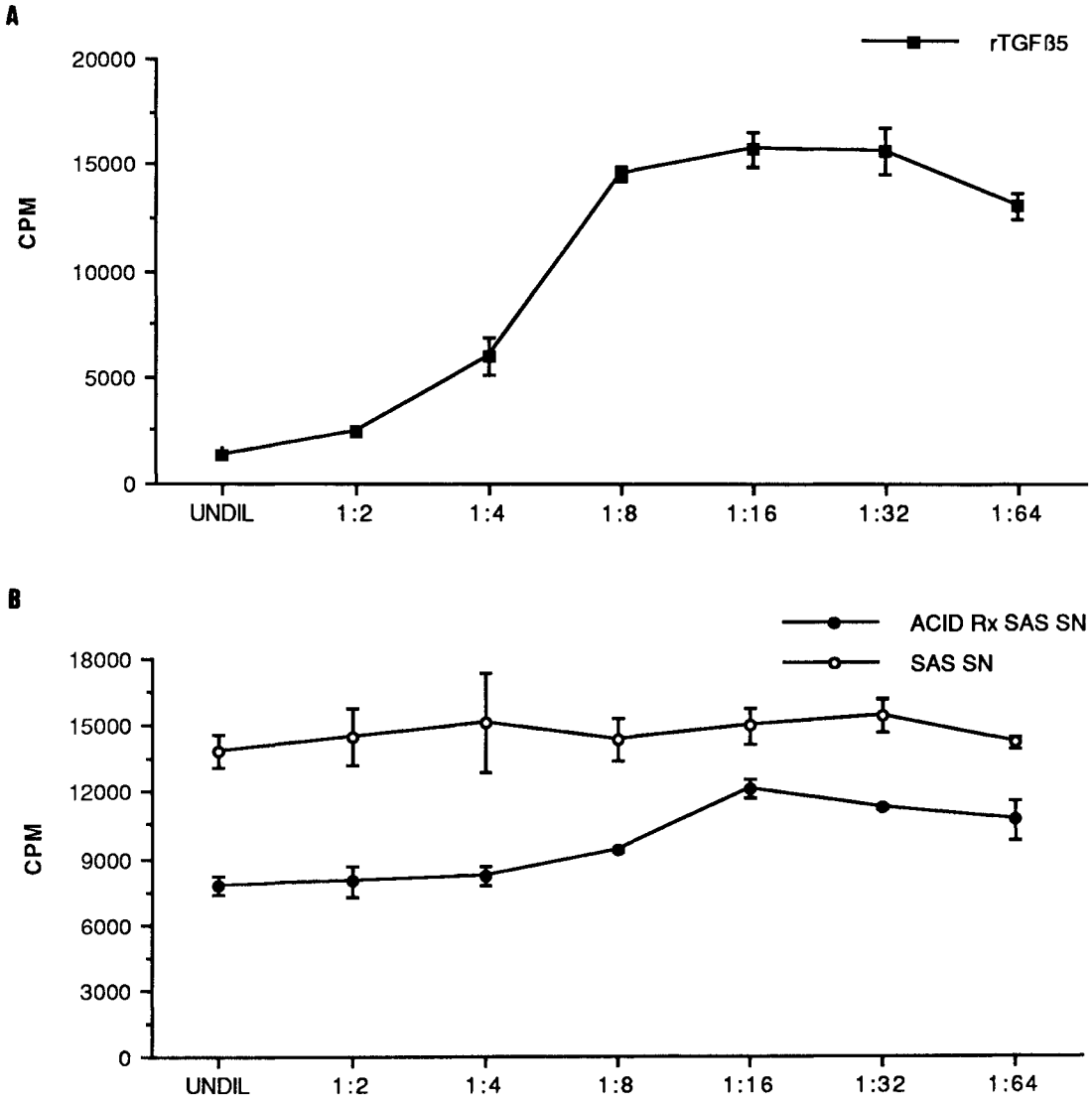

FIGURE 3. The effect of *Xenopus* TGFβ on mink lung fibroblasts. *Xenopus* rTGFβ5 (**A**) (starting concentration=0.5 ng/ml) and *Xenopus* SAS TCGF SN, untreated or acid treated (acid Rx) (**B**) were assayed on cultures of MLF, as detailed in Materials and Methods, for 24 hr and the cultures were then pulsed with ^{3}H-thymidine for 6 hr. Results are expressed as mean CPM±SE. For both experiments, CPM with medium alone was 9013.8±775; acid treatment of medium has no effect.

antiserum treatment. Whether "all" supernatants prepared from mitogen- or antigen-stimulated larval as well as adult *Xenopus* splenocytes also contain only TGFβ5 is currently being investigated. Because TGFβ is involved in development, we are also investigating its role in the immune system during metamorphosis to determine if the downregulation of the immune response that is seen during this period (Flajnik et al., 1987) is, at least in part, attributable to TGFβ.

TGFβ activity in the SAS TCGF SN was demonstrated by the ability of an acid-activated, but not an untreated, supernatant to inhibit the proliferation of mink lung fibroblasts. It is not surprising to find that both human rTGFβ1 and frog rTGFβ5 exhibit biological activity on frog and mammal cells because the three types of TGFβ receptors (I, II, and III) bind all types of TGFβ, albeit with different affinities (Cheifetz et al., 1988; Roberts and Sporn, 1990).

Like mammals, *Xenopus* has T and B lymphocytes and expresses class I and class II MHC molecules that function much like mammalian MHC glycoproteins (Du Pasquier, 1989; Flajnik and Du

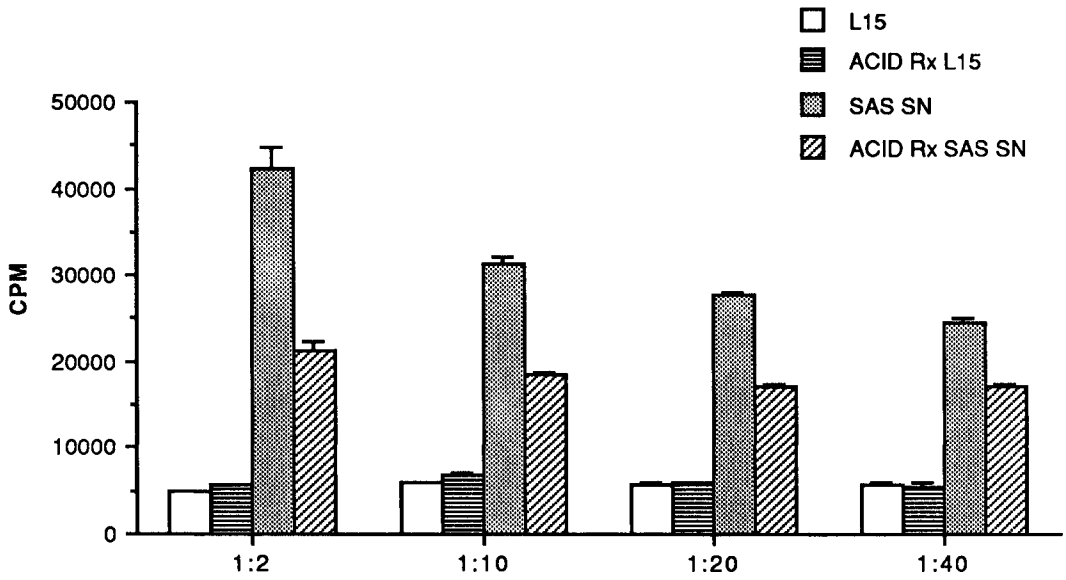

FIGURE 4. SAS TCGF SN contains TGFβ inhibitory activity for *Xenopus* splenic blasts. Untreated or acid Rx ASA TCGF SN, at the indicated concentrations, was assayed on 3-day-old *Xenopus* splenic blasts in a 3-day ^{3}H-thymidine incorporation assay. Results are expressed as mean CPM±SE. L15 is the complete frog medium with 0.25% BSA.

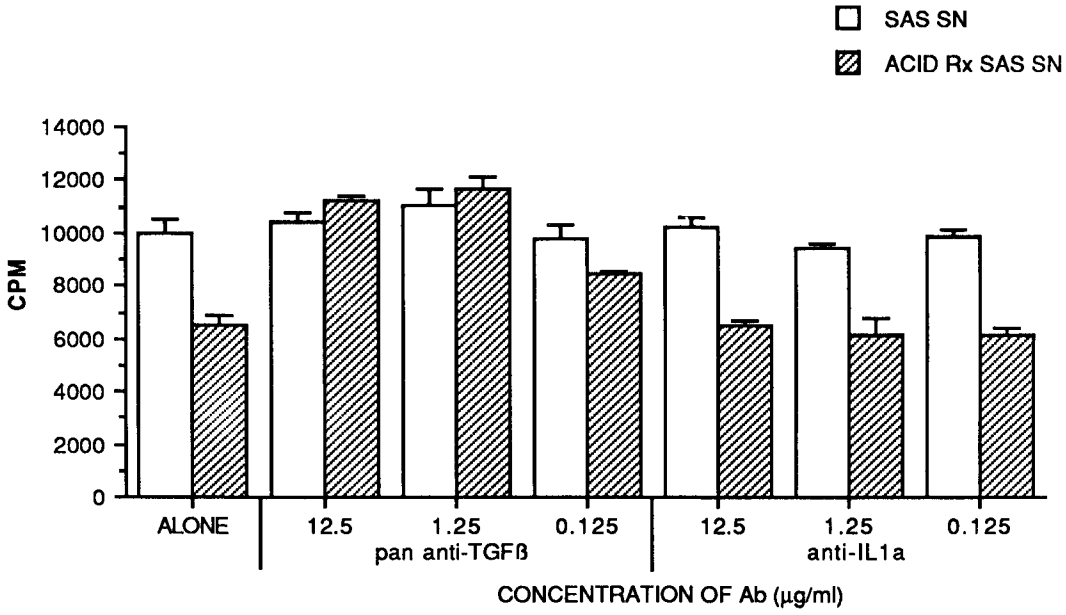

FIGURE 5. Anti-pan TGFβ antiserum reverses TGFβ inhibitory activity found in SAS TCGF SN. Untreated or acid Rx SAS SN (25% per well) with or without rabbit anti-pan TGFβ or control rabbit anti-IL-1a antiserum, at the indicated concentrations, was assayed (as described in Materials and Methods) on 3-day-old *Xenopus* splenic blasts in a 3-day ^{3}H-thymidine incorporation assay. Results are expressed as mean CPM±SE.

Pasquier, 1990). *Xenopus* leukocytes produce an IL-1-like cytokine (Watkins et al., 1987). *Xenopus* T cells produce additional cytokines involved in T- and B-cell proliferation (Cohen and Haynes, 1991) and also exhibit MHC-restricted cytotoxicity (Harding, 1990). *Xenopus* B cells can secrete one of three types of immunoglobulin during an immune response, which are homologous with

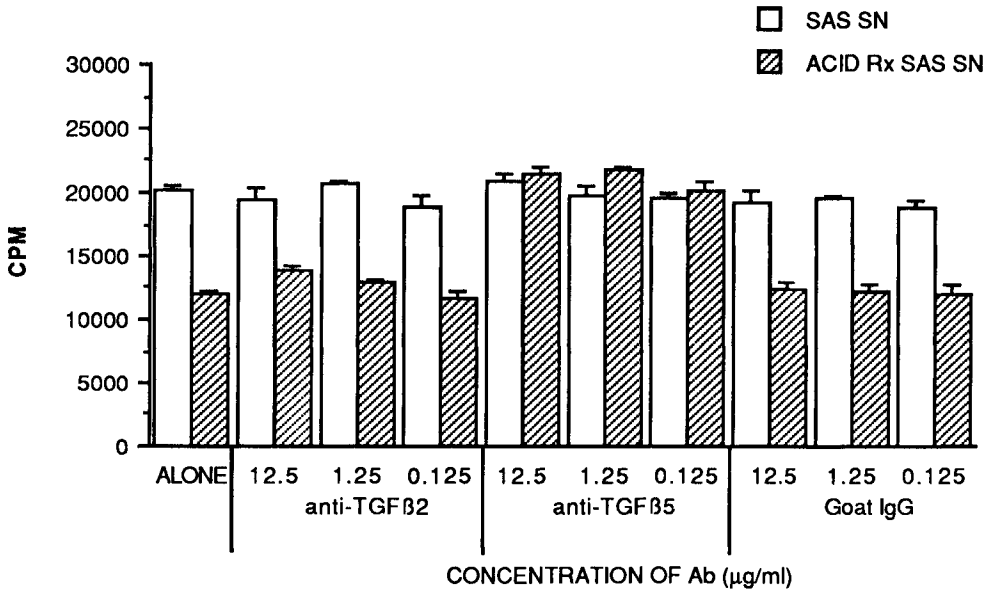

FIGURE 6. Anti-TGFβ5, but not anti-TGFβ2, antiserum reverses TGFβ inhibitory activity found in SAS TCGF SN. Untreated or acid Rx SAS SN (25% per well) with or without goat anti-TGFβ2, goat anti-TGFβ5, or control goat IgG, at the indicated concentrations, was assayed (as described in Materials and Methods) on 3-day-old *Xenopus* splenic blasts in a 3-day ^{3}H-thymidine incorporation assay. Results are expressed as mean CPM±SE. CPM with medium alone is 2061.5±80.1.

mammalian IgM, IgG, and IgA (Du Pasquier, 1989). By demonstrating that *Xenopus* lymphocytes produce TGFβ that can downregulate lymphocyte proliferation, the present study provides additional evidence for the remarkable similarity of the *Xenopus* and mammalian immune systems.

MATERIALS AND METHODS

Animals

Fully grown adult female *Xenopus laevis* were purchased from Xenopus 1 (Ann Arbor, Michigan) or the South African Xenopus Facility (Noordhoek, South Africa).

Production of SAS TCGF SN

SAS TCGF SN was generated as previously described (Watkins and Cohen, 1987). Briefly, 5× 10^6 *Xenopus* splenocytes/ml were incubated at 26°C in complete medium [Leibovitz's L-15 medium (Gibco, Grand Island, New York) adjusted to amphibian osmolarity (220 mOsm) and supplemented with 1.25×10^{-5} M HEPES buffer (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 1×10^{-2} M $NaHCO_3$, 5× 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, Missouri)] with 0.25% bovine serum albumin (BSA) and 1 μg/ml PHA-P conjugated to agarose beads (Sigma). Supernatants were collected from 24-hr and 48-hr cultures, pooled, and the PHA beads were removed from the supernatant by centrifugation. The resulting supernatant was precipitated with saturated ammonium sulfate, dialyzed (Spectra/Por membrane, M_r cutoff 6000–8000) with APBS, and sterile filtered before use. All experiments described in this paper were performed with a single batch of SAS TCGF SN.

To activate any latent TGFβ present in the SAS TCGF SN, it was subjected to acid activation. Five hundred microliters of SN or complete medium was treated with 5 μl of 3N NCl for 1 hr at room temperature; the pH was neutralized with 5 μl of 3N NaOH. Samples were concentrated using Centricon Microconcentrators (Amicon, Beverly, Massachusetts) with a M_r cutoff of 10,000 and then assayed.

Preparation and Assay of Splenic Blasts

Splenocytes (5×10^6/ml) were cultured in complete medium with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, Utah) and

1 to 2 μg/ml PHA-P (Sigma) for 3 days at 26°C in 24-well plates (Costar, Cambridge, Massachusetts). The resulting blasts were then centrifuged (350×g) over Histopaque δ=1.077 (Sigma) and washed twice in complete medium with 1% FBS. TCGF activity was assayed on splenic blasts in a 3-day ^{3}H-thymidine incorporation assay. The samples were incubated with 5×10^4 blasts per well (in complete L-15 with 1% FBS) in 96-well round-bottom plates (Costar). For antibody-inhibition experiments, samples were incubated with antibody for 1 hr in the well before the addition of the cells. The rabbit anti-porcine TGFβ2 polyclonal antiserum (R & D Systems, Minneapolis, Minnesota) specifically neutralizes biological activities of TGFβ2 and 3; rabbit anti-pan-TGFβ polyclonal antiserum (R & D Systems) neutralizes the biological activities of TGFβ1, 2, 3, and 5. Goat anti-purified porcine TGFβ2 and anti-recombinant *Xenopus* TGFβ5, also purchased from R & D Systems, were only used to identify the type of TGFβ in the SAS TCGF SN (see Fig. 6). Rabbit anti-human IL-1α (Genzyme, Boston, Massachusetts) and goat IgG (Sigma) were also used as a negative control. After 48 hr, 1 μCi/well ^{3}H-thymidine (Amersham, Arlington Heights, Illinois) was added. The cultures were harvested after 72 hr and processed for liquid scintillation spectrometry. All cultures were plated in triplicate and the data are presented as mean counts per minute (CPM)±SE.

Mink Lung Fibroblasts (MLF)

MLF (Mv1Lu CCL-64, originally from ATCC; a gift from the laboratory of Dr. David Scott) were maintained at 37°C in 25-cm^2 tissue-culture flasks (Costar) in complete mouse medium [RPMI-1640 (Gibco), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, 1 mM sodium pyruvate, 4.4×10^{-2} M sodium bicarbonate, 0.04 mM 2-mercaptoethanol] with 5% FBS. To assay for TGFβ activity, fibroblasts were aliquoted (2×10^4 per well) in complete medium with 5% FBS in 96-well flat-bottom plates (Costar) and allowed to become confluent. The medium was then replaced with serum-free complete mouse medium and the cells were incubated overnight. The serum-free medium was removed and TGFβ (recombinant human TGFβ1 from R & D Systems was provided by Dr. David Scott; recombinant *Xenopus* TGFβ5 was purchased from R & D Systems) or the supernatants to be tested were added to the cultures that were then incubated for 24 hr. Each well was then pulsed with 1 μCi ^{3}H-thymidine (Amersham) for 6 hr and freeze/thawed before harvesting and processing for liquid scintillation spectrometry. All assays were plated in triplicate and the data are presented as the mean CPM±SE.

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