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Thymosin increases production of T-cell growth factor by normal human peripheral blood lymphocytes

(thymic hormones/lymphokines/immunomodulation/interleukin 2/immunodeficiency)

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ABSTRACT **The *in vitro* incubation of phytohemagglutinin-stimulated peripheral blood lymphocytes with thymosin results in a marked and reproducible increase in production of T-cell growth factor, which is dose dependent and most pronounced in the first 24 hr of culture. Incubation of lymphocytes with thymosin alone failed to induce any production of T-cell growth factor. The biological activity of thymosin fraction 5 cannot be attributed to the activity of thymosin α_1, one of the well-characterized peptide components of fraction 5. These data provide the basis for (*i*) a potential mechanism for the *in vivo* immunorestorative effects of thymosin in primary and secondary immunodeficiencies and (*ii*) identification of an additional, but as yet undefined, immunoregulatory component of thymosin fraction 5.**

The lymphokines are a group of mediators elaborated by activated lymphocytes that have important effects in the cascade of events following lymphocyte stimulation. T-cell growth factor (TCGF), also referred to as interleukin 2 (IL-2), has been widely studied (1) and is known to influence T-cell maturation, production of other lymphokines such as γ-interferon (γ-IFN) (2), and induction of natural killer and T-cell cytolytic activity (3, 4). Recent attention has been focused both on the lack of TCGF production in certain immunodeficiency states and on the immunoregulatory potential of TCGF in enhancing depressed natural killer and virus specific cytotoxicity (5).

The thymosins are a family of thymic humoral factors that have been proposed as hormonal agents with important immunoregulatory effects (6). Thymosins, as well as other thymic preparations, are being tested extensively *in vivo*, as biological response modifiers in clinical trials and experimental models of primary and secondary immunodeficiencies, including acquired immunodeficiency syndrome (AIDS), autoimmune disease, neoplasia, and aging (7–11). The biological basis for the mechanism of action of the thymic hormones is largely speculative; most likely the many thymic factors are not only biochemically distinct but biologically different in their effects and target systems.

Thymosin fraction 5 (F5) is an extract of bovine thymus that contains 40–50 peptides, several of which (thymosins α_1, β_4, β_9, β_{10}, and β_{11}) have been sequenced, synthesized, or both (12–14). The biological properties of two of these peptides, thymosins α_1 and β_4, have been studied extensively (12, 13). It has been suggested from earlier studies that these two peptides do not account for all the biological activity of the parent compound (15–17).

Since F5 increases immune responsiveness in children with primary immunodeficiencies (7) and in cancer patients (10), and is being tested in clinical trials of autoimmune disease (9) and the pre-AIDS complex (8), we asked whether F5 functions, at least in part, by modulating TCGF production. It has been reported that F5 and thymosin α_1 increase production of migration inhibition factor (MIF) in the mouse and guinea pig (18, 19) and production of α- and γ-IFN in mice (20) and humans (21). Thymulin (FTS, facteur thymique sérique), another thymic factor, has been shown to increase TCGF production by cells of nude mice (22). Grinblat *et al.* have reported increased TCGF production by thymic humoral factor (THF) in aged animals (23).

In this paper, we report that F5 substantially and consistently augments production of TCGF by peripheral blood lymphocytes (PBL) from normal human volunteers, in a dose-dependent fashion. These data have important connotations since this system provides (*i*) the rationale for the augmentation and immunological responses observed *in vivo* in immunodeficiencies treated with thymosin; (*ii*) the opportunity to study the mechanisms of action of thymosin in the modulation of lymphokine production; and (*iii*) a quantitative bioassay for isolation and characterization of one or more biologically active components of F5.

MATERIALS AND METHODS

Generation of TCGF. Human PBL were obtained from heparinized blood by separation on lymphocyte separation medium (Litton Bionetics). Cells were washed, resuspended in RPMI 1640 medium supplemented with 25 mM Hepes buffer, 1% human type AB serum, gentamycin, and L-glutamine. The PBL were cultured in 12-well plates on a platform rocker at 37°C, in 5% CO_2/95% air, for 24–96 hr at 1×10^6 per ml in 2 ml containing phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC) at 2 μg/ml and in the presence or absence of thymosin. At the end of the culture, the supernatants were collected and stored at 4°C until assayed.

TCGF Assay. Supernatants were tested at several dilutions (1:2 to 1:32) on the TCGF-dependent C57BL/6-derived CT-6 mouse cell line, as previously described (24). CT-6 cells were grown in HRPMI 1640 medium supplemented with 10% fetal bovine serum (Microbiological Associates, Walkersville, MD), 2-mercaptoethanol (50 μM), glutamine, and penicillin/streptomycin (base medium) and 50% supernatant from concanavalin A-conditioned rat spleen cells, termed "rat factor." For the assay, logarithmic-phase CT-6 cells (originally obtained from J. Farrar) were washed twice, resuspended in base medium, and dispersed in 96-well flat-bottomed microtiter plates at 10^4 cells per well. Unknown supernatants at various dilutions were added to the wells; dilutions of rat

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Abbreviations: TCGF, T-cell growth factor; F5, thymosin fraction 5; γ-IFN, γ-interferon; AIDS, acquired immunodeficiency syndrome; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes.

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factor or base medium were used as positive or negative controls, respectively. After 40 hr of incubation at 37°C, in 5% CO_2/95% air, the cells were pulsed with [^{3}H]thymidine (Schwarz/Mann) at 1 μCi per well (1 Ci = 37 GBq), and well contents were harvested 6 hr later onto glass fiber strips. Results are reported as cpm incorporation of [^{3}H]thymidine or TCGF units/ml as previously described (24). The rat factor standard used for comparison was arbitrarily assigned a value of 1000 units/ml. All tests were performed in triplicate and standard errors never exceeded 10%.

Thymosin. F5 (lots C114080 and BP100), bovine kidney fraction 5, and synthetic thymosin α_1 were provided by Alpha One Biomedicals (Washington, DC). Thymosin lots were clinical grade and endotoxin free as measured by the limulus and rabbit pyrogen assays. β_1 peptide was prepared by T. L. K. Low (The George Washington University, Washington, DC).

Statistics. Data were analyzed for significance by Student's *t* test.

RESULTS

Enhancement of TCGF Production by Thymosin. In our initial studies, PBL from 23 normal donors were screened for PHA-induced TCGF production in the presence or absence of F5 (100 μg/ml) after 72 hr of culture. Forty-eight percent of the individuals tested showed a significant enhancement of TCGF production by F5 (mean fold increase = 2.6 ± 0.7). Since net TCGF detectible in supernatant at 72 hr reflects a balance between production and utilization (25), a kinetic study was performed to determine the time at which thymosin-induced enhancement of TCGF production was optimal (Table 1). Although the peak of TCGF production is between 48 and 72 hr for most individuals, the optimal effect of F5 in increasing PHA-induced TCGF production was seen at the earliest timepoint studied—i.e., 24 hr of culture. Enhancement of PHA-induced TCGF by F5 also was demonstrated in PBL that had been cryopreserved and thawed prior to stimulation with PHA. No effect of F5 or thymosin α_1 on TCGF production has been observed in the absence of PHA (data not shown).

Table 1. Kinetics of thymosin F5-enhanced TCGF production

Donor*	Time, hr	TCGF† cpm × 10^{-3} −F5	TCGF† cpm × 10^{-3} +F5	% increase‡	*P* value§
A	24	36.9	67.1	81.8	<0.001
	48	101.0	119.2	18.0	
	72	102.5	115.2	12.4	
	96	0.9	1.3	44.4	
B	24	61.8	109.8	77.7	<0.001
	48	106.0	126.7	19.5	
	72	4.2	8.7	109.1	
	96	4.9	7.1	44.9	
C	24	25.3	58.2	90.1	<0.001
	48	52.2	80.3	53.8	
	72	90.7	114.1	21.6	
	96	30.9	75.1	143.0	
D	24	82.9	134.8	62.6	<0.001
	48	237.4	240.6	1.3	
	72	254.4	265.2	4.2	
	96	233.8	228.3	−2.4	
E	24	61.2	101.9	39.9	<0.001
	48	135.6	131.5	−3.0	
	72	138.8	141.4	1.9	

*Donor PBL from five different individuals were cultured for the times shown prior to collection of supernatants. PBL from donors A–C were freshly isolated from whole blood. PBL from donors D and E were cryopreserved and thawed prior to testing.
†Cells were cultured in the absence of F5 (−F5) or in the presence of F5 at 100 μg/ml (+F5). Supernatants were tested on CT-6 cells.
‡Percent increase induced by thymosin was calculated as % increase = 100 × [cpm (+F5) − cpm (−F5)]/cpm (−F5).
§Comparison of TCGF production in the presence and absence of F5.

Thymosin Dose and Role of Thymosin α_1. The dose dependence of the thymosin-enhanced production of TCGF is shown in Fig. 1. F5 causes an increase in PHA-induced TCGF production in a linear dose-dependent fashion reaching maximal stimulation at 400 μg/ml. The increases are statistically significant ($P < 0.01$ or 0.001) at all concentrations of F5 ≥25 μg/ml. At high concentrations, thymosin α_1 causes a small increase in TCGF production, which does not account for all the biological activity of F5, since thymosin α_1 is approximately 0.6% of the total protein in F5. The two different lots of F5 (BP100 and C114080) yield virtually identical results. An additional 10 lots of F5 also have been tested, and all show biological activity in this assay (data not shown), although some lots of thymosin α_1 have little or no activity. Neither control kidney fraction 5 nor β_1 peptide results in significant increases of TCGF production.

To assess individual variability of TCGF production in response to thymosin, PBL of 18 individuals were tested; some

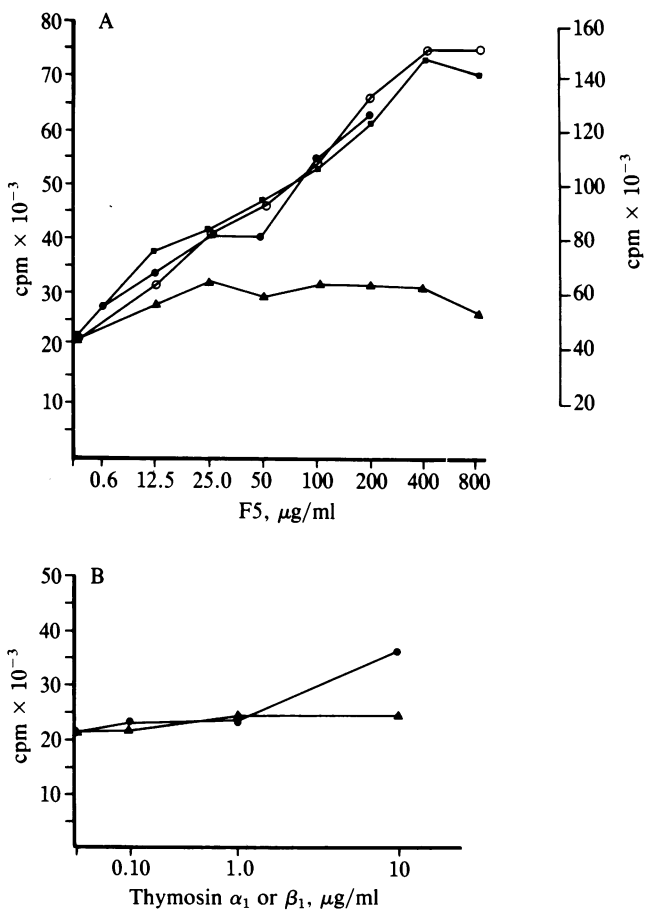

FIG. 1. Thymosin dose-dependent increase of PHA-induced TCGF production. (*A*) Human PBL were cultured for 24 hr in the presence of PHA (2 μg/ml) and various doses of F5 (○, lot C114080; ●, lot BP100) or control kidney fraction (▲). In a second experiment, cells from the same donor were cultured with C114080 (■). Results are expressed as [^{3}H]thymidine incorporation by CT-6 cells; the right-hand *y*-axis gives the scale for the second experiment. (*B*) The same PBL as shown in *A* were cultured with PHA and thymosin α_1 (●) or control peptide β_1 (▲).

Table 2. Thymosin enhancement of TCGF production by PBL from a panel of normal donors

Donor*	Exp.	TCGF† Units/ml	TCGF† % increase by F5‡ 100 μg/ml	TCGF† % increase by F5‡ 200 μg/ml
CG	1	43	350	—
	2	110	191	286
	3	119	—	183
PS	1	162	97	
	2	216	58	
	3	292	102	110
	4	206	55	—
TD	1	198	53	—
	2	229	21	—
JO	1	432	7.5	—
	2	378	37	—
RM	1	650	—	65
KD	1	265	—	75
RC	1	130	—	108
JB	1	212	—	116
CGa	1	136	—	123
JC	1	235	—	134
MZ	1	180	—	150
ME	1	260	—	192
JM	1	125	—	200
SG	1	90	—	267
DC	1	28	—	632
			$\bar{x}$ = 97.3 ±30.8	$\bar{x}$ = 181.7 ±40.1

*PBL of 15 normal individuals were tested one to four times for F5 effect on PHA-induced TCGF production. Multiple tests of the same individual at different times are indicated by experiment numbers.

†Data are expressed as units/ml in supernatants generated after 24 hr in the presence of PHA.

‡When PBL were cultured in the presence of PHA and F5 at either 100 or 200 μg/ml, the percent increase in TCGF production was calculated as % increase = 100 × [units/ml (+F5) − units/ml (−F5)]/units/ml (−F5). A — indicates not tested. The mean increase (±SEM) for each dose of F5 is shown as $\bar{x}$ at the bottom of each column. These increases were significant at $P < 0.01$.

donors were retested multiple times. The data shown in Table 2 demonstrate that virtually all donors' PBL produced more PHA-induced TCGF in the presence of F5 when supernatants were evaluated after 24 hr of culture. The PBL of individuals who were retested on multiple occasions responded in a consistent fashion. These data also suggest that PBL of individuals with low levels of TCGF production may show greater enhancement by F5 than do individuals with high production levels. To facilitate comparisons between individuals and multiple tests on the same individual, data were standardized and expressed as TCGF production in units/ml.

DISCUSSION

In this report we demonstrate that an as yet undefined component of F5 markedly increases PHA-induced TCGF production by PBL of normal individuals. These results differ in several important ways from previous reports of thymic hormone influence on lymphokine production. Data showing induction by thymulin of TCGF production by thymocytes and spleen cells from athymic nude mice suggest that thymulin causes a maturation of immature pre-T cells to TCGF-producing cells (22). A second report (23) also suggests that murine cells must be preincubated with thymic humoral factor in order to detect increased TCGF production. Similarly, enhancement of production of migration inhibition factor by guinea pig PBL has been attributed to the reversal by thymosin (19) of the effects of thymectomy. In contrast, our data suggest an immunoregulatory effect of F5 upon PHA-induced TCGF production by mature PBL, rather than an influence upon pre-T-cell differentiation and maturation.

The biological activity responsible for the induction of both migration inhibition factor and γ-IFN has been attributed to thymosin α_1, and the biological activity in F5 could be accounted for by its α_1 content (19, 20). On the other hand, the present studies indicate that a new component of thymosin F5 other than, or in addition to, α_1 is responsible for the increased production of TCGF by PBL. Several earlier studies have in fact shown that a component of F5 other than thymosin α_1 or β_4 is responsible for phenotypic changes in immature murine thymocytes (16), induction of cGMP in murine thymocytes (17), and increased mixed lymphocyte response and cytotoxic T-cell response by murine thymocytes (6, 15). These latter observations are particularly relevant, since these increased responses could have been mediated by increased TCGF production.

There are several possible explanations for the observed data: (*i*) thymosin may be acting via the macrophage by inducing increased interleukin 1 production or directly inducing a helper T cell to augment or accelerate TCGF production; (*ii*) thymosin could be acting to inhibit utilization of TCGF by modulation of the appearance of TCGF receptors (25); and (*iii*) thymosin may be acting by a combination of these mechanisms. The early appearance of increased TCGF favors the first interpretation.

On the basis of the data presented in this report, it may be possible not only to define additional functionally active components of F5 but also to identify a major activity of thymosin in the maintenance and regulation of T-cell function. Since lymphokine production may be a key step in the immunological events leading to γ-IFN production and generation of effector cells (1–4), and since lymphokine production is reported to be deficient in several clinical states, including aging (26), autoimmune disease, (27) and AIDS (5, 8), modulation of TCGF production by thymosin provides a rational basis for therapeutic intervention. These results also provide an experimental basis for the immunoenhancing effects of thymosin *in vitro*.

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