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Alteration in suppressor cell activity in chronic active hepatitis*

(concanavalin A/lymphocytes/prednisone/cell culture)

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ABSTRACT **We have studied peripheral blood mononuclear cells obtained from 24 patients with acute or chronic active hepatitis to determine if there was an abnormality in concanavalin A-induced suppressor cell activity compared to control subjects. Suppressor cells were generated by preincubation of the mononuclear cells with a mitogenic concentration of concanavalin A (6 μg/ml) for 48 hr followed by treatment with mitomycin C and α-methyl mannoside. Suppressor cell activity was assessed in second cultures by inhibition of concanavalin A-stimulated blast transformation of fresh allogeneic lymphocytes. Concanavalin A-stimulated suppressor activity was not elicited in mononuclear cells from the majority of patients with chronic active hepatitis in contrast to patients with acute hepatitis or acute inflammatory diseases and controls ($P < 0.001$). This finding was demonstrable in chronic active hepatitis patients in remission and relapse, both on and off prednisone therapy, and varied considerably during the course of the disease. The extent of liver injury was not related to the measured suppressor cell activity. These studies suggest that in chronic active hepatitis, a disease in which the host immune response may be involved, there appears to be a defect in concanavalin A-stimulated suppressor cells.**

Recent studies in a number of experimental animal models have indicated that the expression of cellular and humoral immunity by specific antigen-stimulated lymphocytes is regulated by helper and suppressor cells (1–7). These immunoregulatory lymphocytes are T cells (8–10). It has become apparent that similar cells with an immunoregulatory function may be present in man (11, 12). These cells, however, are not exclusively confined to the T-cell population. Thus, it has been postulated that abnormalities in the function of suppressor cells may contribute in part to variable hypogammaglobulinemia (13, 14), multiple myeloma (15), Hodgkin's disease (16), systemic lupus erythematosus (17, 18), and severe fungal infections (19).

Chronic active hepatitis (CAH) is a progressive destructive inflammatory form of liver disease that is often accompanied by clinical and serological evidence of autoimmunity (20). Although the pathogenesis of CAH is poorly understood, current evidence favors a persistent cellular immune response directed against antigens present on liver cells (21–23). Many individuals with CAH have an initial episode indistinguishable from acute viral hepatitis. However, in the majority of patients with acute hepatitis infection, complete recovery is the rule. It seems reasonable to postulate, therefore, that an abnormal control of the immune response may contribute in part to the persistence and progression of the disease. We have investigated patients with acute or chronic active hepatitis and controls by measuring convanavalin A (ConA)-stimulated suppressor cell activity (24). Our investigation demonstrates an altered suppressor cell response in patients with CAH.

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MATERIAL AND METHODS

Patients. Fourteen patients (age range, 21–72 years; mean, 40.1 years) were studied (Table 1). Three men were positive for hepatitis B surface antigen (HBsAg). Three patients were on prednisone therapy (8, 20, and 20 mg/day) at the time of study. An additional three women (patients 1, 13, and 14) had completed a 1- to 2-year course of prednisone and were asymptomatic with normal SGOT, bilirubin, and alkaline phosphatase values at the time of investigation. Liver biopsy in these three demonstrated complete resolution of the disease process. Ten subjects (age range 23–74 years; mean, 37.9 years) with acute hepatitis (four with acute hepatitis B, four with non-B hepatitis, and two with severe halothane hepatitis) were also studied. All were investigated during the acute icteric phase of their disease. However, in three patients with viral hepatitis infection, serial studies were performed very early in the disease process before the SGOT values peaked. In these 10 subjects, mean serum values were: SGOT, 940 IU/liter; bilirubin, 7.4 mg/100 ml; and alkaline phosphatase, 9.2 Bodansky units. The control groups consisted of 18 subjects (age range, 19–72 years; mean, 41.5 years), of whom five were normal, healthy individuals. The 13 others were hospitalized patients with noninflammatory disorders such as peptic ulcer, hiatus hernia, or cardiac arrhythmia. Finally, five subjects (age range 20–72 years; mean, 46.0 years) with an acute inflammatory disease process (three with lobal pneumococcal pneumonia, one with cellulitis, and one with disseminated gonococcal infection) were also investigated.

Suppressor Cell Assay. Mononuclear cells were prepared from 40 to 50 ml of fresh heparinized venous blood by dilution with an equal volume of isotonic saline and centrifugation through a Ficoll/Hypaque sedimentation gradient 400 × *g* for 35 min; the mononuclear cell band at the saline–Ficoll/Hypaque interface was removed. For each assay, cells from a control were included for comparison. Cells were washed three times (400 × *g* for 10 min at 4°) in phosphate-buffered saline and resuspended in RPMI 1640 medium (Microbiological Associates, Bethesda, MD) enriched with 10% heat-inactivated fetal calf serum, 20 mM glutamine, penicillin (1000 units/ml) and streptomycin (100 μg/ml) (complete medium). Under these conditions, cell viability was >95% by trypan blue exclusion. The mononuclear cells were adjusted to a concentration of 3–5

Abbreviations: CAH, chronic active hepatitis; Con A, concanavalin A; HBsAg, hepatitis B surface antigen; IU, international units; MLC, mixed lymphocyte culture; SGOT, aspartate aminotransferase (EC 2.6.1.1) (serum glutamic-oxaloacetic transaminase).

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Table 1. Clinical data on 14 patients with chronic active hepatitis

Patient	Age, years; sex	HBsAg	Bilirubin, mg/100 ml	SGOT,* IU/liter	Alkaline phosphatase, Bodansky units	Prednisone, mg/day
1	21; F	−	0.3	56	4.3	8
2	35; F	−	0.4	56	17.4	0
3	36; F	−	0.1	24	1.9	0
4	56; M	−	1.4	100	26.2	20
5	72; F	−	0.4	142	9.6	0
6	57; M	−	1.4	420	9.5	0
7	42; F	−	4.2	160	18.4	0
8	26; M	+	0.6	72	3.9	20
9	30; M	+	0.3	115	2.6	0
10	26; M	+	0.7	54	11.1	0
11	40; M	−	2.5	264	30.6	0
12	52; F	−	0.9	125	11.8	0
13	28; F	−	0.5	40	4.0	0
14	41; F	−	0.4	36	3.2	0
Mean†	40		1.1 (<1.0)	119 (<45)	12.4 (<4.2)	

* Aspartate aminotransferase, in international units/liter (serum glutamic-oxaloacetic transaminase).
† Normal values in parentheses.

$\times 10^6$/ml and incubated with or without Con A (6 μg/ml) for 48 hr at 37° under 95% air/5% CO_2. After this incubation, control and Con A-activated cells were treated with mitomycin C (50 μg/ml) (Sigma) for 30 min and extensively washed with complete medium. Next, both cell populations were washed three times with 30 mM α-methyl mannoside in phosphate-buffered saline to remove Con A, washed again, and resuspended in complete medium at a concentration of 1×10^6 cells per ml.

Fresh mononuclear cells (responder cells) from a normal donor were prepared as described above and adjusted to a concentration of 1×10^6 cells per ml in complete medium. One of us (H.J.F.H.) served as the source of responder cells in all experiments, unless specified otherwise, to ensure a reproducible Con A-reactive responder cell population. In addition, it was difficult to obtain autologous responder cells from ambulatory CAH patients 48 hr later. For the Con A-induced blast transformation studies, responder cells were incubated for 72 hr in the presence of either the Con A-pretreated cell population or the control cells. In the mixed lymphocyte culture (MLC) experiments, Con A-pretreated cells from CAH patients and controls were incubated with allogeneic responder cells for 90 hr. These studies were performed in quadruplicate in microtiter plates (Cook Engineering Co., Alexandria, VA) to which were added 0.1 ml of responder cells, 0.1 ml of suppressor or control cell suspension, and 0.025 ml of Con A (60 μg/ml) in RPMI medium. RPMI medium (0.025 ml) was added to control cultures to ensure a constant volume. To each well was added 2 μCi of [^{3}H]thymidine (specific activity, 40–60 Ci/mmol, New England Nuclear, Waltham, MA) 18 hr prior to harvesting with a Mash II microtiter automatic cell harvester (Microbiological Associates). Incorporation of [^{3}H]thymidine into cells was measured by liquid scintillation counting.

Data are expressed as mean cpm from quadruplicate cultures, and the degree of suppression was calculated according to the following formula:

$$\text{Percent suppression} = 1 - \frac{\text{cpm}_S\text{·Con A} - \text{cpm}_S\text{·0}}{\text{cpm}_C\text{·Con A} - \text{cpm}_C\text{·0}} \times 100$$

in which cpm_S·Con A is mean cpm of [^{3}H]thymidine incorporation in cultures containing suppressor cells and Con A, cpm_S·0 is [^{3}H]thymidine incorporation in cultures containing suppressor cells but no Con A, cpm_C·Con A is [^{3}H]thymidine incorporation in cultures containing control cells and Con A, and cpm_C·0 is [^{3}H]thymidine incorporation in cultures containing control cells but no Con A. The statistical significance of the suppression was tested with Student's t test.

RESULTS

Characteristics of the Suppressor Cell Assay. Preliminary experiments demonstrated that mitogenic concentrations of Con A in the first culture were necessary to produce the maximal suppression of Con A-induced blast transformation of autologous and allogeneic responder cells. Dose-response curves (range, 0.1–30 μg/ml) of Con A-pretreated cells demonstrated a maximal suppressive effect between 5 and 10 μg/ml (data not shown). This observation is similar to the findings of others (24–26). The reproducibility of the assay as measured in normal individuals was also studied. Serial weekly assays in one individual with the responder cells autologous to the Con A-pretreated cells yielded values of 48, 66, and 32%; in another individual, less variability was observed (68 and 63%). In an additional study, responder cells allogeneic to the suppressor cells but always obtained from the same individual gave successive values of 46, 45, and 47%. Therefore, in the present investigation the responder cells were prepared from a single donor. This was of particular importance in serial studies of patients with CAH because clinical and biochemical aspects of the patient's disease were correlated with the suppressor assay and response to prednisone therapy.

We assessed the effect of Con A-pretreatment at various concentrations on the generation of suppressor cell activity in the MLC reaction. In these experiments, cells in the first culture were pretreated with Con A for 48 hr (concentration range, 0.1–30 μg/ml), washed, and treated with α-methyl mannoside and mitomycin C. Cells were incubated for 90 hr with allogeneic responder cells from a single donor. As shown in Table 2, increasing concentrations of Con A caused progressive suppression of the MLC reaction, reaching a peak response between 5 and 10 μg/ml. This observation is similar to the findings in the Con A-stimulated blast transformation experiments be-

Table 2. Suppressor cell activity generated by various concentrations of Con A and [^{3}H]thymidine incorporation by allogeneic responder cells in the MLC reaction

Con A,* μg/ml	[^{3}H]Thymidine incorporation,† cpm	% suppression
0	15,593	—
0.1	15,751	1.0
0.5	14,502	7.0
1.0	9,241	40.8
2.0	5,413	65.3
5.0	3,816	73.6
10.0	2,882	81.6
20.0	3,019	80.7
30.0	6,187	60.4

* Concentration of Con A used to generate suppressor cells in the first culture.
† Data expressed as mean from four replicate cultures; SEM < 7% for all cultures.

cause mitogenic concentration of Con A was required to produce a maximal suppressor cell effect. Finally, we were concerned about the carryover of Con A from the first culture into the second culture. No difference was observed between unstimulated cultures with Con A-activated cells and control cells.

Con A-Stimulated Suppressor Cell Activity in Patients with Acute or Chronic Active Hepatitis. Con A-generated suppressor activities in various patient groups are depicted in Fig. 1. In CAH, the mean level of suppressor cell activity was substantially reduced as compared to controls, acute hepatitis, and inflammatory disease controls ($P < 0.001$). However, some patients with CAH showed considerable overlap with the

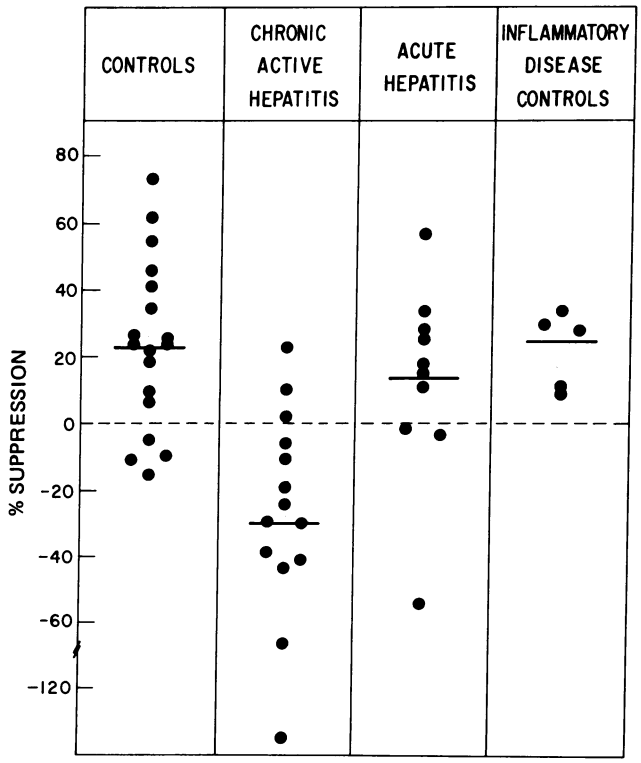

FIG. 1. Con A-induced suppressor cell activity from peripheral blood mononuclear cells from various study groups.

Table 3. Representative examples of suppressor activities of Con A-activated cells on the mitogenic responses of responder cells in CAH and controls

Test*	$\frac{cpm_S\text{-Con A}}{cpm_C\text{-Con A}}$	$\frac{cpm_S\text{-0}}{cpm_C\text{-0}}$	% suppression
P	75,246 / 46,337	12,658 / 8,660	−66.0
C	79,994 / 127,968	16,089 / 31,013	34.1
P	109,742 / 57,000	13,948 / 8,098	−136.0
C	66,236 / 122,485	6,590 / 13,637	45.3
P	95,575 / 79,199	8,470 / 5,131	−22.5
C	66,236 / 122,485	6,590 / 13,637	45.3
P	105,780 / 90,443	23,046 / 24,632	−20.3
C	103,022 / 118,978	17,216 / 18,262	14.8
P	143,167 / 139,836	15,961 / 20,607	−6.7
C	54,081 / 114,341	17,470 / 21,759	60.5

SEM < 7% in all cultures.
* P, patient; C, control.

control group. Table 3 presents representative comparisons of data between controls and patients with CAH to illustrate the magnitude of changes observed in the cultures. Because of the effect of age on the magnitude of Con A-induced suppressor cell activity, we age-matched our study groups as closely as possible (25).

Correlation of the clinical data with the measured suppressor cell activity suggested that hepatic inflammatory disease activity as judged by SGOT values was not necessarily associated with an altered suppressor response. In patients with acute hepatitis the mean SGOT was 940 IU/liter at the time of study, compared to a mean of 119 IU/liter for CAH. Furthermore, if the suppressor cell activity was within the normal range during the acute phase of hepatitis, it remained normal after recovery (data not shown). Of interest were serial investigations on three patients with acute hepatitis infection studied early in the disease process before the peak rise in SGOT. These individuals gave the three lowest values (Fig. 1) for suppressor activity but returned to normal values (mean, 28.4%) with complete resolution of the hepatitis. In contrast, patients with CAH demonstrated variability in the Con A-elicited suppressor cell activity as shown in Table 4; suppressor cell activity was related in part to clinical remission. This observation, however, was not a uniform finding, as illustrated by three female patients (Table 1, patients 3, 13, and 14) with HBsAg-negative CAH who developed positive antinuclear and smooth muscle antibodies and hypergammaglobulinemia during the course of their illness. These three had received prednisone therapy for 1–2 years but were off treatment at the time of study. There was no evidence of disease activity by liver biopsy. Con A-stimulated suppressor cell measurements were −20.3, −32.7, and −40.2%, respectively. This finding at least raises the pos-

Table 4. Representative serial studies on suppressor activities of Con A-activated cells on Con A-induced blast transformation of responder cells in three patients with CAH

Patient	% suppression*	Comment†
1	26.7	Remission
	−1.7	Relapse; Pred., 20 mg/day
	−66.2	Relapse; Pred., 30 mg/day
	21.7	Remission; Pred., 10 mg/day
2	−10.2	Untreated
	43.1	Remission; Pred., 30 mg/day
	20.1	Remission; Pred., 15 mg/day
3	−22.5	Untreated
	17.9	Remission; Pred., 30 mg/day

* Single donor used as source of responder cells.
† Serial studies over 2–10 months. Relapse and remission judged on the basis of clinical and biochemical criteria (26). Pred. = prednisone.

sibility of an intrinsic abnormality in Con A-stimulated suppressor cells in some patients with CAH. Diminished suppressor cell activity was observed in patients during remission and relapse, both on and off prednisone therapy.

Finally, we observed a dissociation in Con A-generated suppressor cell activity when separately comparing the depression of [^{3}H]thymidine incorporation in cells responding to Con A and the MLC reaction. The mean value for Con A-stimulated suppressor cell activity was −30%, compared to 14.0% in the MLC reaction ($P < 0.01$). It is possible that a heterogeneity of suppressor cells or the suppressor cell effect exists in the Con A-generated population.

DISCUSSION

Some properties of the Con A-stimulated suppressor cell population have recently become known. It is apparent that the suppressor effect requires at least a 24-hr incubation with Con A at mitogenic concentrations. Con A-activated cells suppress [^{3}H]thymidine incorporation in both allogeneic and autologous responder cells to Con A and phytohemagglutinin and also suppress a one-way MLC reaction. Furthermore, lymphocyte separation studies have demonstrated that Con A-stimulated T cells, but not B cells, suppress antigen-specific and mitogen-stimulated proliferative responses of both B and T responder cells. The putative suppressor cells have been identified as a high-density T cell that exhibits a poor blastogenic response to Con A (27). Thus, in man, lymphocyte blast transformation is not necessarily a prerequisite for induction of the suppressor cell phenomenon. These studies suggest that the observed suppression is specific and that there exists a resting T-cell population programmed to express suppressor activity even before exposure to Con A (25, 27).

In the present studies, mitogenic concentrations of Con A were necessary to generate the suppressor effect. Because a previous report emphasized the variability of the suppressor response with different allogeneic lymphocytes, we chose to use a single Con A-responsive allogeneic responder cell population. It also seemed important to age-match our study groups as closely as possible because Con A-induced suppressor cells appear to decrease with increasing age (25). Aging has been associated with the appearance of autoantibodies, and it has been suggested that one normal function of suppressor cells is the maintenance of "self-tolerance," because cellular and humoral immune reactivity directed against self-antigens might result from a defect in suppressor cell activity (25, 28). It is noteworthy that many female patients with CAH have circulating antinuclear, antimitochondrial, antismooth muscle, antithyroid, antigastric, and antiadrenal antibodies and thus have lost "self-tolerance" (20).

Animal studies demonstrate that immune responses to thymus-dependent antigens and their modulation by immunoregulatory T lymphocytes are strictly controlled in an antigen-specific manner by the operation of a group of genes located at the major histocompatibility complex (10). Of interest in this regard is the increased incidence of *HLA* haplotypes *B1* and *B8* in female patients with CAH (29). These studies provide some evidence of a genetic predisposition for the acquisition of this disease. In a limited number of female patients with CAH in complete remission, we were unable to elicit a Con A-induced suppressor cell population; *HLA* typing of such patients, therefore, will be of prime importance.

It should be emphasized that in the suppressor cell values obtained from patients with CAH there was considerable overlap with other control groups. Indeed, some CAH patients clearly fell within the control range. More importantly, serial studies on individual patients revealed striking differences in suppressor cell activity, particularly with prednisone therapy and the induction of a clinical remission. These observations illustrate the importance of serial studies because of the variable results of the suppressor cell assay during the course of the illness. Similarly, in acute hepatitis we noted a depression of suppressor cell activity early in the disease, before the appearance of peak SGOT values. Thus, loss of Con A-induced suppressor cell activity may be an early feature in acute viral hepatitis. However, a larger group of patients needs to be studied before this possibility can be firmly established.

It is uncertain whether the lack of Con A-induced suppressor activity is a reflection of the disease state or represents a preexisting abnormality that may predispose to the development of CAH. Serial studies indicate that, during disease relapse, less suppressor cell activity was demonstrable; however, we observed untreated patients and those in remission who had an altered suppressor cell response. It seems unlikely that diminished suppressor cell activity is due solely to hepatic inflammation because most patients with acute hepatitis, with a presumed more severe hepatocellular impairment, demonstrated less of a disturbance in the generation of suppressor cells. Furthermore, the acute inflammatory disease controls showed a normal Con A-induced response.

In most of our patients with CAH, the Con A-generated suppressor cell response was not observed; indeed, the opposite phenomonon was seen. That is, cells preincubated with Con A enhanced Con A-stimulated [^{3}H]thymidine incorporation rather than suppressed it and behaved like helper cells. The mechanism of this reversal is entirely unknown. It is possible that, in the absence of a population or subpopulation of suppressor cells, a normal helper cell population was revealed. Alternatively, the presence of an abnormal helper cell population may have masked suppressor cell activity. Patients with CAH have been shown to have a decreased number of circulating T lymphocytes (30), and thus lack of Con A-induced suppressor cell activity could be due to the absence of a Con A-responsive T-cell population. In a previous study, the mitogenic response of peripheral blood lymphocytes to Con A was no different in CAH patients than in controls (31). Lymphocyte blast transformation, however, is not a necessary prerequisite for the development of T-cell suppressor cell activity (27). As shown by others (24–25, 27, 32) and in the present investigation, suppressor cell activity can be measured in normal subjects by

using these techniques. Our findings indicate that this response is diminished in many patients with CAH.

There is increasing evidence in experimental animal models that suppressor cells do not represent a single homogeneous population. On the contrary, recent data indicate existence of subpopulations of suppressor cells with different antigenic and mitogenic specificities (33–37). It is quite likely that Con A-pretreatment of lymphocytes may activate one or more of these subpopulations to variable degrees. The heterogeneity of suppressor cells may explain in part the observed dissociation between lack of suppression in the Con A-stimulated blastogenic response of responder cells compared to the MLC reaction in CAH. Additional studies are needed in CAH to determine if there is a preferential loss of suppressor influence for the mitogenic response of both B and T cells and, more importantly, specific antigen response of these cells before firm conclusions can be reached regarding the biological significance of altered Con A-induced suppressor activity in CAH.

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