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Modulation of interleukin 2 receptor expression on normal human lymphocytes by thymic hormones

(thymosin/lymphocyte activation/immunoregulation)

MARCELO B. SZTEIN*, SUSANA A. SERRATE†, AND ALLAN L. GOLDSTEIN‡

Departments of *Medicine and ‡Biochemistry, The George Washington University School of Medicine, Washington, DC 20037; and †Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814

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ABSTRACT **The expression of interleukin 2 receptors (IL-2R) is a critical step leading to normal lymphocyte proliferation. Since thymosin fraction 5 (TF5), a thymic hormone preparation, enhances lymphoproliferative responses of human cells, we examined the effects of TF5 on the expression of IL-2R on mitogen-stimulated human lymphocytes. TF5 significantly increased the percentage and antigen density of cells expressing IL-2R after stimulation with an optimal concentration of phytohemagglutinin (PHA) when the cells from the same donor exhibited suboptimal responses to PHA alone. The same effect was observed with a suboptimal PHA concentration and with OKT3 monoclonal antibody stimulation. Thymosin α_1, a synthetic polypeptide originally isolated in its native form from TF5, was also able to increase IL-2R expression in response to PHA, suggesting that it is the active species in TF5. The enhancement of IL-2R expression was paralleled by increased proliferative responses. Increased IL-2R expression appears to be the direct effect of thymic hormones, since abrogation of interleukin 2 production by cyclosporin A did not affect TF5-mediated enhancement of PHA-induced IL-2R expression. These results point to a physiological role of thymic hormones in the maintenance of normal levels of IL-2R expression. This immunoregulatory activity of thymic hormones might be relevant in the treatment of conditions where there is decreased IL-2R expression, such as the acquired immune-deficiency syndrome, or in the restoration of normal IL-2R expression to lymphocytes from aged individuals.**

The induction of lymphocyte proliferation by antigens, mitogenic lectins, and T-cell-specific monoclonal antibodies is dependent upon the secretion of interleukin 2 (IL-2) and the appearance of receptors (IL-2R) specific for IL-2 on the surface of activated cells (1, 2). The regulation of the expression of IL-2R on lymphoid cells is the subject of extensive investigation (3–7). Interleukin 1 (IL-1) and accessory cells contribute to the induction of IL-2R expression on activated cells, and IL-2 can up-regulate the expression of its own receptors (3–9). In addition, inhibition of IL-2R expression has been observed in the presence of glucocorticoids (3, 10). A close correlation between the class and number of IL-2R on lymphocytes and their proliferative responses to IL-2 has been described (2, 4, 11).

Thymic hormones exhibit immunoreconstitutive effects *in vivo* and *in vitro* (12–16). *In vitro*, thymic hormones enhance proliferative responses of both murine and human lymphocytes (12–16). *In vivo*, injection of thymosin α_1, a synthetic peptide originally isolated in its native form from thymosin fraction 5 (TF5), a partially purified calf thymus preparation (12–16), increases IL-2 production *in vitro* and the response to IL-2 of spleen cells from old mice in response to mitogens (17). Further, TF5 increases *in vitro* IL-2 production by human lymphocytes in response to phytohemagglutinin (PHA) (18, 19). Although the increased IL-2 production could account for some of the enhancement of the lymphocyte proliferation induced by thymic hormones, the critical role of the expression of IL-2R in proliferative responses prompted us to examine the modulation of IL-2R expression by TF5 in mitogen-stimulated lymphoid cells.

MATERIALS AND METHODS

Reagents. TF5, bovine kidney fraction 5 (KF5), and synthetic thymosin α_1 were provided by Alpha 1 Biomedicals (Washington, DC) and were prepared as described (16, 20, 21). The TF5 preparation was endotoxin-free as determined by the *Limulus* and rabbit pyrogen assays. Further, we have determined that TF5 is devoid of IL-1 activity, as measured by its ability to enhance PHA-induced proliferation of thymocytes from the C3H/HeJ mouse strain (22); IL-2 activity, as measured by its ability to support the growth of the IL-2-dependent cell line CT6 (23); and interferon activity, as measured by its ability to protect the WISH cell line from a challenge with vesicular stomatitis virus (24). PHA (HA-16) was obtained from Burroughs Wellcome (Research Triangle Park, NC). Monoclonal antibody OKT3 was purchased from Ortho Diagnostics. Anti-Tac monoclonal antibody, which specifically reacts with the human IL-2R (1, 2), was kindly provided by T. Waldmann (Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD). Fluorescein-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin (IgG) antibody was purchased from Tago Immunodiagnostic Reagents (Burlingame, CA). Cyclosporin A (CsA) was a gift from Sandoz Pharmaceutical.

Preparation of Peripheral Blood Mononuclear Cells (PBLs) and Culture Conditions. PBLs were isolated from blood or buffy coats obtained from normal donors by density gradient fractionation in Lymphocyte Separation Medium (Litton Bionetics). Cells were washed and resuspended in RPMI 1640 medium supplemented with 1% human type AB serum (GIBCO), penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine (complete medium). The PBLs were cultured at 10^6 per ml in 24-well plates at 37°C, in 5% CO_2/95% air, for 24–72 hr in 2 ml of complete medium containing PHA (2 or 0.2 μg/ml) or OKT3 monoclonal antibody (25 or 5 ng/ml) in the presence or absence of TF5 or KF5 at various concentrations. In experiments in which the activity of thymosin α_1 was tested, lymphocytes were resuspended in RPMI 1640 without serum and preincubated for 30 min at room temperature in the presence of PHA (0.1 μg/ml) alone or with thymosin α_1 or TF5. After this period,

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Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor(s); TF5, thymosin fraction 5; KF5, kidney fraction 5; PHA, phytohemagglutinin; PBL, peripheral blood lymphocytes; CsA, cyclosporin A; MFCh, mean channel number of log fluorescence intensities.

human AB serum was added (final concentration 1%), and the cultures were incubated as described above.

IL-2 Bioassay. Supernatants were collected 24 hr after initiation of the cultures and their IL-2 content was determined by their ability to support the growth of an IL-2-dependent murine cell line (CT6), as described (23). Serial dilutions of the samples, ranging from 1:4 to 1:32, were made in 96-well plates, in RPMI 1640 containing 5% fetal bovine serum and antibiotics. Before each assay, the CT6 cells were extensively washed in RPMI 1640 with no serum and then incubated in 50 ml of the same medium in order to remove any IL-2 carried over from the culture flask. Cells (10^4) were added to each well. A standard IL-2 preparation was included in every assay, diluted from 1:4 to 1:256. The activity of each dilution was tested in triplicates. Also, three wells containing cells plus medium were included to estimate background proliferation. After 24 hr of incubation at 37°C in 5% CO_2, 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine was added to each well and the plates were incubated for 6 hr. The cells were then harvested with a MASH II (Microbiological Associates) into filter pads, which were then transferred to scintillation vials. Scintillation liquid was added to each vial, and radioactivity was measured in a scintillation counter. The data (cpm) were analyzed with an Apple II computer by probit analysis (23). Since the same IL-2 standard preparation was used in every assay, results obtained in separate experiments were compared directly. Results are expressed as units/ml, as compared to the BRMP human IL-2 (Jurkat) reference reagent (BRMP, National Cancer Institute–Frederick Cancer Research Center, National Institutes of Health).

Fluorescence-Activated Cell Sorter (FACS) Analysis. Cells were collected after 48–72 hr, washed once in 0.15 M NaCl/10 mM phosphate, pH 7.2, containing 1% bovine serum albumin, and stained with the monoclonal antibody anti-Tac (1, 2) and a fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG antibody. Cells were fixed in 1% formaldehyde and analyzed in a FACS IV (Becton Dickinson). Ten thousand cells, gated to exclude nonviable cells, were accumulated for each histogram. The percentage of IL-2R$^+$ cells (% Tac$^+$ cells) was estimated against a background of nonspecific labeling of the cell populations with MOPC-21 (a nonspecific IgG obtained from mouse myeloma cells) (Litton Bionetics) and fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG. Comparisons of fluorescence intensities (a measurement of the relative density of the Tac antigen) between different populations were done by comparing the mean channel numbers of the logarithms of fluorescence intensities (MFCh) in the different Tac$^+$ populations. Logarithms of fluorescence intensities in individual histograms were distributed over 256 channels.

Proliferation Assays. Proliferative responses, as measured by [^{3}H]thymidine incorporation, were determined on parallel cultures 72 hr after initiation of the assays. Cells (10^5) in 0.1 ml of complete medium were seeded in triplicate in 96-well plates and incubated with PHA or monoclonal antibody OKT3 in the presence or absence of TF5 or KF5 as indicated.

RESULTS

Modulation of PHA-Induced IL-2R Expression by TF5. The induction of IL-2R by an optimal concentration of PHA (2 μg/ml), as measured by the percentage of Tac$^+$ cells, was evaluated in PBLs obtained from 47 donors. The mean percentage of Tac$^+$ cells was 49.4 ± 3.2 (SEM). However, the individual responses exhibited by PBLs from different donors in the presence of an optimal concentration of PHA fell within two groups that were significantly different ($P < 0.001$): those with optimal induction of IL-2R (mean % positive cells >49.4 − 2 SEM, or 43%) and those with significantly lower induction of IL-2R (<49.4 − 2 SEM, or 43%). The mean percentage of Tac$^+$ cells of these groups was 61.4 ± 2.4 ($n = 34$) and 37.7 ± 1.4 ($n = 13$), respectively. TF5 did not have any effect on the responses exhibited by the cells within the former group, as evaluated by [^{3}H]thymidine incorporation or IL-2R expression, although increased IL-2 production was observed (data not shown). However, TF5 did significantly increase ($P < 0.05$) the IL-2R expression in response to optimal PHA concentration by PBLs from 8 out of the 13 donors whose PBLs exhibited suboptimal responses (Fig. 1*A*). TF5-induced enhancement of IL-2R expression was manifested by increased percent of cells expressing IL-2R and increased IL-2R antigen density on the positive cells (Fig. 1*A*). TF5 did not induce the appearance of IL-2R in nonactivated normal human PBLs (data not shown).

Since the suboptimal expression of IL-2R was readily modulated by TF5, we investigated the effects of TF5 on PBLs activated by PHA at 0.2 μg/ml, a concentration that induces suboptimal mitogenic responses. Under these conditions, PHA induced suboptimal IL-2R expression (<27% Tac$^+$ PBLs) and little or no detectable IL-2 production.

The expression of IL-2R by human PBLs activated with suboptimal doses of PHA was significantly stimulated by TF5

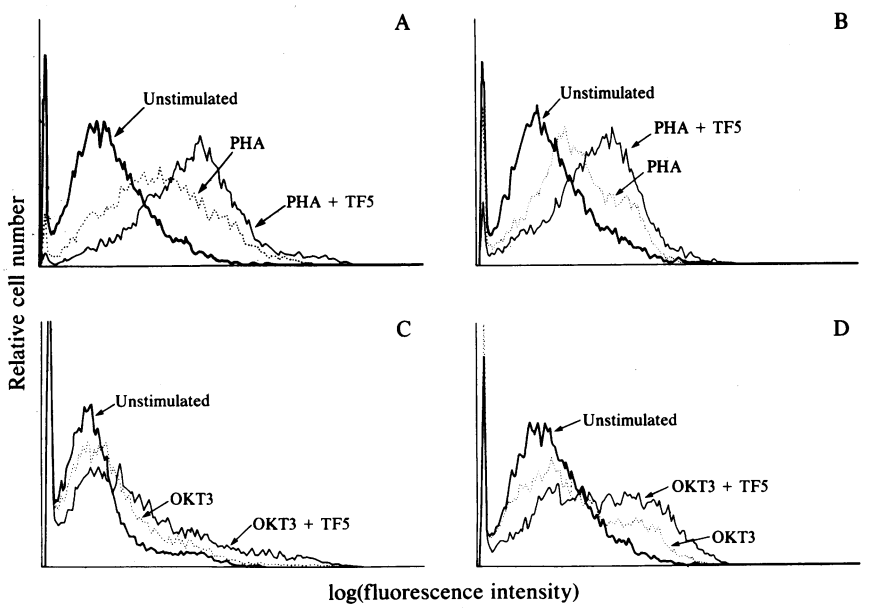

FIG. 1. Enhancement of IL-2R expression by TF5 in PBLs stimulated with PHA or monoclonal antibody OKT3. PBLs were treated with PHA or OKT3 in the presence or absence of TF5 (200 μg/ml). Unstimulated cells were incubated in medium alone. After 72 hr, cells were collected and stained with anti-Tac and a fluorescein-conjugated goat anti-mouse IgG antibody and analyzed in a FACS IV. (*A*) Suboptimal response to optimal PHA concentration (2 μg/ml). PHA: 36.9% Tac$^+$ cells, MFCh 97. PHA + TF5: 59.4% Tac$^+$, MFCh 122. (*B*) Response to suboptimal PHA concentration (0.2 μg/ml). PHA: 26.6% Tac$^+$, MFCh 78. PHA + TF5: 46.2% Tac$^+$, MFCh 96. (*C*) Suboptimal response to optimal OKT3 concentration (25 ng/ml). OKT3: 14.7% Tac$^+$, MFCh 73. OKT3 + TF5: 32.9% Tac$^+$, MFCh 97. (*D*) Response to suboptimal OKT3 concentration (5 ng/ml). OKT3: 17.3% Tac$^+$, MFCh 89. OKT3 + TF5: 38.7% Tac$^+$, MFCh 101. Data are from one representative experiment of several with similar results (see text).

($P < 0.05$) in 15 out of 21 experiments (Fig. 1*B*). As observed with suboptimal responses to optimal PHA concentration, both the percent of Tac^+ cells and their antigen density were enhanced by TF5 (Fig. 1*B*). These effects were dose-dependent, with optimal enhancement observed with 100 μg of TF5 per ml (Fig. 2). KF5, a kidney preparation obtained by following a purification procedure identical to the one employed to prepare TF5, did not exhibit stimulatory effects (Fig. 2).

Thymosin α_1 Enhances PHA-Induced IL-2R Expression. Experiments were performed in order to determine whether thymosin α_1, which has been shown to have immunoregulatory activities in other systems (12–17, 21), is able to modulate IL-2R expression on human lymphocytes stimulated by suboptimal PHA concentration. A representative experiment of five with similar results is included in Fig. 3. Thymosin α_1 significantly increased PHA-induced IL-2R expression by human lymphocytes stimulated with PHA at suboptimal concentration. Similar increases of IL-2R expression were observed with TF5 at 200 μg/ml and thymosin α_1 at 50 ng/ml.

TF5 Modulation of IL-2R Expression Induced by Monoclonal Antibody OKT3. The lymphoproliferative responses to specific antigens have been shown to be enhanced by thymic hormones (12–16). These responses involve activation through the T-cell antigen receptor, IL-2 production, and IL-2R expression. The T-cell antigen receptor is a single complex consisting of the T3 molecule and a clonotypic, disulfide-linked heterodimer (T3–Ti). Anti-T3 antibodies (e.g., OKT3), which trigger the proliferation of T cells in a way similar to antigen (25), induce IL-2 production and IL-2R expression (6). To determine whether IL-2R expression can be modulated by thymic hormones in cells activated through the T-cell receptor, and to confirm and extend the observations made with TF5 on PHA-stimulated cells, we studied the effects of TF5 on IL-2R expression and proliferation induced by OKT3 monoclonal antibodies.

Cultures were as described above for PHA stimulation. OKT3 induced maximal IL-2R expression (>mean % Tac^+ cells − 2 SEM, or 21%) in 11 out of 15 donors. Addition of TF5 did not result in enhanced IL-2R expression in these experiments. Significant enhancement ($P < 0.02$) of IL-2R expression by TF5 was observed in responses to an optimal OKT3 concentration (25 ng/ml) in which less than 21% of the cells expressed IL-2R (3 out of 4 donors) and in responses to a suboptimal OKT3 concentration (5 ng/ml) in 12 out of 15 donors (Fig. 1 *C* and *D*). Both the percent of PBLs expressing IL-2R and the IL-2R antigen density on the cell surface were significantly enhanced by TF5 (Fig. 1 *C* and *D*). Taken together, these results indicate that the IL-2R expression induced by mitogens and by activation through the T-cell antigen receptor can be modulated by thymic hormones.

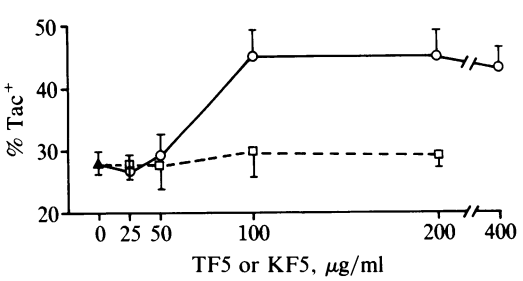

FIG. 2. Dose–response of TF5-mediated enhancement of IL-2R expression. PBLs were incubated with PHA (0.2 μg/ml) and various concentrations of TF5 (○—○) or KF5 (□---□), harvested after 72 hr, stained with anti-Tac, and analyzed by flow cytometry. Results are expressed as % Tac^+ cells. Each point represents the mean ± SEM of 5–9 independent experiments.

TF5-Induced Enhancement of IL-2R Expression Is Not Mediated by Increased Levels of IL-2 Production. The IL-2-dependency of the IL-2R expression on human cells has been demonstrated (3–9). Purified IL-2 can directly up-regulate synthesis and expression of its own receptors on PHA-activated T cells during the early phase of mitogen-induced IL-2R expression (10) and on cells whose IL-2R expression had declined (4). Since TF5 augments IL-2 production in response to PHA (18), it was possible that the increases in IL-2R expression observed in the presence of TF5 were due to IL-2-mediated up-regulation of IL-2R. To investigate this possibility, we incubated PBLs with PHA or OKT3 and TF5 in the presence of CsA. CsA is able to directly inhibit IL-2 production but not IL-2R expression by human lymphocytes (26–29). In three experiments, significant increases in IL-2R expression occurred in cells activated with a suboptimal concentration of PHA in the presence of TF5 and CsA, while IL-2 production was completely abrogated (Fig. 4). Similar results were obtained with OKT3-stimulated cells (data not shown). Thus, these results indicate that TF5 modulation of IL-2R expression is not due to IL-2R up-regulation by IL-2 and suggest that TF5 might have a direct effect on the expression of IL-2R.

TF5-Increased IL-2R Expression Correlates with Increased Proliferation Rates. The number of cells expressing IL-2R and the IL-2R surface antigen density on PHA-activated lymphocytes directly correlates with the proliferative rate of the

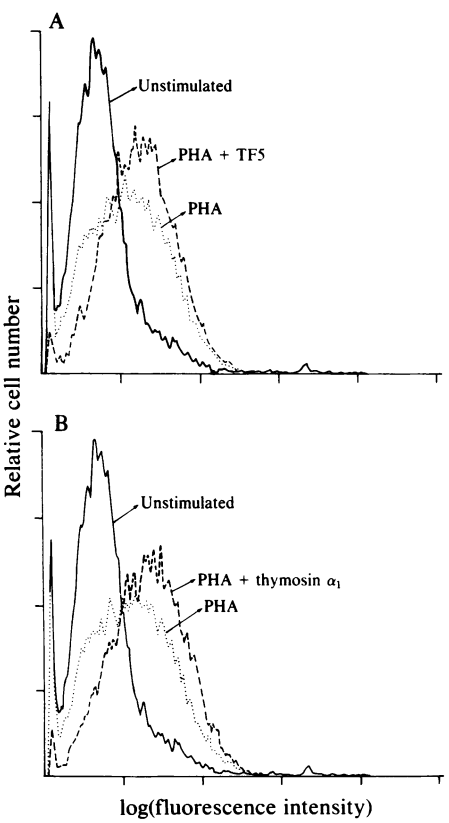

FIG. 3. Enhancement of IL-2R expression by thymosin α_1 in PHA-stimulated lymphocytes. PBLs were incubated for 30 min at room temperature in the absence (unstimulated) or presence of PHA (0.1 μg/ml), alone or with TF5 (200 μg/ml) (*A*) or thymosin α_1 (50 ng/ml) (*B*) in RPMI medium without serum. After this period, human AB serum was added to a final concentration of 1%, and the cultures were incubated at 37°C for 48 hr. Cells were harvested, stained with anti-Tac, and analyzed by flow cytometry. In this experiment, PHA induced 34.4% Tac^+ cells, MFCh 67; PHA + TF5 induced 51.9% Tac^+ cells, MFCh 80; and PHA + thymosin α_1 induced 49.2% Tac^+ cells, MFCh 86. Similar results were observed in four additional experiments.

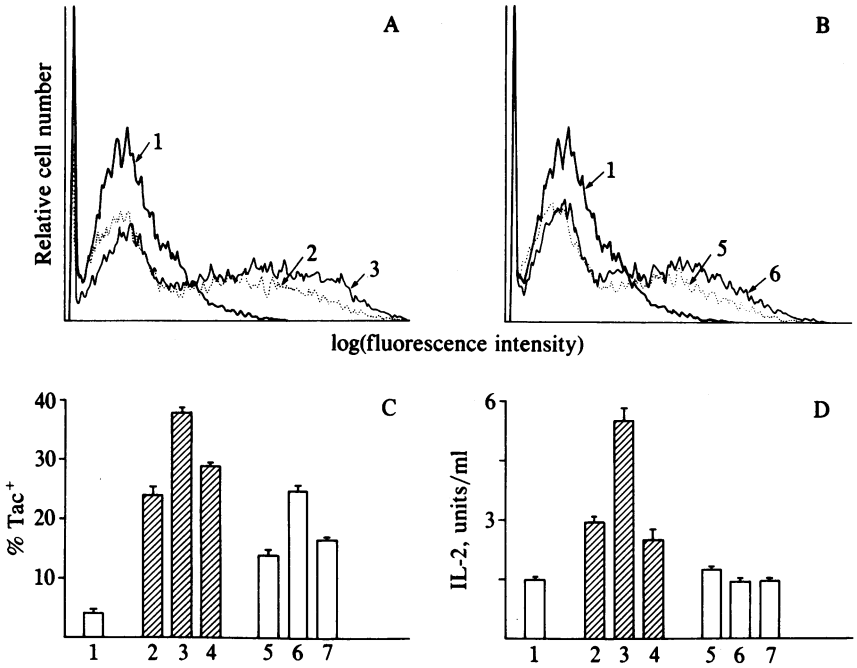

FIG. 4. Enhancement of IL-2R expression in the presence of CsA. PBL were obtained and cultured in duplicate for 48 hr in medium alone (unstimulated) or in the presence of PHA (0.2 μg/ml) with or without TF5 (200 μg/ml) or KF5 (200 μg/ml) (*A*, *C*, and *D*). Replicate plates were cultured in the presence of CsA (1 μg/ml) (*B*, *C*, and *D*). Cells were harvested and stained with anti-Tac, and the results (*A–C*) expressed as described in *Materials and Methods*. Supernatants for the determination of IL-2 levels were collected after 24 hr in culture; results (*D*) are expressed as units/ml. Histograms (*A* and *B*) and bars (*C* and *D*) are labeled as follows: 1, unstimulated, no CsA; 2, PHA; 3, PHA + TF5; 4, PHA + KF5; 5, PHA + CsA; 6, PHA + CsA + TF5; 7, PHA + CsA + KF5. In this experiment, PHA induced 23.8% Tac^+ cells, MFCh 143; PHA + TF5 induced 36.8% Tac^+ cells, MFCh 163; and PHA + KF5 induced 27% Tac^+ cells, MFCh 146. In the presence of CsA, PHA induced 14.1% Tac^+ cells, MFCh 123; PHA + TF5 induced 24.6% Tac^+ cells, MFCh 146; and PHA + KF5 induced 17% Tac^+ cells, MFCh 128. Similar results were observed in two additional experiments.

population (11). To determine the functional implications of the increased expression of IL-2R induced by TF5 on mitogen- or antigen-activated cells, we simultaneously studied the modulation of IL-2R and the proliferative responses of cells stimulated with PHA or OKT3 in the presence or absence of TF5 (Table 1). The TF5-mediated increase in IL-2R expression induced by either PHA or OKT3 was accompanied by a significant augmentation of lymphocyte proliferation. These data indicate that the expression of IL-2R can be modulated by thymic hormones and that this effect is associated with increased lymphocyte proliferation.

Table 1. Effect of TF5 on PBL IL-2R expression and proliferation induced by PHA and monoclonal antibody OKT3 at suboptimal concentrations

Donor	TF5	IL-2R expression		Proliferation	
		% Tac^+	Increase	cpm (SEM)	Increase
		PHA-induced			
1	−	25.7		75,300 (509)	
	+	52.3*	103%	113,666 (235)*	51%
2	−	34.3		7,942 (1,920)	
	+	43.7†	27%	21,763 (4,023)*	174%
3	−	22.3		3,535 (588)	
	+	35.6*	62%	20,255 (871)*	473%
4	−	24.2		15,652 (2,054)	
	+	38.1*	57%	35,527 (2,893)*	127%
		OKT3-induced			
5	−	11.4		2,804 (338)	
	+	20.7*	82%	22,757 (2,523)*	712%
6	−	7.0		616 (84)	
	+	15.4*	120%	7,382 (782)*	1353%
7	−	2.7		508 (102)	
	+	11.2*	315%	2,766 (73)*	444%

PBLs were cultured in replicates with PHA (0.2 μg/ml) or OKT3 monoclonal antibody (5 ng/ml) in the presence or absence of TF5 (200 μg/ml) as described in *Materials and Methods*. Parallel cultures were set up in 96-well plates to determine proliferation. IL-2R expression was determined 48 hr after initiation of the cultures, and proliferation was determined at day 3. Results are expressed as percent $IL2R^+$ (Tac^+) cells and as incorporation of [^{3}H]thymidine (mean cpm of triplicate cultures ± SEM).
*$P < 0.02$ (Student's t test).
†$P < 0.05$.

DISCUSSION

As suggested by Depper *et al.* (4), IL-2R expression on the surface of activated cells appears to be modulated by a number of signals, including IL-2. The data presented here indicate that IL-2R expression is also subject to modulation by thymic hormones. Further, we have identified thymosin α_1, a synthetic polypeptide originally isolated from TF5 in its native form (21), as the active molecule in TF5 responsible for the enhancement of PHA-induced IL-2R expression.

Unstimulated cells require two or more signals to initiate IL-2R expression (4), but, like IL-2, TF5 is not an "initiating" signal by itself, since it did not induce the appearance of IL-2R in the absence of mitogen or antigen. When the cells are already activated or "competent," IL-2 induces up-regulation of IL-2R but only in the presence of small concentrations of mitogen or antigen (4, 6). This appears to be also the case for thymic hormone up-regulation of IL-2R, because TF5 enhanced the expression of IL-2R when this expression was suboptimal and had been triggered by the competent signals. The contribution of IL-2R up-regulation, due to enhanced IL-2 production in the presence of TF5, to the TF5-modulation of IL-2R expression was addressed in the experiments performed in the presence of CsA. TF5 up-regulated IL-2R expression in the absence of measurable concentrations of IL-2, suggesting that it could affect the expression of IL-2R by two complementary pathways, one directly modulatory of IL-2R on activated cells, and the second through increased production of IL-2, which in turn would up-regulate its own receptors.

The mechanisms involved in the modulation of IL-2R on activated lymphocytes by thymic hormones, either directly or through thymic hormone-mediated increased IL-2 production, are not known. The expression of high numbers of high-affinity IL-2R on the surface of activated cells correlates with the proliferative rate of the cells in the presence of saturating concentrations of IL-2 (30), whereas the functional implications of the interaction of IL-2 with low-affinity IL-2R are not understood. Since TF5 was able to increase the proliferative rates of activated lymphocytes, and proliferation is closely associated with expression of high-affinity IL-2R, it is possible that TF5 could modulate the expression of high-affinity IL-2R on activated lymphocytes. However, we cannot rule out an effect of TF5 on the expression of

low-affinity IL-2R, since the anti-Tac monoclonal antibody reacts with both high- and low-affinity IL-2R (30). In addition, thymic hormones might also affect some mechanisms involved in the proliferative process not directly related to the interaction of IL-2 with its receptors.

Although most of the thymic hormone activities studied to date deal primarily with induction of T-cell maturation and differentiation markers (12–16), it is clear that thymic hormones might also affect the activity of mature lymphocytes. For example, enhancement of lymphokine production, specific cytotoxic responses, mixed lymphocyte reactions, and autologous mixed lymphocyte reactions by thymic hormones has been described (12–16, 18, 19, 31–35). Since the adequate expression of IL-2R is an important requirement for the normal development of these responses, it is possible that the modulation of these receptors is one of the mechanisms through which thymic hormones contribute to maintaining normal immune responses. It is noteworthy that TF5 and thymosin α_1 increased the suboptimal but not the optimal expression of IL-2R in normal human lymphocytes, suggesting that they might be useful in helping to counteract the decreased T-cell functions (including decreased IL-2 production and IL-2R expression) associated with the aging process (36) and in clinical situations associated with depressed expression of IL-2R, such as the acquired immune-deficiency syndrome (37). The immunoregulatory activity of thymic hormones described in this report may therefore have important functional implications in immune responses mediated by mature T cells.

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