Suppressor Function of Peripheral Blood Mononuclear Cells in Normal Individuals and in Patients with Systemic Lupus Erythematosus

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ABSTRACT Normal peripheral blood mononuclear cells demonstrated increased DNA synthesis and secretion of newly synthesized protein when suboptimal concentrations of Concanavalin A (Con A) were added to the cultures after 24-h incubation in vitro. Cells stimulated by Con A, 1 μg/ml, after 24-h incubation demonstrated 3.0 times more tritiated thymidine incorporation, and 4.4 times more ^{14}C-amino acid incorporation into newly synthesized secreted protein, than cells stimulated at 0 h ($P < 0.001$). The acquisition of increased responsiveness was not abrogated by washing and resuspending the cells in fresh medium. Since the increased responsiveness could be inhibited by the addition to the cultures of small numbers of cells previously activated by Con A it is suggested that the enhanced reactivity acquired in culture represents the loss of a subpopulation of suppressor cells that modulate the T-lymphocyte response. Cells from nine patients with active, untreated systemic lupus erythematosus demonstrated normal responses to optimal concentrations of Con A added at 0 h, but an impaired response to Con A, 1 μg/ml. When these cells were incubated for 24 h, a significant increased response to Con A was not observed. This observation suggests that patients with active SLE lack circulating suppressor cells. When seven SLE patients were again studied after corticosteroid therapy had led to clinical improvement, the response to Con A, 1 μg/ml, added after 24-h incubation was similar to that observed in normal controls, suggesting that suppressor function in SLE returns as disease activity declines.

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INTRODUCTION

It is now well established that a subpopulation of lymphocytes can function as suppressor cells in the regulation of the immune response (1). A wide variety of model systems in rodents have been used to describe the properties of such cells. It has been shown that suppressor cells may be induced by any of several immunological stimuli, including specific antigens (2–5), nonspecific mitogens (6–9), graft-versus-host disease (10, 11), mixed lymphocyte reaction (12), carrier priming of T cells (13), and chronic allotype suppression (14). Spontaneous in vitro induction of suppressor cells has also been described (15).

Despite numerous animal models, description of suppressor cells in the human is limited. Abnormally increased suppression has been observed in patients with common variable hypogammaglobulinemia (16), multiple myeloma (17), and some patients with fungal infections (18); while in normal individuals suppressor cells have been described in the spleen (19). In the present report suppressor cell activity has been studied in human peripheral blood mononuclear cells (PBMC)[1] with a technique based on the observation that murine suppressor T cells are relatively short-lived in vitro (6). Thus, Dutton observed that the addition of Concanavalin A (Con A) to mouse spleen cells responding to sheep erythrocytes at the initiation of culture led to a suppression of their antibody response, but when the cultures were incubated for 24 h before addition of Con A an enhanced response ensued. This was interpreted as indicating a depletion of suppressor cells during the

[1] *Abbreviations used in this paper*: Con A, concanavalin A; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen; SK-SD, streptokinase-streptodornase; SLE, systemic lupus erythematosus; [^{3}H]TdR, tritiated thymidine.

incubation period. The application of this observation to normal human PBMC led us to the observation that 24-h incubation before the addition of suboptimal concentrations of Con A to cultures resulted in an enhanced cellular proliferative response and an increase in the amount of newly synthesized protein secreted by the cells. The possibility that this enhancement was due to the loss of a population of suppressor cells during the incubation period was examined and the results indicated that the enhanced response could be inhibited by the addition of PBMC previously activated with Con A. In addition, it was shown that the enhanced response was not obtained when PBMC from patients with active, untreated systemic lupus erythematosus (SLE) were incubated for 24 h before stimulation, suggesting the possibility that these patients lacked circulating short-lived suppressor cells.

METHODS

Subjects. 20 healthy adults aged between 18 and 42 yr were used as donors of normal lymphocytes. Most of these subjects were studied on several occasions. Nine patients with active SLE not receiving either corticosteroids or immunosuppresive drugs were studied. All satisfied the preliminary criteria of the American Rheumatism Association for the diagnosis of SLE (20). Serum antibodies to native DNA were measured with a millipore filter assay (21) and total hemolytic complement was determined by the method of Nelson et al (22). Seven of the SLE patients were reinvestigated 5–11 mo later when treatment with corticosteroids had led to clinical improvement. A group of six patients aged 18–35 yr with acute bacterial infections was also studied within 48 h of their hospital admission. All had previously been well and were febrile at the time of study. Four of these patients had pneumonia, one had endocarditis, and one had a urinary tract infection with septicemia. A further group of 5 patients receiving corticosteroid therapy for at least 2 wk for illnesses other than the rheumatic or connective tissue diseases were investigated. Two of these patients had asthma, two had pustular acne, and one had allergic dermatitis. Their ages ranged between 22 and 42 yr and they were receiving between 15 and 40 mg prednisone daily.

Culture conditions. Venous blood was obtained in a syringe containing preservative-free heparin (10 U/ml blood) and diluted in an equal volume of sterile 0.9N NaCl. 20-ml portions were carefully layered onto 10 ml of Ficoll-Hypaque (23) and centrifuged at 400 *g* for 40 min at room temperature. The cells at the interface were removed, washed three times in Hank's Balanced Salt Solution (Grand Island Biological Co., Grand Island, N. Y.), and resuspended in RPMI 1640 tissue culture medium (Grand Island Biological Co.), supplemented with L-glutamine, 10% fetal calf serum (Grand Island Biological Co.) previously heated at 56°C for 30 min, Penicillin G (1,000 U/ml), and Gentamycin (50 μg/ml). Test cultures were set up in triplicate in 16 × 25 mm tissue culture tubes (Falcon Plastics, Division of Bioquest, Oxnard, California), each tube containing 1.5×10^6 cells in 2 ml medium. The cultures were incubated at 37°C in an atmosphere containing 95% air and 5% CO_2. The cells were stimulated either at initiation of culture or after a 24-h period of incubation. Cell viability, as determined by exclusion of trypan blue dye, was examined at 24 h and at the termination of culture.

Con A (Pharmacia Fine Chemicals Inc., AB, Piscataway, N. J.) was dissolved in RPMI 1640 and sterilized by passage through a micropore filter, 0.45 μm in pore diameter (Millipore Corp., Bedford, Mass.). Portions of 3 mg/ml concentration were prepared as stock and stored at −20°C. Appropriate dilutions of the stock were prepared as required. PHA P (Difco Laboratories, Detroit, Mich.) was similarly dissolved in RPMI 1640 and portions of 1 mg/ml concentration prepared and stored at −20°C. Fresh dilutions were prepared for each experiment. Pokeweed mitogen (PWM) (Grand Island Biological Co.) was dissolved in saline and diluted to the required concentration in RPMI 1640 immediately before use. Streptokinase-streptodornase (SK-SD) (3,500 SK U/mg) (Lederle Laboratories, Pearl River, N. Y.) was stored at 4°C and diluted immediately before use. Mixed lymphocyte cultures were performed by mixing 0.75×10^6 cells from each of two randomly selected normal donors in 2 ml of medium. Similar numbers of cells from the same donors were also mixed after incubation for 24 h.

Measurement of DNA synthesis and newly synthesized secreted protein. The incorporation of tritiated thymidine ([^{3}H]TdR) (New England Nuclear, Boston, Mass.) into DNA was assayed by adding 2.0 μCi (1 Ci/mM) to the cultures for a 2-h period 3 days after the addition of mitogen. Thus, whether stimulated at 0 or at 24 h, cells were in contact with mitogen for 72 h before addition of [^{3}H]TdR. When cells were stimulated by SK-SD [^{3}H]Tdr was added 5 days after simulation and incubation continued for an additional 12 h. Similarly, in mixed lymphocyte cultures [^{3}H]TdR was added 5 days after mixing and incubation continued for 12 additional h.

Upon completing their incubation with [^{3}H]TdR the cells were centrifuged and washed. They were then cultured for a further 21 h in 1 ml of specially prepared Eagle's minimal essential medium lacking L-arginine, L-lysine, L-leucine, and L-valine (Grand Island Biological Co.) and supplemented with L-glutamine, 5% human AB serum previously heated at 56°C for 30 min, 5 μCi of ^{14}C-labeled amino acid mixture containing L-arginine 460 mCi/mM, L-lysine 300 mCi/mM, L-leucine 312 mCi/mM, and L-valine 200 mCi/mM (Schwarz-Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y.) (24). At the end of the culture period, the cells and supernate were separated after centrifugation. The cells were washed twice with cold saline solution and resuspended in 1 ml of 10% normal rabbit serum. The DNA was precipitated by adding 1 ml of 10% trichloroacetic acid. The suspension was centrifuged at 800 *g* for 20 min, the supernate decanted, and the precipitate dissolved in 1 ml 0.5N NaOH. The radioactivity was measured by transferring 0.1 ml of the solution to counting vials and diluting with 15 ml Bray's phosphor (25) containing thixotrophic gel (Cab-O-Sil, Packard Instruments Co., Inc., Downers Grove, Ill.), in a Beckman scintillation counter LS-330 (Beckman Instruments, Inc., Fullerton, Calif.). The amount of [^{3}H]TdR incorporated into cells was calculated by using ^{3}H and ^{14}C standards and was expressed as total counts per 1×10^6 cells in culture. To determine the amount of ^{14}C-amino acids incorporated into secreted newly synthesized protein, the culture supernates were centrifuged at 75,000 *g* for 1 h and dialyzed in large volumes of phosphate buffered saline at 4°C. Newly synthesized protein secreted during the last 21 h of culture was measured by precipitation with 10% trichloroacetic acid (26). The precipitate was dissolved in 0.5 ml of 0.5N NaOH, transferred to counting vials and diluted in Bray's phosphor. The incorporation of ^{14}C-labeled amino acids was measured in a Beckman scintillation counter LS-330 and expressed as total counts per 1×10^6 cells.

Calculation of results. The base-line counts in the unstimulated cultures were subtracted from the counts in the stimulated cultures and the difference expressed as Δ cpm. The increased response of cells cultured for 24 h before addi-

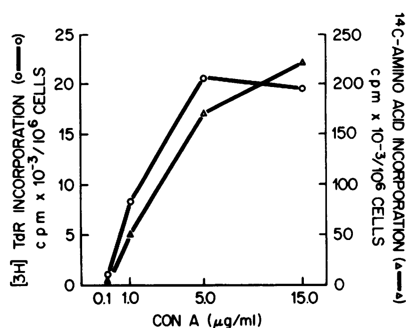

FIGURE 1 Comparison between [^{3}H]TdR incorporation into DNA and ^{14}C-amino acid incorporation into newly synthesized secreted protein. Con A was added at the initiation of culture. DNA synthesis (O−O) and protein secretion (△−△) showed a similar response to increasing concentrations of Con A.

tion of mitogen was expressed as the ratio of Δ cpm incorporated by cells stimulated after 24 h incubation to Δ cpm of cells stimulated at 0 h (Δ24/Δ0).

Analysis of data. Student's *t* test for paired data was used to analyze the statistical significance of the responses to stimulation at 0 h and at 24 h. Student's *t* test for nonpaired data was used to compare the response of the normal individuals with the responses of patients.

RESULTS

The responses of lymphocytes to mitogen have been studied by measuring two different functions: (*a*) incorporation of [^{3}H]TdR into DNA as a measure of cellular proliferation, (*b*) incorporation of ^{14}C-labeled amino acids into trichloroacetic acid precipitable protein in culture supernates as a measure of total protein synthesized and secreted by the cells during the final 21 h in culture. Fig. 1 compares the two parameters studied in normal lymphocytes stimulated by increasing concentrations of Con A and indicates that the pattern of the response for both functions was similar. Con A at concentrations of 5–15 μg/ml resulted in a maximal response when added at 0 h. Con A at a concentration of 1 μg/ml led to a submaximal response. When cells were incubated at 37°C for 24 h before the addition of Con A an increased response resulted, particularly to suboptimal concentrations of Con A (Tables I and II). The mean increase in the response after addition of Con A, 1 μg/ml, after 24-h incubation was threefold (Δ24/Δ0 = 3.0±0.3) when DNA synthesis was measured, and greater than fourfold (Δ24/Δ0 = 4.4±0.5) when secreted protein was measured. Thus, Con A at a concentration of 1 μg/ml added after 24-h incubation led to a significantly greater response than Con A added at 0 h at the same concentration ($P < 0.001$ for both [^{3}H]TdR incorporation and ^{14}C-amino acid incorporation), whereas higher concentrations of Con A added at 0 or 24 h led to responses that were not significantly different from each other. It was noted that where Con A at a concentration of 0.1 μg/ml resulted in absent or only a trivial response after stimulation at 0 h this concentration led to a substantial response when added at 24 h. Con A 5 μg/ml led to the maximum response of cells cultured for 24 h; however, the relative increase in the response of cultured cells (Δ24/Δ0 = 1.7±0.2 for DNA synthesis, 2.1±0.4 for protein secretion) was less than that observed at the lower concentrations.

Viable cell recovery. The average viable cell recovery of unstimulated cells after 24-h incubation in 11 experiments was 1.1×10^6 out of an initial 1.5×10^6

TABLE I
[^{3}H]Thymidine Incorporation by Normal Peripheral Blood Mononuclear Cells after Con A Stimulation at 0 or at 24 h

Time of addition of Con A	Con A concentration, μg/ml*				
	0	0.1	1	5	15
h					
0	3,806±711‡	1,223±1,227	8,814±1,435	20,709±8,814	19,116±5,096
24	5,465±965	13,445±9,062	21,806±4,044	39,847±19,583	25,206±5,052
Δ_{24}/Δ_0§	—	—ǁ	3.0±0.3	1.7±0.2	1.4±0.4
No. of exp.	28	4	28	6	4

* Counts in Con A containing cultures have been corrected by subtraction of base-line counts of unstimulated cultures and the responses expressed as net cpm (±SEM)/10^6 cells.
‡ Numbers refer to mean cpm (±SEM)/10^6 cells in unstimulated cultures (base-line counts).
§ Values Δ_{24}/Δ_0 represent mean (±SEM) of the ratios calculated for individual experiments, and not the ratio of the mean Δ_{24} to mean Δ_0 values given above.
ǁ The addition of Con A 0.1 μg/ml at 0 h led to absent, or only trivial, responses. Thus, where Δ_0 was 0, the ratio Δ_{24}/Δ_0 became infinite in a number of experiments. For this reason Δ_{24}/Δ_0 is not included.

TABLE II

[14]C-Amino Acid Incorporation into Newly Synthesized Protein Secreted by Normal Peripheral Blood Mononuclear Cells after Con A Stimulation at 0 or at 24 h

Time of addition of Con A	Con A concentration, μg/ml*				
	0	0.1	1	5	15
h					
0	50,926±4,577‡	392±3,368	51,276±5,340	169,426±52,430	224,331±45,089
24	59,229±5,678	60,724±29,999	248,639±20,727	400,384±98,482	289,600±95,624
Δ_{24}/Δ_0§	—	—‖	4.4±0.5	2.1±0.4	1.3±0.3
No. of exp.	33	4	33	7	8

* Counts in Con A containing cultures have been corrected by subtraction of base-line counts of unstimulated cultures and the responses expressed as net cpm (±SEM)/10^6 cells.

‡ Numbers refer to mean cpm (±SEM)/10^6 cells in unstimulated cultures (base-line counts).

§ Values Δ_{24}/Δ_0 represent mean (±SEM) of the ratios calculated for individual experiments, and not the ratio of the mean Δ_{24} to mean Δ_0 values given above.

‖ The addition of Con A 0.1 μg/ml at 0 h led to absent, or only trivial, responses. Thus, where Δ_0 was 0 the ratio Δ_{24}/Δ_0 became infinite in a number of experiments. For this reason Δ_{24}/Δ_0 is not included.

(73%). Of the cells recovered after 24 h, over 90% were viable. When unstimulated cells were harvested at the termination of culture an average of 1.0 × 10^6 viable cells were recovered (67%). Cells stimulated at 0 h by Con A, 1 μg/ml, yielded a recovery of 1.1 × 10^6 viable cells (73%), while cells stimulated at 24 h yielded 1.2 × 10^6 viable cells when harvested (80%).

Comparison of Con A, PHA, and PWM. In four experiments the effects of two other mitogens, PHA, and PWM, were compared to that of Con A. Suboptimal or optimal concentrations were added to cultures at 0 or after 24-h incubation. The mean submaximal responses to stimulation with Con A, 1 μg/ml, PHA, 1 μg/ml, or PWM, 5 μg/ml, added at 0 h were approximately of the same order. Fig. 2 indicates that when a suboptimal concentration of mitogen was added to cultures after 24-h incubation the mean response to PHA, 1 μg/ml, was substantially increased over the response to stimulation at 0 h (Δ24/Δ0 = 2.6±0.9 for DNA synthesis; 3.1±1.0 for secreted protein). In contrast, the mean response to PWM, 5 μg/ml, added at 24 h showed little increase (Δ24/Δ0 = 1.7±0.6 for DNA synthesis; 1.5±0.2 for secreted protein).

Effect of antigen and mixed lymphocyte culture. Four experiments were performed to examine whether stimulation with SK-SD after 24-h incubation led to increased [^{3}H]TdR incorporation relative to that after stimulation at 0 h. Various concentrations of antigen were used, ranging from 100 to 5 U/ml. The response to stimulation with SK-SD, at three different concentrations added after 24-h incubation did not exceed the response to stimulation at 0 h. Three further experiments examined the effect of mixing PBMC from two unrelated donors after incubating the cells for 24 h. Whether cells were mixed at 0, or after 24-h incubation, the response was similar.

Time dependency of enhanced response to Con A. Fig. 3 demonstrates the time required for the acquisition of increased responsiveness to Con A, 1 μg/ml. When Con A was added to the cultures at 2 and 6 h a progressive increase in responsiveness was noted. When Con A was added at 18 h the increased responsiveness was equal to that observed at 24 h.

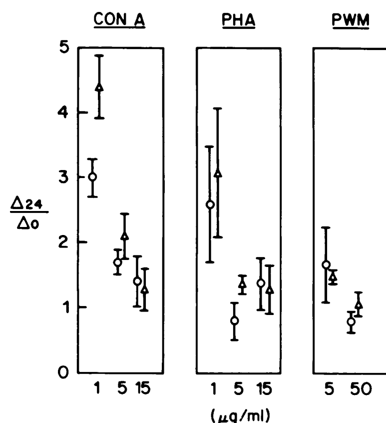

FIGURE 2 Comparison of effects of Con A, PHA, and PWM on cultures stimulated after 24-h incubation. Optimal or suboptimal concentrations of mitogen were added to cultures of normal peripheral blood mononuclear cells at 0 or at 24 h. Both DNA synthesis (○) and secreted newly synthesized protein (△) were measured. The relative increase in the response of cultures stimulated at 24 h is represented by the mean ratio Δ24/Δ0 (±SEM). For each mitogen the lowest concentration led to approximately similar responses after stimulation at 0 h.

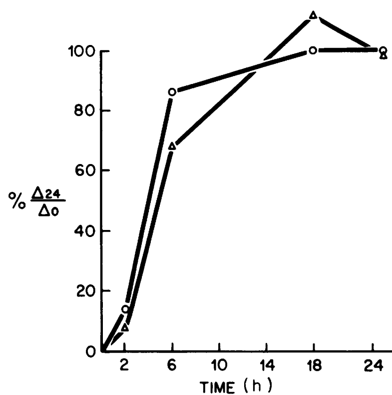

FIGURE 3 Time dependency of the enhanced response to Con A. Con A, 1 μg/ml, was added to cultures after varying periods of incubation. DNA synthesis (O) and newly synthesized secreted protein (Δ) were measured. The increased responsiveness acquired during the various intervals of incubation is expressed as the percentage Δ24/Δ0. Most of the increased responsiveness developed between 2 and 6 h after the initiation of culture.

Absence of soluble enhancing factors. Experiments were performed to examine whether the increased response to suboptimal concentrations of Con A added at 24 h might be due to the liberation of enhancing factors into the culture medium during the first 24 h of incubation. Medium from unstimulated 24-h cultures was removed after centrifugation and the cells washed in RPMI 1640. They were then resuspended in fresh medium and Con A, 1 μg/ml, added. The enhanced responsiveness of cells after 24-h incubation, as measured by [^{3}H]TdR incorporation, was not decreased by removing the culture medium and resuspending the cells in fresh medium (Table III). Furthermore, the response to Con A of fresh autologous cells incubated in the supernate obtained from 24-h cultured cells was only 10 and 18% greater than the control cells incubated in fresh medium (Table IV). Measurement of newly synthesized secreted protein gave identical results. Thus, the culture medium appeared to play little role in mediating the enhanced response of cells stimulated at 24 h.

TABLE III
Effect of Fresh Medium on Increased Responsiveness after 24-h Incubation

Exp. no.	Δ_0	Δ_{24} Medium not changed	Δ_{24} Medium* changed	Difference
				%
1	16,633	35,597	38,481	+7
2	2,453	10,055	12,490	+22
3	9,674	31,713	34,048	+7
4	5,670	20,002	15,760	−30
Mean		24,329	25,194	2

* Supernate was separated from cells after centrifugation at 24 h and cells resuspended in fresh medium immediately before addition of Con A, 1 μg/ml.

Inhibition of enhanced response to Con A by addition of preactivated autologous cells. Several experiments were designed to investigate whether the enhanced response of cells cultured for 24 h could be suppressed by the addition of fresh or preactivated autologous cells. Cells were cultured for 24 h with or without the addition of Con A, 1 μg/ml. Con A was then removed by washing the cells in 40 mM methyl α-D-mannopyranoside solution. Nonactivated cells were similarly washed, and portions of either activated or nonactivated cell suspensions were added to freshly obtained autologous cells, making a final cell concentration of 1.5 × 10^6 per culture. Suboptimal concentrations of Con A were added to such mixed cultures either at 0 or 24 h. Preliminary experiments established that optimal suppression of the increased responsiveness after 24-h incubation resulted from mixing 0.2 × 10^6 preactivated cells with 1.3 × 10^6 fresh cells. Addition of 0.2 × 10^6 previously activated cells resulted in significant suppression of the response observed in similar cultures to which nonactivated cells had been added (Table V). The results are expressed as percentage suppression of the enhanced response to stimulation after 24-h incubation. In five of these experiments (nos. 2–6) the increased response to Con A, 1 μg/ml, was suppressed; in experiments 1 and 7, suppression of the increased response to Con A, 0.5 μg/ml and Con A, 5.0 μg/ml, respectively, was demonstrated. Suppression of cell proliferation, as measured by the rate of [^{3}H]TdR incorporation, was generally more marked than suppression of newly synthesized protein secretion. Thus, in five out of six experiments suppression of proliferation was greater than 50%. With greater concentrations of Con A to activate the transferred cells the degree of suppression achieved was similar to that suppression observed when the transferred cells were activated by Con A, 1 μg/ml.

In these experiments the possibility was also considered that the inhibition of the enhanced response was not due to the activated cells but to contamination of the fresh cells with Con A transferred with the preactivated cells. In two experiments, Con A, 0.2 μg, a concentration greater than the maximum amount that could have been transferred with 0.2 × 10^6 preactivated cells, was added at the initiation of culture. In both experiments there was no inhibition of the response to Con A, 1 μg/ml, subsequently added after 24 h. In other experiments, fresh autologous cells were added to cultured cells immediately before 24-h stimulation but no suppression of the response was observed.

TABLE IV
Effect of Supernate from 24-h Cultured Cells on Responsiveness of Fresh Cells

Exp.	Δ_0 Cells in fresh medium	Δ_0 Cells in cultured medium*	Enhancement
			%
1	28,823‡	34,029	18
2	14,666	16,102	10

* Autologous cells were cultured without stimulation for 24 h. Supernates were removed after centrifugation and pooled. 1.5×10^6 fresh cells were suspended in 2 ml of the cultued medium and stimulated with Con A, 1 μg/ml.
‡ Results represent net [^{3}H]TdR cpm/10^6 cells.

Enhanced responsiveness of PBMC from patients with systemic lupus erythematosus after 24-h incubation. Since the increased responsiveness observed when PBMC were incubated for 24 h could be due to the loss of suppressor lymphocytes, it was decided to investigate the responsiveness of PBMC from patients with SLE. Suboptimal concentrations of Con A were added after 24-h incubation to determine, in this way, whether suppressor lymphocytes were present. Table VI describes the most prominent clinical features of the SLE patients during phases of disease activity. All patients had active disease and were hospitalized. Only two were receiving nonsteroidal anti-inflammatory therapy at the time of study. One patient (R. S.) had previously received corticosteroids as the diagnosis of SLE had been made 6 mo previously at another hospital. Prednisone 10 mg on alternate days had been prescribed, which the patient had discontinued 1 wk before her admission. None of the other patients were receiving immunosuppressive therapy. PBMC from these patients with active, untreated SLE when stimulated at 0 h demonstrated maximal responses to Con A, 5–15 μg/ml, which were not significantly different from normal. However, at a suboptimal concentration (1 μg/ml) the responses were significantly decreased when secreted protein was measured ($P < 0.05$), though not when DNA synthesis was measured. This supports the observation of Malavé et al. (27), who described decreased responses of PBMC from SLE patients to suboptimal concentrations of PHA. In contrast to the finding in normal subjects, when the effect of incubation before stimulation of SLE mononuclear cells was examined, either at optimal or suboptimal concentrations, there was little or no increase in the response. Fig. 4 demonstrates that at suboptimal concentrations the mean increase in the rate of [^{3}H]TdR incorporation after stimulation at 24 h ($\Delta 24/\Delta 0$), was 1.2 ± 0.2 in active SLE. This was significantly lower than the normal ratio of 3.0 ($P < 0.005$). When ^{14}C-amino acid incorporation into newly synthesized secreted protein was measured, PBMC from active un-

TABLE V
Suppression of the Increased Responsiveness after 24-h Incubation by the Addition of Preactivated Autologous Lymphocytes

	[^{3}H]TdR incorporation				^{14}C-amino acid incorporation			
		Δ_{24}				Δ_{24}		
Exp.	Δ_0*	Control culture‡	Preactivated cells added§	Inhibition	Δ_0*	Control culture‡	Preactivated cells added§	Inhibition
			%	%				%
1	11,512	23,836	16,897	56	40,816	104,337	82,845	34
2	7,748	13,377	10,206	56	68,362	164,321	116,794	50
3	3,883	18,968	16,120	19	49,470	196,209	161,927	23
4	ND‖	—	—	—	88,693	215,106	135,113	63
5	1,584	3,867	2,474	61	26,860	80,993	59,006	41
6	4,233	6,912	4,719	82	76,513	119,163	103,220	37
7	15,204	25,519	19,468	59	ND	—	—	—
Mean				56				41

* Numbers represent net cpm/10^6 cells where Con A was added at 0 h. The concentration of Con A added at 0 and 24 h was 1 μg/ml in experiments 2–6, 0.5 μg/ml in experiment 1, and 5 μg/ml in experiment 7.
‡ 0.2×10^6 nonactivated cells, previously incubated for 24 h and washed with methyl-α-D-mannopyranoside, were added to 1.3×10^6 fresh cells and stimulated with Con A added after 24 h.
§ 0.2×10^6 cells previously incubated with Con A, 1 μg/ml, for 24 h and washed with methyl-α-D-mannopyranoside, were added to 1.3×10^6 fresh cells and stimulated with Con A added after 24 h.
‖ ND, Not done.

TABLE VI
Clinical Characteristics of Patients with SLE

					Initial testing				Follow-up testing				
							Δ_{24}/Δ_0§					Δ_{24}/Δ_0§	
Patient	Sex	Race	Age	Clinical features	Anti-n-DNA*	CH50‡	[^{3}H]Tdr	^{14}C-amino acid	Drug therapy	Anti-n-DNA*	CH50	[^{3}H]TdR	^{14}C-amino acid
						U			*mg/day*	*μg/ml*	*U*		
M. Z.	F	LA	28	Arthritis, cutaneous vasculitis, proteinuria	20	15	ND	1.5	Prednisone, 10	11	8	3.5	5.1
B. M.	M	W	16	Arthritis, malar rash, MPGN‖	2	28	ND	1.7	Prednisone, 25 Cyclophosphamide, 50	0	90	3.3	3.6
W. P.	F	B	17	Arthritis, pleurisy, seizure	30	60	1.6	0.8	Prednisone, 40	0	60	2.8	3.1
L. S.	M	B	20	Arthritis, MPGN	31	35	1.3	2.2	Prednisone, 25 Azathioprine, 100	12	90	2.6	1.7
L. F.	F	W	16	Arthritis, rash, MPGN	44	ND	0.7	1.0	Prednisone, 25	5	55	1.0	4.5
G. F.	M	B	47	Arthritis, pleurisy	15	55	1.6	3.3	Prednisone, 30	8	90	4.3	5.3
B. H.	F	B	17	Arthritis, alopecia, pleurisy pericarditis, myocarditis, MPGN	16	10	1.0	1.8	Prednisone, 60	5	70	1.7	5.0
R. S.	F	W	25	Arthritis MPGN	34	18	1.0	2.2					
C. G.	F	LA	16	Arthritis MPGN	28	55	1.2	1.2					

* Normal value, <1.5 μg DNA bound/ml serum.
‡ Normal value, 60–110 U.
§ Δ_{24}/Δ_0, ratio derived from responses to Con A, 1 μg/ml.
‖ MPGN, Membrane-proliferative glomerulonephritis.

treated SLE patients again demonstrated a relative inability to acquire an increased responsiveness to Con A, 1 μg/ml, added after 24-h incubation (Fig. 5). The ratio Δ24/Δ0 of 1.7±0.3 was significantly less than the normal ratio of 4.4 ($P < 0.02$). Similarly, at higher concentrations of Con A, PBMC from the SLE patients did not demonstrate increased responsiveness when stimulated after 24-h incubation.

It is of interest that PBMC from four out of six patients with acute bacterial infections failed to demonstrate the acquisition of increased responsiveness to Con A, 1 μg/ml, added after 24-h incubation. However, the base-line values of [^{3}H]TdR incorporation were substantially higher than in normals and SLE patients. Moreover, the increased rate of proliferation of the unstimulated cells from these acutely ill patients may have compromised their capacity to demonstrate an increased responsiveness. However, when ^{14}C-amino acid incorporation into newly synthesized secreted protein was measured, the base-line values in the control patients with infections were identical to the base-line values observed in the normal subjects and SLE patients. In contrast to the findings on measurement of DNA synthesis cells from these patients consistently demonstrated their ability to acquire increased responsiveness in culture, the average Δ24/Δ0 being 6.8±2.5. This was significantly higher than the value observed in SLE patients ($P < 0.02$).

Effect of removal of antilymphocyte antibodies. In experiments with three SLE patients, unstimulated cultures were washed at 37°C after 24-h incubation and the supernates discarded to remove antilymphocyte antibodies (28). This procedure did not lead to any increase in responsiveness.

The responsiveness of PBMC from SLE patients receiving corticosteroid therapy. Seven of the nine SLE patients were reinvestigated 5–11 mo after commencing corticosteroid therapy. In addition, one patient with glomerulonephritis received cyclophosphamide and another received azathioprine (Table VI). All patients had improved clinically although in only two had there been a complete return to normal of both anti-DNA titer and serum complement level. The response to Con A, 1 μg/ml, added at 0 h to PBMC from

these treated patients was not increased. This is consistent with the fact that some degree of disease activity persisted. Again, normal responsiveness was observed with optimal concentrations of Con A. Important, however, was the observation that there was a striking increase in the capacity of PBMC from these patients to acquire an increased responsiveness during 24-h incubation similar to that of normal individuals and corticosteroid treated control patients with nonrheumatic diseases. Fig. 6 demonstrates increased Δ24/Δ0 ratios for Con A, 1 μg/ml, in six of the seven patients. Thus, after treatment, despite the unchanged diminished response to Con A, 1 μg/ml, added at 0 h, the response to 24-h stimulation was significantly greater ($P < 0.02$ for [^{3}H]TdR incorporation; $P < 0.005$ for ^{14}C-amino acid incorporation).

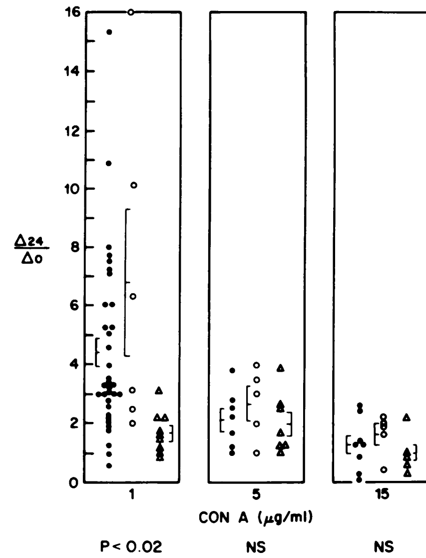

FIGURE 5 Effect of 24-h incubation on ^{14}C-amino acid incorporation into newly synthesized secreted protein by blood mononuclear cells of controls and patients with active SLE. Cells from normal subjects (●), control patients (○) and patients with active untreated SLE (△) were stimulated with Con A at 0 or at 24 h. The increased responsiveness acquired after 24-h incubation is expressed as the ratio Δ24/Δ0. Con A, 1 μg/ml, led to a mean ratio of 4.4±0.5 for normal subjects. For SLE patients the ratio was significantly lower at 1.7±0.3 ($P < 0.02$). All six patients with acute infections acquired an increased response to Con A, 1 μg/ml, after 24 h in culture, as indicated by the mean ratio of 6.8±2.5.

DISCUSSION

Many rodent experimental models have been described characterizing a subpopulation of lymphocytes which function as suppressors of other potentially reactive cells. However, in the human the role of suppressor cells in regulating the normal immune response has yet to be established. In three diseases it has been suggested that an abnormal increase of suppressor function plays a pathogenic role. Purified peripheral blood T lymphocytes from patients with common variable hypogammaglobulinemia suppressed the response of normal B lymphocytes to PWM (16). Similarly, peripheral blood mononuclear cells from patients with multiple myeloma suppressed polyclonal immunoglobulin synthesis of normal lymphocytes (17). Finally, when circulating lymphocytes from some patients with disseminated fungal infections were allowed to remain in culture for a 7-day period they acquired increased responsiveness to mitogen and antigen stimulation, suggesting that a cell with suppressor function had been eliminated during the period

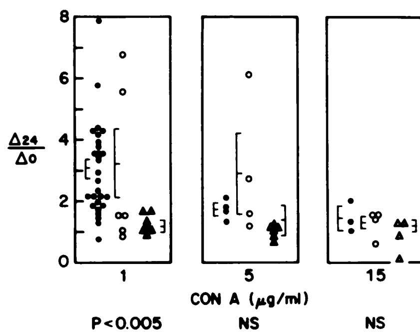

FIGURE 4 Effect of 24-h incubation on [^{3}H]TdR incorporation by blood mononuclear cells of controls and patients with active SLE. Cells from normal subjects (●), control patients (○), and patients with active untreated SLE (△) were stimulated with Con A at 0 or at 24 h. The increased responsiveness acquired after 24 h incubation is expressed as the ratio Δ24/Δ0. Con A, 1 μg/ml, led to a mean ratio of 3.0±0.3 for normal subjects. For SLE patients the ratio was lower at 1.2 ±0.2 ($P < 0.005$). Four of the six patients with acute infections also had low ratios.

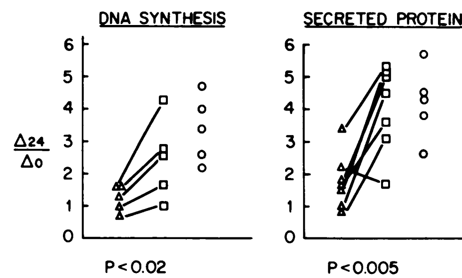

FIGURE 6 Responses of cells from SLE patients before and after treatment. Patients with active SLE (△) were tested for their ability to acquire increased responsiveness to Con A, 1 μg/ml, after 24-h incubation. The patients were again tested 5–11 mo after institution of corticosteroid therapy (□). The relative increase in responsiveness is expressed as Δ24/Δ0. There was a significant increase in the ability of the cells to acquire increased responsiveness in culture after corticosteroid therapy ($P < 0.02$ for [^{3}H]TdR incorporation, $P < 0.005$ for ^{14}C-amino acid incorporation). The values observed in a control group of corticosteroid treated patients are shown for comparison (○).

of incubation (18). To date, no method has been described for investigating the suppressor function of PBMC from normal individuals, or from patients with diseases where suppressor function might be decreased or absent. However, recent work by Shou et al. (29) suggests that Con A activation of normal human blood lymphocytes induces suppressor activity on mitogen and antigen mediated lymphocyte responses.

The data presented here indicate that normal human peripheral blood mononuclear cells acquired a substantial increase in their capacity to proliferate and to secrete protein in response to stimulation with suboptimal concentrations of Con A added after the cells had been cultured for 24 h. This observation was not confined to experiments with Con A inasmuch as PHA at suboptimal concentrations could produce a similar effect. One possible explanation for this phenomenon might be that during incubation before the addition of mitogen the cells were subjected to facilitating influences imposed by the culture conditions, or, perhaps allowed optimal cell contact for stimulation. This would seem an unlikely explanation since the increased responsiveness of cultured cells was not compromised in experiments in which the cells were washed and resuspended in fresh medium immediately before 24-h stimulation. This observation would appear to exclude aggregation of cells and secretion of an enhancing factor as being responsible for the increased reactivity. Furthermore, cultured cells did not develop increased responses to all forms of stimulation, but demonstrated a degree of specificity. Thus, the response of cultured cells to PWM was only slightly greater than the response of fresh cells. Also, cultured cells did not develop increased responsiveness to SK-SD or to allogeneic cells in a mixed lymphocyte culture.

Another possible explanation for the increased response of cultured cells is that some inhibitory or suppressive influence, which might be activated in freshly stimulated cells, is lost or diminished during incubation. That this is the most likely explanation is suggested by the observation that when cells were cultured with small numbers of preactivated cells before stimulation, the increased responsiveness was significantly inhibited. Thus, since the increased responsiveness acquired during 24-h incubation was suppressed by the addition of activated autologous cells it seems most likely that these added cells were replacing suppressor cells whose function was altered in culture. That the Con A-activated suppressor cells were short lived in vitro is also strongly suggested by the inability of the nonactivated transferred cells to decrease responsiveness in spite of Con A activation 24 h later.

The cell functioning as the suppressor of the increased responsiveness acquired in culture has not been identified. However, as DNA synthesis measured after 3 days in culture in response to Con A stimulation is a measure of T-cell proliferation (30), it may be suggested that the data demonstrate suppression of Con A-responsive T lymphocytes. As demonstrated in Fig. 1, the amount of secreted newly-synthesized protein closely paralleled the measurements of DNA synthesis. Recent experiments in this laboratory indicate that protein secretion in response to Con A is also a measure of T-lymphocyte activation.[2]

The increased responsiveness demonstrated by normal human peripheral blood mononuclear cells after 24 h in culture is in contrast to the observation of Stobo et al. (18) who found little increase in the reactivity of normal lymphocytes cultured for 3 or 4 days before stimulation. However, the data described by these authors relate only to optimal concentrations of mitogen. It is possible that despite the activity of suppressor cells in vitro, a maximal, or near maximal response is achieved when fresh lymphocytes are stimulated with optimal concentrations of Con A or PHA, and that the restraining influence of suppressor cells becomes clearly evident only with suboptimal concentrations.

If the ability to acquire increased responsiveness to suboptimal concentrations of Con A after 24-h incubation is an indication that there is a subpopulation of mononuclear cells in peripheral blood which suppress other potentially reactive cells, then it should be possible to study this phenomenon in disease states where a lack of suppressor function may exist. In SLE there is a marked rise in total immunoglobulin synthesis, as well as the appearance of antibodies directed against a variety of self-antigens, particularly nuclear antigens (31). This apparent increased B-cell activity is compatible with a loss of normal regulation. Similar abnormalities have been observed in the New Zealand Black/White hybrid mouse in which the females develop with age a disease associated with appearance of antinuclear antibodies and renal lesions identical to lupus nephritis (32). The disease in these mice further resembles SLE in that they also develop impaired T-lymphocyte function. Of particular importance in regard to the present discussion is the observation that while spleen cells from NZB/W female mice under 2 mo of age can suppress the thymus dependent graft-versus-host reaction, the ability to suppress is lost as the mice age (33). Thus, in this animal model of human SLE convincing evidence has accumulated to suggest that defective cellular immunity includes the loss of regulatory suppressor cells and contributes to the development of autoimmune disease.

The data presented here demonstrate that mono-

[2] Bresnihan, B., C. Lavalle, and H. E. Jasin. Manuscript in preparation.

nuclear cells from patients with active SLE respond as well as cells from normal subjects to optimal concentrations of Con A, while at suboptimal concentrations their response was less than normal. The normal ability of PBMC to acquire an increased responsiveness to Con A, 1 μg/ml, added after 24 h in culture, was markedly decreased. That the failure to acquire an increased responsiveness in culture was due to the presence of lymphocytotoxic antibodies is unlikely since removal of such antibodies by washing after incubation at 37°C did not lead to increased responsiveness. Furthermore, the failure of SLE cells to acquire added responsiveness in culture is unlikely to be simply a reflection of the decreased response observed at suboptimal concentrations as it was noted that normal PBMC demonstrating trivial or no response when stimulated by Con A, 0.1 μg/ml, demonstrated a response to the same concentration added after 24 h which was equivalent to that of fresh cells responding to ten times the amount of Con A (Tables I and II). This would again argue that while normal cells lose their suppressor function in culture, cells from patients with active SLE have lost that function from the outset. Furthermore, when the patients were responding to corticosteroid therapy, their PBMC did acquire a substantial increase in responsiveness during 24 h incubation. It should be emphasized that the observed increase in responsiveness occurred despite the absence of any increase in the response of these cells when stimulated at 0 h. The decreased reactivity of PBMC from the patients receiving corticosteroid therapy is compatible with the fact that disease activity persisted in this group, and that in only two did anti-DNA and complement values return to normal. Nevertheless, these cells acquired an increased responsiveness during 24 h incubation similar to that of the normal population and of a group of other corticosteroid treated control patients. This suggests that the events resulting in a remission in SLE include the return of immunological suppressor function despite a persisting impairment of T-lymphocyte reactivity.

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