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Thymosin: an immunomodulator of antibody production in man

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Summary. Thymosin (TFX) stimulates polyclonal antibody synthesis of human peripheral blood, lymph node and spleen lymphocytes. This stimulation is observed only with T-cell-dependent polyclonal activators of immunoglobulin production and its mechanism appears to be associated with TFX-induced activation of T-helper cells. On the other hand, antibody production *in vitro* of lymphocytes already involved in high humoral responses is inhibited by thymosin. TFX has similar activity both *in vitro* and *in vivo*.

INTRODUCTION

The recent progress in thymic hormone research has offered new approaches in the clinical management of patients with disorders of the immune system. A number of reports have been published which showed the effectiveness of thymic hormones and their synthetic analogues, one of the most promising being Goldstein's thymosin fraction V (Low & Goldstein, 1979). Almost all papers reporting studies on thymosin dealt with its efficacy in restoring cell-mediated immunity in man, while its effects on antibody production received little attention. It has been shown that thymosin inhibits *in vitro* pokeweed mitogen (PWM)-driven immunoglobulin synthesis (Wolf, Goldstein & Ziff, 1978). However, clinical experience with an analogue of thymosin fraction V, TFX-Polfa (Polfa Pharmaceuticals, Jelenia Góra, Poland) suggests that it may correct immunoglobulin levels in serum when given to patients with various types of hypogammaglobulinaemia (Aleksandrowicz, Blicharski, Czyzewska-Wazewska, Jasinski, Lisiewicz, Skotnicki, Skierczyńska, Turowicz & Szmigiel, 1975). Moreover, other authors (Sredni, Gopas & Rozenszajn, 1978) have demonstrated that thymic cells produce factor(s) capable of triggering terminal differentiation of mouse B lymphocytes to immunoglobulin-producing cells. In the present communication, we analyse the mechanisms of thymosin action on antibody synthesis *in vitro* by human lymphocytes.

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MATERIALS AND METHODS

Blood donors

Patients treated with thymosin had primary glomerulopathies without renal insufficiency or nephrotic syndrome, most of them having IgA nephropathy. TFX was given i.m. 10 mg every day during the first 3 weeks, every second day during the next 3 weeks and twice weekly thereafter. As controls we used age-matched normal blood donors who were not receiving any treatment.

Lymphocyte cultures

Mononuclear cells were isolated from heparinized

blood using standard Ficoll-Isopaque gradient centrifugation and washed three times with phosphate-buffered saline (PBS). In some experiments, lymph node and splenic lymphocytes (obtained during nephrectomy for renal transplantation) were also used. Purified populations of T- and B-enriched cells were obtained taking advantage of T-cell rosetting with sheep red blood cells (SRBC), as described earlier (Kamiński, Nowaczyk, Skopínska-Różewska, Kamínska & Bem, 1981). The viability of cells was at least 85%. The cells were cultured in a final volume of 1 ml at a concentration of 10^6/ml in medium RPMI 1640 (Gibco) with 20% AB serum, 1-glutamine and 25 μg/ml gentamicin in fully humidified atmosphere with 5% CO_2 (Assab). In co-cultures of purified lymphocyte populations, the proportion of T:B cells was 1:1. Terminal differentiation of B cells to immunoglobulin-containing plasmacytes was triggered with the T-cell dependent or independent PWM 10 μl/ml, Gibco or *Staphylococcus aureus* Cowan I strain, respectively. The bacteria were killed with formaldehyde, heat treated at 80° for 5 min and used in a concentration of 2×10^7/ml found to produce the highest number of plasma cells. Mixed lymphocyte cultures (MLC) were established by co-culturing lymphocytes of two HLA-mismatched donors in two way or one way combinations.

Testing of TFX activity

TFX was added in concentrations ranging from 10 to 1000 μg/ml to the cultures. Control cultures contained only lymphocytes with medium or an equivalent concentration of splenic fraction V (a gift of Professor A. Goldstein of Washington University School of Medicine) or a similar product obtained from Polfa Pharmaceuticals.

In some experiments, the cells were preincubated with TFX for 1 hr at 37° and washed thoroughly before subsequent culture.

Assay for immunoglobulin synthesis

Following 7 (10 for MLC) days of culture the cells were cytocentrifuged and processed for the detection of intracytoplasmatic immunoglobulins (Ic-Ig) as described by Miyawaki, Kubo, Nagaoki, Moriya, Yokoi, Mukai & Taniguchi (1981) using FITC-labelled antibodies to human γ-globulins (Kent Laboratories and Tago Immunodiagnostics, U.S.A.). The number of Ic-Ig positive plasma cells was determined with aid of a fluorescence microscope (Opton).

RESULTS

The effect of TFX on antibody synthesis of PWM- and Cowan-activated blood lymphocytes

TFX added *in vitro* to PWM-stimulated lymphocyte cultures caused a marked enhancement of immunoglobulin synthesis. Optimal stimulation was caused by the 100 μg/ml dose, although doses up to 1000 μg/ml were also active. Cultures containing only lymphocytes or lymphocytes with TFX without PWM or Cowan formed only marginal numbers of Ic-Ig positive cells. The stimulation of antibody synthesis of a similar range was also seen when TFX was added on days 1–5 of culture instead of being added at the beginning. No stimulation of antibody production was observed in cultures stimulated with Cowan (Fig. 1, left). The preincubation of lymphocytes with thymosin before culture with PWM also resulted in an increase of the number of Ic-Ig positive plasma cells, but the effect of preincubation was always weaker than that caused by a continued presence of TFX in the cultures.

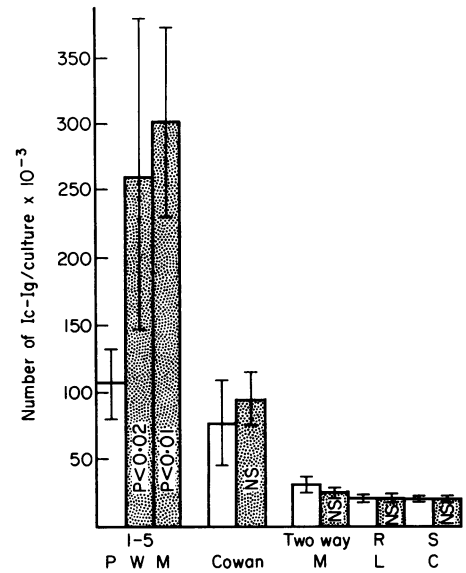

Figure 1. The effect of TFX on PWM and Cowan (left) and MLC-driven (right) antibody synthesis of peripheral blood lymphocytes. Mean values of ten experiments ± SD (for MLC, mean values were derived of four experiments). 1–5 Denotes mean value of Ic-Ig number in cultures where TFX was added on day +1, +2, +3, +4 and +5 of culture (three experiments for each day). R, responder, S, stimulatory function in MLC. No TFX added (□), +TFX 100 μg/ml (▨). The splenic extract was without effect causing sometimes inhibition of immunoglobulin synthesis.

Thymosin and antibody production in MLC

In subsequent experiments we determined the effect of TFX on alloantigen-induced immunoglobulin synthesis in MLC. HLA-mismatched lymphocytes were cultured in the presence or absence of TFX and assayed for Ic-Ig as described above. Furthermore, we tested the effect of TFX on stimulatory (S) and responding (R) functions of lymphocytes in MLC. To assess S, mitomycin-treated lymphocytes were incubated with TFX for 1 hr at 37°, washed three times with PBS and mixed with normal allogeneic cells, while R function was assayed by mixing mitomycin-treated normal lymphocytes with TFX-treated (see above) allogeneic cells. TFX did not influence antibody production in MLC nor did it affect S or R functions of lymphocytes in MLC (Fig. 1, right).

TFX enhances immunoglobulin synthesis of lymph node and spleen lymphocytes

Having learned that TFX causes a marked stimulation of antibody synthesis of peripheral blood lymphocytes, we turned next to the question of TFX action on lymphocytes from lymphoid organs. Figure 2 shows that TFX was also effective in enhancing the PWM-driven immunoglobulin synthesis of lymph node and splenic B cells. Although this stimulation was polyclonal, IgM synthesis appeared to be affected mostly.

In vivo action of TFX

An important question remained whether TFX acts

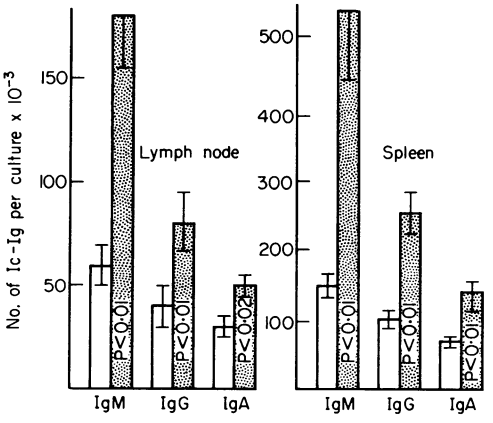

Figure 2. The influence of TFX on PWM-driven immunoglobulin synthesis of lymph node and spleen lymphocytes. Mean values of four experiments ± SD. No TFX (□), with TFX (▩).

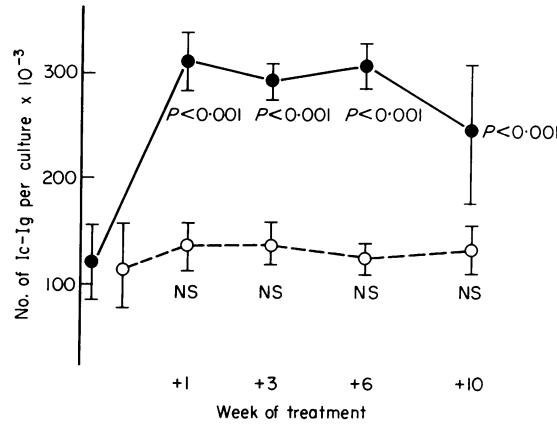

Figure 3. The effect of TFX treatment on *in vitro* lymphocyte response to PWM (●) and Cowan (○). Data presented as mean values from ten patients ± SD.

solely *in vitro* or also *in vivo*. To answer this, lymphocytes of patients receiving TFX were cultured and processed for Ic-Ig as described above. During TFX treatment, patients' lymphocytes acquire a markedly enhanced humoral responsiveness as measured by the number of Ic-Ig positive cells secondary to PWM stimulation (Fig. 3). Again, this enhancement is associated only with T-cell-dependent responses, as direct B-cell response (Ic-Ig production evoked by Cowan) remains unchanged.

TFX stimulates T-helper cells

The data obtained up to this moment strongly suggested that thymosin produces a T-cell dependent stimulation of antibody synthesis. Further evidence of such a mechanism was offered by the results of cocultures of purified T and B cells. Figure 4 shows that cocultures of enriched populations of T and B cells of patients treated with TFX yielded significantly more Ic-Ig than did the cocultures of normal donors' lymphocytes. Moreover, purified populations of T cells from TFX-treated patients caused a markedly enhanced helper effect when cocultured with normal B cells, while the cocultures of normal T cells with patients' B cells produced normal amounts of Ic-Ig. In another series of experiments, we tested the effect of TFX on helper cells *in vitro*. Purified populations of T cells were preincubated with TFX 100 μg/ml, washed and co-cultured with B cells. Such co-cultures produced $220 \pm 42 \times 10^{-3}$ Ic-Ig positive cells per culture in response to PWM, while the co-cultures of T cells

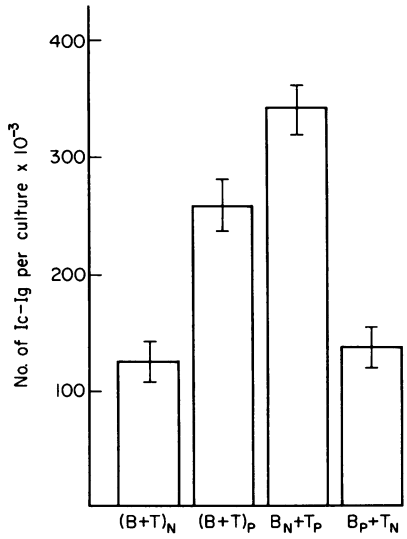

Figure 4. The effect of TFX on immunoglobulin synthesis by purified populations of lymphocytes. N, normal, p, patient treated with TFX. Data presented as mean values of five experiments ± SD.

incubated with medium and B cells produced $100 \pm 30 \times 10^{-3}$ ($P < 0{\cdot}02$) and the co-cultures of T cells preincubated with splenic fraction V and B cells $60 \pm 17 \times 10^{-3}$ ($P < 0{\cdot}01$). Moreover, concanavalin A-activated (48 hr culture with 50 μg/ml of Con A followed with wash with α-methyl-mannoside) T cells of TFX-treated patients were still able to inhibit PWM-driven Ic-Ig synthesis (unpublished observations). This finding suggests that TFX-induced augmentation of immunoglobulin synthesis is indeed caused by its activation of T-helper cells rather than impairment of the T-suppressor cell compartment.

TFX inhibits immunoglobulin synthesis of 'high' responders

Assaying immunoglobulin production of lymphocytes from patients treated with TFX whose cells produced high numbers of Ic-Ig positive cells in response to PWM, we noted that the addition of TFX to such cultures produces an inhibition rather than a further stimulation of humoral responses. Therefore, we attempted to characterize further this phenomenon providing a further TFX stimulus to cultures of lymphocytes already stimulated with thymosin *in vitro* and *in vivo*. When PWM-activated TFX-containing cultures were rechallenged with TFX, 10 or 100 μg/ml, at a time when first Ic-Ig positive plasma cells appear, this is, on day +4 or +5 of culture, the number of Ic-Ig positive cells per culture dropped to $144 \pm 94 \times 10^{-3}$, which was within the same range as the number of Ic-Ig positive cells generated in TFX-free cultures ($82 \pm 40 \times 10^{-3}$, $P > 0{\cdot}2$). The cultures which received only one dose of TFX on day +5 produced $310 \pm 145 \times 10^{-3}$ Ic-Ig, $P < 0{\cdot}02$ when compared with TFX-free cultures, which is in agreement with previous observations that TFX stimulates antibody production when added on days +1 to +5 of culture. Similar observations, although less consistent, were also made *in vivo*. Thus, the addition of as little as 10 μg/ml of TFX to PWM-stimulated lymphocyte cultures of patients treated with TFX produced in many cases a profound decrease of the number of developing Ic-Ig positive cells; likewise, lymphocytes of patients actively rejecting their renal allografts synthesizing high numbers of Ic-Ig positive cells lost this ability when cultured with TFX (data not shown).

DISCUSSION

The results of our studies indicate that TFX is a potent stimulant of antibody synthesis, enhancing the activity of T-helper cells. These findings are in accord with our previous observations that TFX, both *in vitro* and *in vivo*, enhances the growth of B lymphocyte colonies (Górski, Skotnicki, Gaciong & Korczak, 1981) as well as T-cell-dependent humoral responses in mice (Gaciong, Paczek & Górski, submitted for publication). Likewise, others have demonstrated that thymosin fraction V stimulates increased IgG synthesis (Mutchnick, Lederman, Missiman & Johnson, 1981). On the other hand, there is a report suggesting that thymosin suppresses PWM-driven terminal differentiation of B lymphocytes (Wolf, Goldstein, & Ziff, 1978). Our data showing that TFX may also decrease elevated antibody responses (either resulting from prior TFX activation *in vitro* and *in vivo* or caused by an unknown agent *in vivo*) suggests that TFX is in fact an immunomodulating agent, whose final effect depends on whether an individual is a 'high' or 'low' responder. Such an effect was already reported previously in regard to TFX action on T-lymphocyte colony growth in man (Górski *et al.*, 1981). Why the humoral inhibitory action of TFX was less consistent being undetectable in a few patients remains to be elucidated; in fact, there are suggestions that thymosin is capable of inducing both suppressor and helper function and the response is quite unpredictable (Kaufman, 1980). Similar data were reported in

patients with systemic lupus erythematosus (Horowitz, Borcherding, Moorthy, Chesney, Schultze-Wisserman, Hong & Goldstein, 1978). That indeed immune status determines, at least to some extent, the final effect of TFX administration, may be supported by the observation that the observed effect is not dose dependent, that is, even very high concentrations of TFX still stimulate antibody synthesis in 'low' or 'normal' responders, while lower concentrations are able to inhibit immunoglobulin production in 'high' responders. A similar situation occurs in patients treated with levamisole, a drug whose activity is in some aspects similar to thymosin (Giełdanowski, 1981; Giełdanowski & Ślopek, 1981): there appears to exist a group of 'high' responders whose enhanced immune parameters are not affected by the drug (Hodinka, Meretey, Zahumensky & Bozsoky, 1981). Our data thus suggest that TFX may be a useful agent in the treatment of patients with deficiency of humoral immunity especially associated with dysfunctions of helper T cells. Moreover, our findings also explain the reported efficacy of thymosin in correcting humoral immune abnormalities with hypergammaglobulinaemia (e.g. in lupus erythematosus): this subgroup of patients being in fact 'high' responders reacts to thymosin with inhibition of antibody synthesis.

The findings that TFX does not enhance alloantigen-induced antibody synthesis appears to be of a special interest. MLC-activated antibody production seems to be at least partially T-cell independent (Górski *et al.*, unpublished observations). These observations along with above reported preliminary findings that TFX may be able to diminish immunoglobulin synthesis of lymphocytes from patients rejecting their renal allografts suggests that TFX may be a useful agent in the treatment of kidney allograft recipients with deteriorating renal function due to humoral rejection which is frequently resistant to presently available therapy and ends in graft failure.

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