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EARLY EVENTS IN THYMOCYTE ACTIVATION. I. STIMULATION OF PROTEIN SYNTHESIS BY A THYMUS-DEPENDENT HUMAN SERUM FACTOR

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SUMMARY: Human serum contains a thymus-dependent factor that raises cyclic AMP levels in thymocytes. We found that this factor stimulates protein synthesis in thymocytes cultured in vitro. This activity of serum factor is thymus-dependent, because it is absent in sera from thymectomized donors; furthermore, this effect is predominantly found on precursors of mature T cells. Incubation of thymocytes with other agents that increase cyclic AMP, induces an increase in protein synthesis similar to that observed with serum factor. Most likely, the increase in protein synthesis is one of the events following stimulation of adenylate cyclase in thymocytes that leads to cell differentiation.

It has been reported that the thymus produces humoral factors active in the maturation of precursors of T lymphocytes (1). The acquisition of immune competence is the result of a series of biochemical events, one of which involves an increase in protein synthesis (2). The induction of T-cell-specific antigens by the thymic extracts thymosin and thymopoietin, as well as the restoration by thymic humoral factor (THF) of the capability of spleen cells from neonatally thymectomized mice to induce a graft-versus-host reaction in vitro, is blocked by cycloheximide, a specific inhibitor of protein synthesis (3,4,5). Therefore, it is likely that thymic factors induce protein synthesis in immature T cells.

ABBREVIATIONS

THF, thymic humoral factor; SF, thymus-dependent serum factor; MEM, minimum essential medium; HC, hydrocortisone; PGE_1, prostaglandin E_1.

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We described a low molecular weight thymus-dependent factor, present in human serum (Serum Factor, SF), which has the capacity to induce an increase of intracellular cyclic AMP levels in precursors of mature T cells (6). SF can induce maturation of thymocytes as evaluated by the acquisition of resistance to hydrocortisone (HC) (7). This property can be induced by other thymic factors (8,9) as well as by cyclic AMP-increasing agents, such as isoproterenol (7).

The aim of the present study is to show that in the series of events leading to differentiation induced by SF, an intracellular cyclic AMP increase is followed by an increase in protein synthesis, selectively in precursor T cells.

MATERIALS AND METHODS

Preparation and assay of Serum Factor (SF) and its inhibitor. SF was prepared and assayed as previously described (7). In brief, serum from defibrinated blood was ultrafiltered to remove high molecular weight inhibitors. The capacity of the ultrafiltrate to raise intracellular cyclic AMP levels in thymocytes was tested as reported earlier (6). In this study all the SF fractions from healthy donors and from non-thymectomized patients had a cyclic AMP increasing activity >30 pmoles/10^7 thymocytes. Control serum ultrafiltrate preparations (inactive SF) were obtained by inactivating the human serum at 56°C for 1 h before ultrafiltration and in all the experiments, the same batch of inactive SF was used. Inactive SF was tested in the cyclic AMP assay and found not to induce any cyclic AMP increase.

The high molecular weight serum fraction retained above the Amicon filters (see above) was heat-inactivated at 56°C for 1 h and tested for its capacity to inhibit cyclic AMP stimulation induced by SF. All the inhibitory fractions, when used at a dilution <1:10, inhibited completely the stimulation of cyclic AMP induced by SF. All serum fractions studied (SF, inactive SF and inhibitor) were used in different concentrations given as percentage (vol/vol) of the final solution.

Preparation of cell suspensions. The thymus, spleen and lymph nodes from C57BL/6 mice, aged 6 weeks, were collected in petri dishes containing Earle's solution and gently pressed through a metal sieve to provide cell suspensions. Cells were resuspended in leucine-free minimum essential medium (MEM) (Gibco, Glasgow, U.K.) supplemented with a mixture of non-essential amino acids 1 ml/100 ml MEM (Gibco), 2 mM glutamine, penicillin 100 U/ml, streptomycin 100 µg/ml and 12.5 mM Hepes (Flow Laboratories, Irvine, U.K.), final pH 7.3. HC-resistant thymocytes were collected from C57BL/6 mice 40 h after injection with 2.5 mg HC-acetate per mouse as previously described (7).

Fragments of human thymus were obtained (with parental consent) from children undergoing cardiac surgery. The thymic fragments were cut

into small pieces (about 1 mm^3) and the thymocytes were collected by extensive washing with Earle's solution. Aggregates were removed and cells were resuspended as described above.

Human peripheral blood lymphocytes were isolated by buoyant-density centrifugation (10).

Protein synthesis assay. The cells were incubated in microtiter plates, each well contained 150 µl of cell suspension (final concentration $2x10^6$/ml) in leucine-free MEM supplemented as described above. Because serum ultrafiltrate (both SF and inactive control preparations) contains leucine, cells were cultured in the presence of a constant amount of serum ultrafiltrate (final concentration 20%), but with different ratios of active and inactive preparations. Cultures were incubated 2 to 4 h at 37°C in humidified air with 5% CO_2. At the start 0.6 µCi of L-(4.5-^{3}H)-leucine (specific activity 40-60 Ci/mmol, The Radiochemical Centre, Amersham, UK) was added to every well. Each sample was tested in triplicate. After incubation, the cells were harvested on filters and the radioactivity of the incorporated ^{3}H-leucine was measured with a liquid scintillation counter.

The effect of a β-adrenergic activator and of prostaglandin E_1 (PGE_1) on protein synthesis was also tested. To this end, isoproterenol in a concentration of 10^{-5} M and PGE_1 in a concentration of 10^{-6} M were added to the cultures containing 20% inactive SF. Inhibition of protein synthesis was obtained by addition at time 0 of cycloheximide 2 µg/ml. Stimulation index of protein synthesis was calculated as follows:

$$\text{Stimulation index} = \frac{\text{mean cpm stimulated culture}}{\text{mean cpm unstimulated culture}}$$

Individual results were compared by Student's t-test and dose response curves by the Fischer transformation test.

RESULTS

Fig. 1 shows the level of cyclic AMP and protein synthesis in mouse thymocytes incubated with SF and inactive SF. Whereas the level of cyclic AMP reaches its maximum after 5 min and decreases thereafter, the stimulation of protein synthesis continues to increase after 4 h.

Cycloheximide, a specific inhibitor of protein synthesis, fully prevents increase in the uptake of leucine by thymocytes, indicating that we are indeed measuring protein synthesis (Table I). Furthermore, the high molecular weight inhibitor of SF, present in human serum, reduces protein synthesis. The cyclic AMP elevating agents isoproterenol and PGE_1 also induced a consistent increase in protein synthesis, similar to that induced by low concentrations of SF (Table I).

The cyclic AMP-increasing activity of SF is thymus-dependent (6). To prove that the stimulation of protein synthesis by SF is a thymus-depen-

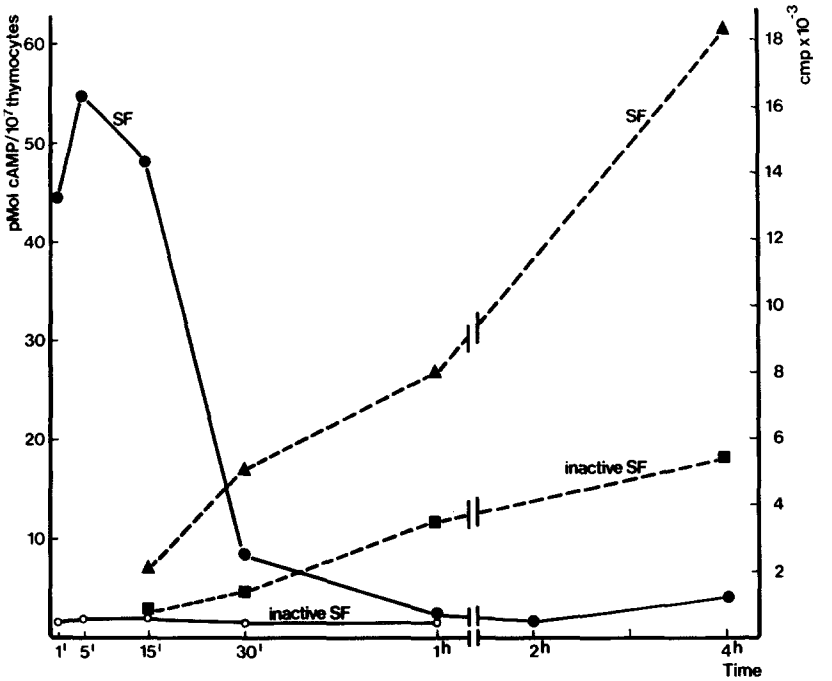

FIGURE 1

Effect of 20% SF on cyclic AMP levels (o——●) and protein synthesis (▲---■) in mouse thymocytes.

dent phenomenon, we tested the activity of SF obtained from sera of 10 non-thymectomized and 10 thymectomized myasthenia gravis patients. Except for one, all the sera from thymectomized patients tested in this study had no such cyclic AMP-increasing activity. Table II shows that SF from non-thymectomized patients stimulates protein synthesis as SF from healthy donors ($p>0.9$), whereas sera from thymectomized patients induce an increase of protein synthesis only when used at 20% concentration and to a significantly lower extent than sera from normals and non-thymectomized patients. The dose-response relationship found with SF from healthy donors and non-thymectomized patients is higher than that obtained with sera from thymectomized patients. The serum from the thymectomized patient, whose cyclic AMP-increasing activity remained high after thymectomy (42 pmol/10^7 thymocytes), stimulates protein synthesis as SF from normal donors (s.i. = 2.8) (Data not shown).

Table I

Stimulation indices of protein synthesis in mouse thymocytes incubated for 4 h with various agents

Substanced added	Stimulation index
SF 10% + inactive SF 10%	1.6 ± 0.03
SF 20%	2.5 ± 0.09
Cycloheximide 2 μg/ml + inactive SF 20%	0.2 ± 0.02
SF 10% + inactive SF 10% + cycloheximide 2 μg/ml	0,2 ± 0.03
SF 20% + cycloheximide 2 μg/ml	0.2 ± 0.03
High m.w. inhibitor 20%	0.3 ± 0.03
SF 10% + inactive SF 10% + high m.w. inhibitor 20%	0.6 ± 0.05
SF 20% + high m.w. inhibitor 20%	0.7 ± 0.02
PGE_1, 10^{-6} M + inactive SF 20%	1.4 ± 0.09
Isoproterenol 10^{-5} M + inactive SF 20%	1.4 ± 0.11

Results are expressed as mean stimulation index ± SEM of 3-18 experiments.

We demonstrated that the cyclic AMP-increasing activity of SF in thymocytes is restricted to HC-sensitive cells (6,7). To determine whether SF induces protein synthesis predominantly in HC-sensitive thymocytes, we investigated the leucine incorporation in different types of mouse lymphocytes. Table III shows that SF induces protein synthesis in HC-resistant thymocytes to a much lower extent than in normal mouse thymocytes. The stimulation of protein synthesis induced by SF in mouse spleen and lymph node cells was lower than that found in thymocytes; SF also increased protein synthesis in human thymocytes to a higher extent than in human peripheral blood lymphocytes, in agreement with our findings on the cyclic AMP-increasing activity of SF (6).

Table II

Thymus dependency of protein synthesis stimulating activity of SF in mouse thymocytes

	source of SF		
	normal donors	Tx patients	non-Tx patient
SF 1% + inactive SF 19%	1 1 ± 0.01	0.9* ± 0.06	1.1 ± 0.02
SF 3% + inactive SF 17%	1.2 ± 0.03	0.8* ± 0.06	1.2 ± 0.04
SF 5% + inactive SF 15%	1.3 ± 0.02	1.0* ± 0.05	1.4 ± 0.04
SF 10% + inactive SF 10%	1.6 ± 0.03	1.1* ± 0.07	1.7 ± 0.06
SF 20%	2.7 ± 0.12	1.8 ± 0.19	3.1 ± 0.25

Results are expressed as mean stimulation index ± SEM of 10-25 experiments. Mouse thymocytes were incubated for 2-4 h. All values are significantly different from stimulation index. 1 except those indicated with *.

Fischer transformation test: normal donors versus Tx patients $p < 0.001$

non-Tx patients versus Tx patients $p < 0.001$

DISCUSSION

The data presented in this study indicate that SF stimulates protein synthesis, in both mouse and human thymocytes. From our data, it may be concluded that SF used in final concentrations up to 10%, predominantly stimulates protein synthesis in HC-sensitive thymocytes, i.e. in precursors of mature T cells. We found that the protein synthesis stimulating activity of SF, at least when used in such low concentrations, is thymus-dependent. The stimulation of protein synthesis found with 20% SF is probably partially due to non-specific factor(s). In fact, when used at such high concentration, SF stimulates protein synthesis (but not cyclic AMP (7)) also in HC-resistant thymocytes and a stimulation of protein synthesis (but not of cyclic AMP (6)) occurs using sera from thymectomized donors. In addition, the dose-response relationship of protein synthesis stimulation induced by SF is linear for the SF dose range 1%-10%; the

Table III

Effect of SF on different lymphoid cells

	mouse				human	
	thymocytes	HC-resistant thymocytes	spleen cells	lymph node cells	thymocytes	peripheral blood lymphocytes
SF 1% + inactive SF 19%	1.1 ± 0.01	n.d.	1.0 ± 0.06	1.0 ± 0.05	1.2 ± 0.07	1.0 ± 0.07
SF 3% + inactive SF 17%	1.2 ± 0.03	n.d.	1.1 ± 0.06	n.d.	1.2 ± 0.05	0.9 ± 0.10
SF 5% + inactive SF 15%	1.3 ± 0.02	1.1 ± 0.03	1.1 ± 0.05	1.1 ± 0.05	1.3 ± 0.04	0.9 ± 0.07
SF 10% + inactive SF 10%	1.6 ± 0.03	1.2 ± 0.04	1.2 ± 0.05	1.3 ± 0.08	1.4 ± 0.02	1.1 ± 0.09
SF 20%	2.7 ± 0.12	1.6 ± 0.18	1.6 ± 0.13	1.5 ± 0.13	2.2 ± 0.11	1.2 ± 0.08

Results are expressed as mean stimulation index ± SEM of 4-25 experiments. Cells were incubated with SF of normal healthy donors for a period of 2-4 h.

Fischer transformation test: protein synthesis induction in: mouse thymocytes versus Hydrocortisone-resistant thymocytes $p<0.01$
mouse thymocytes versus spleen cells $p<0.001$
mouse thymocytes versus lymph node cells $p<0.01$
human thymocytes versus peripheral blood lymphocytes $p<0.01$

stimulation found with 20% SF was much higher than what would be expected from extrapolation. This indicates the presence of additional factor(s) increasing protein synthesis. Since thymectomy induces a marked decrease in the protein synthesis stimulating activity in human serum, it may be assumed that most of this activity is under the control of the thymus. Protein synthesis is one of the events occurring after stimulation of the cellular adenylate cyclase (11,12). Therefore, it is possible that the stimulation of protein synthesis by SF is a result of the increase in intracellular cyclic AMP. This is sustained by the finding that the stimulation of protein synthesis is preceded by the increase of cyclic AMP, and that cyclic AMP stimulating agents, such as PGE_1 and isoproterenol, induce stimulation of protein synthesis although to a lower extent than SF. Furthermore, SF stimulates cyclic AMP as well as protein synthesis to a higher extent in HC-sensitive thymocytes than in other lymphocytes.

So far, only THF and SF have been found to increase cellular levels of cyclic AMP in thymocytes (6,13). Thymosin, which has no effect on

cyclic AMP levels in thymocytes in vitro, induces the appearance of SF when injected into patients lacking SF activity (14).

It has been reported that cyclic AMP plays a role in the maturation of lymphocytes (15). In particular, exogenous cyclic AMP, and cyclic AMP elevating agents induce T-cell maturation in vitro as described for different thymic factors (16-19). Therefore, it is likely that T-cell maturation steps, such as appearance of membrane markers and induction of HC-resistance, are mediated by the cyclic AMP-increasing activity of thymic factors. It has been speculated that the changes induced by thymic factors might be due either to membrane rearrangement or to irreversible differentiation by gene activation (1). The finding that SF stimulates protein synthesis in thymocytes, probably as a consequence of increased cellular cyclic AMP, indicates that, most likely, an active intracellular process is induced by thymic factors.

Since it is known that for cell maturation synthesis of specific groups of proteins is required (12,20), qualitative studies on the proteins synthetized under the influence of SF are now currently being performed in our laboratories.

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