Increased Thymic Hormone Responsive Suppressor T Lymphocyte Function in Chronic Active Hepatitis

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Mitogen-induced suppressor T lymphocyte function was evaluated in patients with chronic active hepatitis (CAH). The in vitro *effect of the biological response modifier, thymosin fraction 5, on the suppressive activity of peripheral blood mononuclear cells (PBM) was also assessed. Suppressor cell activity was significantly decreased in patients with CAH when compared to controls* ($P < 0.001$)*. In the absence of the inducing mitogen, thymosin-treated PBM from both patients and controls promoted enhancement of tritiated thymidine uptake by cocultured allogeneic lymphocytes. When thymosin-treated mononuclear cells were mitogen-activated; patients, but not the controls, showed a marked increase in suppressor activity* ($P < 0.001$)*. These results indicate that the polypeptides contained in thymosin fraction 5 can promote a helper effect in patients and controls. Furthermore, PBM from patients with CAH contain a subset of lymphocytes that can express a suppressive function following thymosin treatment. We conclude that thymosin fraction 5 can promote an* in vitro *restoration of suppressor T cell function in patients with CAH.*

Chronic active hepatitis (CAH) represents the histological expression of diverse etiology associated with progressive destruction of hepatocytes. An immune-mediated disorder may be responsible for the perpetuation of hepatic injury observed in some patients with CAH (1–7). Abnormalities in immunoregulatory T lymphocytes have been described in a variety of immune-associated disorders. These regulatory lymphocytes, termed suppressor and helper T cells, appear to modulate and regulate both cell-mediated and humoral immune responses (8–10). Defects in nonspecific suppressor cell function have been reported in CAH (11–14), which suggests that the normal state of tolerance to hepatocyte autoantigens may be compromised, resulting in increased autoreactive hepatocytotoxic effector cell activity (14, 15).

In previous work we have shown that thymosin fraction 5, an extract of calf thymus glands, decreases the *in vitro* cytotoxic activity of peripheral blood mononuclear cells (PBM) obtained from patients with CAH (16). Other investigators have utilized biologically active thymus-derived factors to treat patients with chronic virus-B hepatitis and observed *in vivo* improvements in T cell counts and in immune reactivity (17).

The thymus gland is critical for the development, growth, and function of lymphoid tissue and for the maintenance of immune balance (18). The mechanism by which the thymus exerts its control over T

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cell development is not clear, but a critical part of this control involves the elaboration of hormones which regulate the differentiation of precursor T cells and influence the function of already differentiated peripheral T cells (19–22). Thymosin fraction 5 is composed of at least 16 polypeptides with differing biologic activities that can act individually, sequentially, or in concert to influence the development of T cell subpopulations (18). Thymosin has also been shown to exert specific effects on regulatory T cell function (18, 23–26).

Since thymosin fraction 5 contains polypeptides which can, at the same time, influence helper or suppressor activity, the present studies were undertaken to assess thymosin effects on the helper and suppressor activity of PBM obtained from patients with CAH.

MATERIALS AND METHODS

Patients. Eighteen patients had CAH by clinical, biochemical, and histological criteria (27). The clinical and biochemical characteristics of these patients are summarized in Table 1. There were 12 men and six women with an age range of 17–60 years (35.0 ± 3.0, means ± SEM). All patients had symptoms and/or biochemical abnormalities in tests of liver function for at least six months prior to biopsy and inclusion in the study. Histologic findings included the presence of piecemeal necrosis and bridging necrosis in all patients. Four individuals had concomitant early cirrhosis. None of the 12 patients lacking hepatitis B virus markers in their serum had clinical or morphological evidence to suggest alcohol abuse or exposure to known hepatotoxic agents. At the time of evaluation four patients were taking prednisone and 14 patients were untreated. Five untreated patients were subsequently placed on prednisone and evaluated once again one to four months after. Eighteen age- (35.4 ± 3.0 years) and sex-matched healthy volunteers not known to have current or previous liver disease were also studied. Patients and controls were excluded if they had malignancy, previous known immunodeficiency, a history of recent viral upper respiratory illness (28), or were recipients of blood transfusions within two months of the evaluation.

Isolation of PBM. Mononuclear cells were isolated from the peripheral blood of patients and controls using Ficoll-Hypaque gradients as previously described (16). PBM were resuspended at 5×10^6 cells/ml in RPMI-1640 medium (Gibco, Grand Island, New York) enriched with vitamins, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum (complete medium). Purified bovine thymosin fraction 5 was prepared (29) and stored at −20° C. Concentrations ranging from 50 to 400 μg/ml were used.

Helper and Suppression Assays. To measure helper and suppressor activity promoted by thymosin in the presence or absence of concanavalin A (Con A; Pharmacia Fine Chemicals, Piscataway, New Jersey), a mixed-lymphocyte-culture proliferation assay was used (23, 30) with modifications as previously described (31). In the initial culture, 0 or 20 μg of thymosin in a constant volume (0.1 ml) of complete medium was added to 1 ml of the PBM suspension and incubated at 37° C for 24 hr in a humidified 5% CO_2 and air atmosphere. Parallel tubes containing 1 ml of the PBM suspension plus 0.1 ml of complete medium were prepared for immediate use. The final volume in each tube was 1.1 ml. Following the 0- or 24 hr period of incubation, the PBM were resuspended, and 0.55 ml of each tube was incubated in the presence or absence of Con A (5 μg/ml) for an additional 48 hr. These constituted the 2-day and 3-day initial cultures, respectively. After incubation the control and Con A-activated cells were treated with 50 μg/ml of mitomycin C (Sigma Chemical Co., St. Louis, Missouri) for 30 min, and washed twice in 30 mM α-methylmannoside and once again in complete medium. The PBM were resuspended at 1×10^6 cells/ml in complete medium.

Fresh PBM (responder cells) from a healthy donor were adjusted to 2×10^6 cells/ml in complete medium. For the coculture experiments, cells from the 2- and 3-day initial cultures were incubated with fresh allogeneic responder cells from the same donor for 90 hr. The studies were performed in quadruplicate in flat-bottom microtiter plates (Costar, Cambridge, Massachusetts) to which were added 0.05 ml of responder PBM (1×10^5 cells), 0.1 ml of precultured PBM (1×10^5 cells), and 0.05 ml of complete medium containing Con A (40 μg/ml). In control wells 0.05 ml of complete medium without Con A were added such that the total volume in each well was 0.2 ml. Tritiated thymidine, 2 μCi per well (New England Nuclear, Waltham, Massachusetts), was added 18 hr prior to harvesting with an automatic multiple sample harvester (Otto Hiller Co., Madison, Wisconsin). The incorporation of [^{3}H]thymidine into cells was measured in a Beckman liquid scintillation system.

Effect of Preincubation on Suppressor Activity. Recent studies have reported a decrease in Con A-induced suppressor activity when PBM in initial culture are incubated for 24 hr prior to activation with Con A (32–34). The influence of a 24-hr preincubation period on suppressor activity was determined to assess *in vitro* loss of suppressor cell activity (SCA) and to evaluate the suppressor cell response to preincubation in the presence of thymosin.

Calculations of Helper/Suppressor Activity. The suppression of allogeneic responder cells by PBM from the 2- and 3-day initial cultures not containing thymosin was calculated as: % suppression = 1 − (Δ counts per minute (cpm) suppressor cells/Δ cpm control cells) × 100; where Δ cpm = cpm of Con A stimulated cocultures − cpm unstimulated cocultures; suppressor = Con A activated PBM from initial cultures; control = PBM not activated with Con A in initial cultures. Thymosin effect in the 3-day initial cultures was calculated as: % suppression = 1 − (Δ cpm thymosin pretreated suppressor cells/Δ cpm thymosin pretreated control cells) × 100. The effect of thymosin alone on SCA in the absence of Con A induction in the initial cultures was determined as: % suppression = 1 − (Δ cpm thymosin pretreated PBM/Δ cpm untreated PBM) × 100. A positive value indicated suppression of blast transformation in responder cells, and a

TABLE 1. CLINICAL DATA OF 18 PATIENTS WITH CAH

Patient	Age	Sex	Liver histology*	Therapy†	$HB_sAg/HB_sAb/HB_cAb$‡	ASMA/ANA§	SGPT‖ (IU/liter)	γ-globulin¶ (gm/dl)
1	17	M	CAH	O	−/−/−	+/+	680	2.4
2	32	F	CAH	O	−/−/−	−/−	365	3.0
3	33	M	CAH	O	−/−/−	−/−	200	1.9
4	24	M	CAH	O	−/−/−	−/+	880	2.1
5	22	M	CAH	O	−/−/−	−/−	110	1.4
6	19	F	CAH	O	−/−/−	+/−	640	4.6
7	26	M	CAH-C	O	−/−/−	−/−	144	2.2
8	27	F	CAH	P	−/−/−	+/ND	81	2.9
9	47	M	CAH	P	−/−/−	−/−	65	ND
10	47	F	CAH-C	O	−/+/−	−/−	23	1.9
11	54	M	CAH	O	+/−/+	−/−	156	2.3
12	60	M	CAH	P	+/−+	−/−	405	3.8
13	47	F	CAH	P	−/−/−	−/−	550	2.1
14	52	F	CAH-C	O	−/+/+	+/+	302	2.7
15	32	M	CAH	O	+/−/+	+/−	199	2.0
16	36	M	CAH-C	O	−/−/−	+/−	112	3.0
17	30	M	CAH	O	+/−/+	−/−	49	1.6
18	25	M	CAH	O	+/−/+	−/−	109	2.3

*CAH, chronic active hepatitis; CAH-C, chronic active hepatitis with cirrhosis.
†P, prednisone; O, none.
‡HB_sAg, hepatitis B surface antigen; HB_sAb, hepatitis B surface antibody; HB_cAb, hepatitis B core antibody; +, positive; −, negative.
§ASMA, anti-smooth muscle antibody; ANA, antinuclear antibody; ND, not determined.
‖Serum glutamic-pyruvic transaminase.
¶Serum γ-globulin.

negative value reflected enhancement of responder cell transformation. Statistical evaluation of SCA in patients and controls was accomplished with Student's t test. The significance of the modulation in SCA in the presence of thymosin was determined using Student's paired t test.

RESULTS

Determination of Optimal Thymosin Concentration. We have previously shown that thymosin does not exert a mitogenic effect on PBM and, in healthy volunteers, maximum suppression of responder cells is observed when PBM in initial cultures are incubated with 10 and 20 μg/ml of thymosin (31). Similar results were seen when PBM from six patients with CAH were incubated in the absence of thymosin (−0.53 ± 2.4; % suppression ± SEM) or with 5 μg/ml (−21.4 ± 11.1), 10 μg/ml (9.8 ± 2.7), 20 μg/ml (15.4 ± 3.2), and 40 μg/ml (6.2 ± 2.9) of thymosin. The effect of thymosin alone on the suppressive function of PBM was determined by omitting Con A from the initial cultures. A dose-response was observed in patients and controls with enhancement rather than suppression of [^{3}H]thymidine incorporation by Con A-stimulated responder cells. Maximal enhancement was observed at 10 and 20 μg/ml of thymosin. A thymosin concentration of 20 μg/ml was therefore used for the remaining experiments.

Con A-Induced Suppression of Mixed-Lymphocyte-Culture Proliferation. The suppression of fresh allogeneic responder cells by PBM from the 2-day initial cultures is depicted in Figure 1 and Table 2. A significant decrease in SCA was observed in patients with CAH, irrespective of seropositivity for the hepatitis B virus. No differences were noted between patients with CAH on prednisone treatment (−0.5 ± 4.6%; mean ± SEM) and those not receiving medication (−0.8 ± 3.4%; P = NS). SCA in the controls was substantially decreased in the 3-day initial cultures not containing thymosin as compared to the 2-day initial cultures (Table 2). There was no corresponding change in the low SCA observed in the patient group.

Thymosin Effect on Suppressor and Helper Activity. Thymosin pretreatment of PBM in the 3-day initial cultures markedly increased SCA in the patient group as compared to parallel untreated cultures. The responses were the same when CAH patients were stratified by seropositivity for the hepatitis B virus or by the presence or absence of steroid therapy. Moreover, the suppression promoted by thymosin in the CAH group was virtually identical to that seen in the controls, in whom no

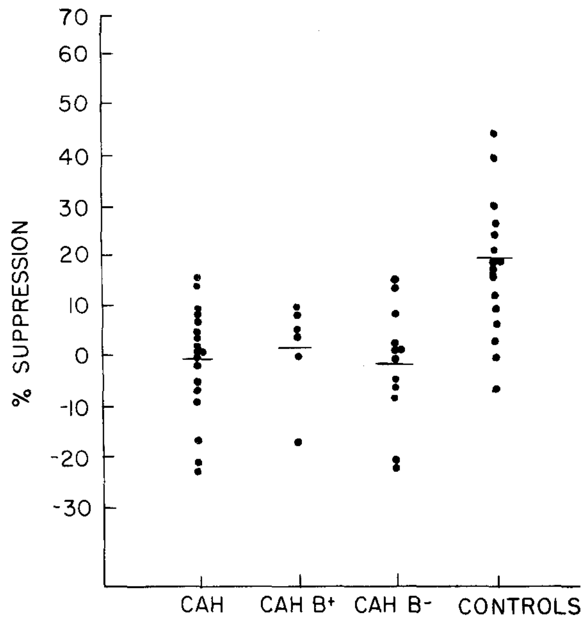

Fig 1. Con A-induced suppressor cell activity of peripheral blood mononuclear cells in 2-day initial cultures. CAH, total group of patients with CAH; CAH B+, hepatitis B-positive subgroup; CAH B-, hepatitis B-negative subgroup.

significant change in SCA was observed after thymosin treatment (Table 2).

The influence of thymosin alone on SCA was determined by omitting Con A activation of suppressor cells in the 3-day initial cultures. Enhancement rather than suppression of Con A-stimulated blast transformation of allogeneic responder cells was observed in both the patient and control groups (Table 2).

Influence of Prednisone Treatment on SCA. Five patients were examined prior to and from one to four months after initiation of prednisone therapy at 10–20 mg daily. SCA in the 2-day initial cultures were not different before and after initiation of treatment (2.5 ± 4.0%, before; −0.1 ± 8.7%, after). In a similar manner the response to thymosin did not differ before and after initiation of treatment.

Correlation Between SCA and Biochemical Tests of Liver Function. There were no individual correlations between the SCA observed in the 2-day initial cultures, the suppressor cell response to thymosin in the 3-day cultures, the level of enhancement promoted by thymosin alone, and biochemical tests of liver function including total bilirubin, SGOT, SGPT, alkaline phosphatase, and serum gamma globulin levels.

DISCUSSION

Our previous observation that thymosin fraction 5 decreased the *in vitro* cytotoxic activity of PBM from patients with CAH suggested that this biological response modifier influenced a subset of lymphocytes that directly mediated cytotoxic activity or that exerted immunoregulatory control over the actual effector cells (16). The initial report of a decrease in Con A-induced SCA in patients with CAH (11) prompted the present study to determine the effect of thymosin on suppressor T cell function. Subsequent investigations have also described alterations in suppressor activity in patients with CAH employing assays that monitor T cell suppression of pokeweed mitogen-induced immunoglobulin

TABLE 2. EFFECT OF INCUBATION TIME AND THYMOSIN ON SUPPRESSOR CELL ACTIVITY

	Initial cultures				
Study group	*Duration (Days)*	*PBM treated with thymosin*	*Con A activation*	*Suppression (%, mean ± SEM)*	P *value*
CAH (18)*	2	0	+	−0.9 ± 2.7	<0.001
Controls (18)	2	0	+	19.8 ± 4.3	
CAH (18)	3	0	+	−1.3 ± 4.0	<0.05
Controls (18)	3	0	+	7.7 ± 1.9†	
CAH (18)	3	+	+	12.9 ± 3.1‡	NS
Controls (18)	3	+	+	11.2 ± 1.9	
CAH (18)	3	+	0	−13.8 ± 2.8	NS
Controls (18)	3	+	0	−8.6 ± 3.3	

*Number of subjects tested given in parenthesis
†$P < 0.02$ compared to 2-day initial cultures of controls, Student's *t* test.
‡$P < 0.001$ compared to 3-day initial cultures in the absence of thymosin, Student's paired *t* test.

synthesis (12), mitogen (Con-A) -induced suppressor T cells (14), short-lived suppressor T cells (13), prostaglandin-producing suppressor (monocyte) cells (35), and spontaneous suppressor (monocyte) cells (14).

In preliminary work we noted that PBM from patients with CAH required a 24-hr incubation with thymosin in order to elicit an increase in SCA. This requirement for a 24-hr preculture introduced the possibility that a loss in short-lived suppressor cells might occur in the 3-day initial cultures. These cells appear to constitute a distinct group of Con-A inducible suppressor cells (33, 34, 36).

As shown in Table 2, there was a significant decrease in SCA in the controls between the 2-day and 3-day initial cultures with no appreciable change in SCA seen in the patient group. Thus, if there were few suppressor cells present in the PBM of patients with CAH, a 24-hr preculture period would not have influenced the already low suppressor activity. Conversely the "normal" suppressor activity in the controls would decrease with preincubation. Thymosin did not maintain the functional capacity of suppressor cells from the controls, but it did increase SCA in patients with CAH.

It is possible that there are increased numbers of precursor suppressor cells (37, 38) in patients with CAH, but not in healthy controls, that differentiate into suppressor cells in the presence of thymosin with subsequent induction by Con A into activated cells. This hypothesis is supported by studies reporting a suppression of the proliferative responses of human PBM to a variety of mitogens by a thymosin-induced suppressor T cell generated from a precursor population (39, 40). Furthermore, it is not likely that thymosin decreased a helper T cell effect in patients with CAH, resulting in an altered helper–suppressor balance, as we have shown that PBM incubated in the presence of thymosin alone promoted enhancement of proliferation in the responder cells (Table 2). Moreover, while thymosin has been shown to mediate a predominantly helper effect in normal human peripheral blood T cells, Con A induction can unmask the equivalent suppressor activity in thymosin-pretreated PBM to that observed in PBM from the same individual not pretreated with thymosin (31). Alternatively, thymosin may have corrected intrinsic suppressor cell defects in patients with CAH or countered the *in vivo* influence of extrinsic factors such as immune complexes and antibodies to suppressor cells which might impair suppressor cell function (41, 42). Conceivably, thymosin may have modulated the function of a suppressor T cell subset which in turn regulates an effector–suppressor T subset (43).

Although conclusive evidence is lacking, the target lymphocyte influenced by thymosin is most likely a T cell. This hypothesis is based on the following observations. First, thymosin-treated human null cells possessing T but not B cell antigens will form T cell E rosettes (ER) (44). Furthermore, B cell and monocyte numbers or function are not directly altered by thymosin (45, 46). Second, *in vitro* studies have shown that thymosin can increase both T cell ER and Con A-induced suppressor activity in patients with systemic lupus erythematosus (SLE) (23, 45). Thymosin-induced increases in T cell ER, both *in vitro* and *in vivo*, have also been reported in patients with CAH (17, 47). Third, studies in animals indicate that thymosin fraction 5 can induce *in vitro* suppressor cells which then inhibit the induction of cytotoxic T cells in a mixed lymphocyte–tumor cell culture. These suppressor cells have been characterized as Thy 1-positive, nonadherent, short-lived T cells (48).

A variety of immunopathologic disorders are associated with perturbations in immunoregulatory balance. Thus autoimmunity may result from deviations in the function of helper or suppressor cells (49). Interpretation of the suppressor cell dysfunction in CAH as reported in previous studies as well as the present study is difficult. All published reports have underscored the lack of an association between suppressor cell function and biochemical changes, clinical status, or the presence of steroid therapy. This inability to relate suppressor cell function in CAH to other parameters of disease activity (7) may imply that the described regulatory cell changes represent the consequences of, rather than the causes for, perpetuation of the disease state (50). On the other hand, the lack of a correlation between SCA and disease indicators may suggest that the suppressor cell defect in CAH is genetically predetermined and is a predisposing factor to initiation or perpetuation of the disease. Support for this viewpoint has been shown in studies where family members of patients with SLE, in whom suppressor cell defects are regularly found, also have suppressor cell defects in the absence of clinical disease (51).

It is apparent that the relationship between defective suppressor cell activity and the pathogenesis of CAH requires further examination. The present study suggests that the PBM of patients with CAH

contain a subset of T lymphocytes which, in the presence of thymic hormone, can express suppressor function following Con A induction. This raises the intriguing prospect that there may be an alteration in thymic regulation of T cell differentiation and function in patients with CAH.

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