

# Induction of Suppressor Activity in the Autologous Mixed Lymphocyte Reaction and in Cultures with Concanavalin A

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**ABSTRACT** T lymphocytes that are activated in the autologous mixed lymphocyte reaction (MLR) have suppressor activity. Concanavalin A (Con A) augments the suppressor activity generated in cultures containing both T and non-T lymphocytes and can induce suppressor activity in T-lymphocyte preparations that contain too few (10%) non-T cells to generate a significant autologous MLR. However, when such T-lymphocyte preparations are further depleted of adherent cells and contain <2% non-T cells, Con A fails to induce suppressor activity. These findings support the concept that an autologous MLR may play an important role in generation of suppressor cells by Con A.

## INTRODUCTION

Human T lymphocytes are stimulated by autologous non-T lymphocytes to proliferate in culture (1, 2). This phenomenon, termed the autologous mixed lymphocyte reaction (MLR),<sup>1</sup> suggested a mechanism by which T lymphocytes might maintain self-tolerance. This thesis is compatible with the observation (3, 4) that the autologous MLR is impaired in patients with systemic lupus erythematosus. The defect in the autologous MLR in these patients is a failure of non-T lymphocytes to stimulate the proliferation of autologous T lymphocytes (4). The autologous MLR is also impaired in the murine model of this disease in NZB mice (5).

Autoimmune phenomena associated with systemic lupus erythematosus in man and in diseased NZB mice

have been related to a loss of suppressor T-cell activity (6, 7). The autoantibodies found in these states may reflect the expression of autoreactive clones of non-T lymphocytes normally inhibited by suppressor T lymphocytes. The loss of the autologous MLR and of suppressor T-cell activity in humans with systemic lupus erythematosus and in NZB mice suggested that suppressor T lymphocytes might be generated during the autologous MLR. This report shows that T lymphocytes activated in the autologous MLR suppress the generation of human plaque-forming cells (PFC) in culture. Furthermore, evidence is offered that an autologous MLR may play an important role in the generation of suppressor T lymphocytes by concanavalin A (Con A).

## METHODS

*Preparation of Lymphocytes.* 50 ml of venous blood from healthy volunteers were drawn into a plastic syringe containing 50 U of heparin (Upjohn Co., Kalamazoo, Mich.). Heparinized blood was diluted with an equal volume of calcium- and magnesium-free Hanks' balanced salt solution (HBSS, Microbiological Associates, Walkersville, Md.). 35–40 ml of the diluted blood was layered over 12 ml of a mixture of Ficoll (Pharmacia Fine Chemicals, Piscataway, N. J.) and sodium diatrizoate (Hypaque, Winthrop Laboratories, Sterling Drug, Co., New York) in sterile 50-ml polypropylene tubes (No. 25330, Corning Glass Works, Science Products Div., Corning, N. Y.). The Ficoll-Hypaque solution was prepared by mixing one part of 50% Hypaque with four parts of 8% (wt/vol) Ficoll in water. The density of the Ficoll-Hypaque mixture was adjusted to a 1.078–1.080-sp grav with distilled water and passed through an 0.45- $\mu$ m millipore filter. Tubes containing the diluted blood layered on Ficoll-Hypaque were centrifuged at 400 g for 40 min at 20°C. The mononuclear cells removed from the interface were washed three times with HBSS or with HBSS containing 10% heat-inactivated fetal bovine serum (Microbiological Associates) if they were to be fractionated into T- and non-T-lymphocyte preparations. The cells were collected by centrifugation at 150 g for 10 min at 20°C. The cells were then resuspended in medium RPMI 1640 (Microbiological Associates) with 100 U penicillin/ml, 100  $\mu$ g streptomycin/ml (Grand Island Biological Co., Grand Island, N. Y.), L-glutamine 2 mM (Microbiological Associates), and 20% fetal bovine serum at a concentration of

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<sup>1</sup> *Abbreviations used in this paper:* Con A, concanavalin A; FSA, formalinized staphylococcus aureus; HBSS, Hanks' balanced salt solution; MLR, mixed lymphocyte reaction; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

2 million lymphocytes/ml if they were to be rosetted. Unfractionated mononuclear cells were resuspended in this medium RPMI 1640 supplemented with 20% human AB serum that had been heated to 56°C for 30 min.

**Fractionation of T- and non-T enriched populations.** Sheep erythrocytes (SRBC, Flow laboratories, Inc., Rockville, Md.) were washed twice with HBSS, and three drops of packed SRBC were added to 10 ml HBSS. Equal volumes of the human lymphocyte and SRBC suspensions were mixed in 50-ml polycarbonate tubes, and the tubes were centrifuged at 50 g for 5 min at 20°C and incubated overnight at 4°C.

The cell pellet was gently resuspended, and 40 ml of the SRBC lymphocyte suspension was layered over 12 ml of Ficoll-Hypaque, and centrifuged at 400 g for 40 min at 4°C. Unrosetted lymphocytes were removed from the interface. The lymphocyte preparation that did not form rosettes with SRBC contains B lymphocytes, K lymphocytes, monocytes, and possibly other cell types. We will refer to this preparation as non-T lymphocytes. Rosetted T lymphocytes in the pellet beneath the Ficoll-Hypaque were washed with 0.83% ammonium chloride-0.17 M Tris buffer, pH 7.2, to lyse the SRBC, and collected by centrifugation (150 g for 10 min at 20°C). The T- and non-T-enriched lymphocyte preparations were washed three times with HBSS and resuspended at a concentration of 1 million lymphocytes/ml in the final culture medium, RPMI 1640 with 100 U penicillin/ml, 100 µg streptomycin/ml, 2 mM L-glutamine, and 20% heat-inactivated human AB serum.

**Induction of suppressor activity.** Lymphocyte cultures were established in tissue culture flasks (No. 3013; Falcon Labware, Oxnard, Calif.) by combining equal volumes of the T-lymphocyte preparation at  $2 \times 10^6$  liters/ml and the irradiated (3,000 R, cesium source) non-T lymphocyte preparation. The mixed lymphocyte cultures were incubated for 144 h at 37°C in a 5% CO<sub>2</sub> and 95% air, humidified environment. T-lymphocyte preparations, T-lymphocyte preparations depleted of adherent cells, and mononuclear cell preparations were also preincubated for 144 h. Suppressor activity generated in each of these preparations in the presence or absence of 50 µg/ml Con A was assessed. All cultures containing Con A were incubated for 72 h.

Suppressor activity developed during the lymphocyte culture was quantitated by the ability of precultured cells to suppress the PFC response of fresh lymphocytes cultured with formalinized staphylococci of the Cowan strain.

**Generation of PFC.** Formalinized staphylococci were used as polyclonal activators of human PFC (8).  $5 \times 10^6$  mononuclear cells were suspended in 1 ml of RPMI 1640 with 10% AB serum supplemented with 1% trypticase soy broth, 0.02 M L-glutamine, 100 U penicillin/ml, and 100 µg streptomycin sulfate. The human AB serum used in the cultures was absorbed with one-half its volume of packed SRBC. 0.5 ml of fresh cells and  $2.5 \times 10^6$  precultured cells in 0.5 ml medium or media were mixed and incubated in 35-mm plastic Petri dishes (No. 1008; Falcon Labware) with a final concentration of 0.03% (vol/vol) formalinized staphylococcus aureus (FSA) of the Cowan strain.

These cultures were incubated for 6 d on a rocker platform (7 oscillations/min) at 37°C in a 5% CO<sub>2</sub> and 95% O<sub>2</sub> 100% humidity environment. Every day, 0.1 ml of complete medium was added to the culture. At the end of the culture period the cells were harvested from the Petri dishes using rubber policemen. The cells were then collected by centrifugation (200 g) for 10 min, washed once with unsupplemented medium RPMI 1640 and anti-SRBC PFC determined by a slide modification (9) of the Jerne plaque assay. PFC were calculated per million viable cells recovered at the end of the culture. The percentage of suppressor or helper activity was

calculated by the following formula: PFC fresh cells + precultured cells + FSA/PFC fresh cells + media + FSA. This is an underestimate of the suppressor activity as the number of viable cells recovered in unstimulated or suppressed cultures was very much less than the number of viable cells recovered in stimulated lymphocyte cultures.

## RESULTS

**Suppression of human PFC by cells activated in autologous MLR.** Human mononuclear cells incubated for 6 d with FSA produce 300–1,000 anti-SRBC PFC/ $10^6$  viable cells recovered at the end of culture. As the number of cells increase during the 6 d in cultures with staphylococci, these results underestimate the PFC response of the lymphocytes placed into culture. Lymphocytes cultured in the absence of staphylococci generated only 10–20 PFC/ $10^6$  cells.

T cells activated in the autologous MLR suppress the PFC response of lymphocytes cultured with staphylococci (Table I). Thus, T cells cultured for 6 d with autologous non-T cells reduce the PFC response of autologous lymphocytes cultured with staphylococci by 50–100%. In contrast, T lymphocytes cultured alone for 6 d frequently augmented the PFC response of lymphocytes cultured with staphylococci (Table I). The precultured T lymphocytes did not form PFC but appeared to augment the PFC response of autologous lymphocytes cultured with staphylococci. Thus, precultured T lymphocytes generated no PFC when cultured with staphylococci, although their addition to lymphocytes cultured with staphylococci increased their PFC response as much as threefold (Table II). In contrast, T lymphocytes preincubated with autologous non-T lymphocytes suppressed the PFC response by 70%. Suppressor activity was generated during the autologous MLR. No suppressor activity was found when T and non-T cells were precultured separately and mixed just before their addition to cultures of lymphocytes and FSA (data now shown). The suppressor activity generated during the autologous MLR showed no histocompatibility restriction. Thus, lymphocytes activated in the autologous mixed lymphocyte cultures suppressed the PFC response of autologous and allogeneic lymphocytes from 50 to 90% (Table III).

Suppressor activity generated in the autologous MLR was eliminated by 3,000 R irradiation. When T cells activated in an autologous MLR were irradiated before their addition to lymphocytes cultured with FSA, suppressor activity was lost and significant helper activity revealed (Table IV). No suppressor factor was demonstrated in the supernatant medium obtained after 6 d from the autologous MLR cultures. The PFC response of lymphocytes cultured with FSA was not suppressed when supernatant media from autologous mixed lymphocyte cultures were added in equal amounts to fresh media.

**TABLE I**  
*Suppression of PFC Response by T Lymphocytes Activated in the Autologous MLR*

Addition to lymphocyte cultures		PFC/10 <sup>6</sup> cells	Helper (↑) or suppressor (↓) activity
			%
<b>Experiment 1</b>			
C.F.	Media	452	—
	Preincubated T cells	823	82 ↑
	Preincubated T + non-T cells	217	52 ↓
<b>Experiment 2</b>			
B.J.	Media	954	
	Preincubated T cells	581	38 ↓
	Preincubated T + non-T cells	10	99 ↓
J.I.	Media	367	
	Preincubated T cells	404	10 ↑
	Preincubated T + non-T cells	57	85 ↓
<b>Experiment 3</b>			
D.B.	Media	346	
	Preincubated T cells	415	20 ↑
	Preincubated T + non-T cells	96	73 ↓

Mononuclear cells were incubated with complete culture medium containing 0.03% (vol/vol) FSA for 6 d. The anti-SRBC PFC generated were measured by the Jerne technique. Where indicated, an equal number of autologous T cells preincubated for 6 d alone or with irradiated autologous non-T lymphocytes were added at the initiation of culture.

These studies did not distinguish between the generation of suppressor cells during preincubation and the generation of regulatory cells which activate suppressor T cells in the lymphocyte preparation cultured with FSA. If the suppressor activity seen when preincubated

cells were mixed with unfractionated lymphocytes was due to preincubated cells that activated suppressor T cells in the unfractionated preparation, no suppression would be expected when cells activated in the autologous MLR were mixed with non-T cells. When cells activated in the autologous MLR were added to unfractionated cells or non-T lymphocytes, suppression of the PFC response was seen (Table V). Thus, regulatory cells generated in the autologous MLR can overcome the helper activity of preincubated T cells.

**TABLE II**  
*Regulatory Influence of Precultured T Lymphocytes on PFC Response of Unfractionated Lymphocytes*

Addition to lymphocyte cultures	Unfractionated lymphocytes	PFC/10 <sup>6</sup> cells	Helper (↑) or suppressor (↓) activity
Media	Present	328	—
Preincubated T cells	Present	910	↑290%
Preincubated T + non-T cells	Present	92	↓72%
Preincubated T cells	Absent	11	—
Preincubated T + non-T cells	Absent	10	—

T cells were preincubated for 6 d alone or with autologous non-T cells. These regulatory cells ( $2.5 \times 10^6$  cells in 0.5 ml of medium) were added to either an equal volume of unfractionated lymphocytes containing  $2.5 \times 10^6$  cells or an equal volume of medium alone. All cultures contained 0.03% (vol/vol) FSA and were incubated 6 d. The number of PFC generated in these cultures during 6 d was measured by the modified Jerne technique.

**TABLE III**  
*Suppressor Activity Generated in Autologous MLR Effects Allogeneic Lymphocytes*

Lymphocyte donor	PFC response/10 <sup>6</sup> cells		
	Addition to lymphocyte culture		
	Media	Allogeneic T lymphocytes activated in autologous MLR	Allogeneic preincubated T lymphocytes
I	446	195	Not done
II	275	127	437
III	301	33	273

Mononuclear cells were incubated with FSA for 6 d. Where indicated, an equal number of allogeneic T cells preincubated for 6 d alone or with autologous non-T cells were added at the initiation of the cultures.

**TABLE IV**  
*Suppressor Activity of T Lymphocytes Activated in Autologous MLR:  
Effect of Irradiation and Supernatant Medium*

Addition to cultures		PFC/10 <sup>6</sup> cells	Helper (↑) or suppressor (↓) activity
		%	
Experiment 1	Media	954	
	Preincubated T + non-T <sub>x</sub> cells	10	99 ↓
	Irradiated preincubated T + non-T <sub>x</sub>	1458	53 ↑
Experiment 2	Media	173	
	Preincubated T + non T <sub>x</sub> cells	33	79 ↓
	Supernatant preincubated T + non-T <sub>x</sub>	330	91 ↑
	Irradiated preincubated T + non-T <sub>x</sub>	580	235 ↑
Experiment 3	Media	452	—
	Preincubated T + non-T <sub>x</sub> cells	217	53 ↓
	Supernatant preincubated T + non-T <sub>x</sub>	415	10 ↓

Mononuclear cells were incubated with FSA for 6 d. Where indicated an equal number of irradiated or unirradiated cells previously activated in the autologous MLR were added. In some experiments, supernatant medium obtained after 6 d of an autologous MLR made up 50% of the medium, in which the mononuclear cells were cultured in the FSA. Non-T cells were irradiated (non-T<sub>x</sub>) with 3,000 R.

*Lymphocyte preparations required for the induction of suppressor activity by Con A.* Human mononuclear cells incubated with Con A suppress mitogen-induced lymphocyte proliferation (10). The repeated

failure of T lymphocytes incubated with Con A to suppress mitogen-induced proliferation (data not shown) suggested the need for a mixture of T and non-T cells for the induction of suppressor activity by

**TABLE V**  
*Effect of Preincubated T Cells in PFC Response of Unfractionated or  
Non-T Lymphocyte Enriched Preparations*

Responding preparation		Addition to lymphocyte cultures	PFC/10 <sup>6</sup> cells
Experiment 1	Unfractionated lymphocytes	Media	261
		Preincubated T lymphocytes	1538
		T lymphocytes activated in autologous MLR	154
	non-T-enriched lymphocytes	Media	13
		T lymphocytes	86
		T lymphocytes activated in autologous MLR	4
Experiment 2	Unfractionated lymphocytes	Media	1879
		Preincubated T lymphocytes	1798
		T lymphocytes activated in autologous MLR	294
	non-T-enriched lymphocytes	Media	14
		Preincubated T lymphocytes	437
		T lymphocytes activated in autologous MLR	19

Mononuclear cells were prepared by centrifuging diluted blood through Ficoll-Hypaque. B lymphocytes were obtained by passing mononuclear cells through column containing anti-human F(ab)<sub>2</sub> antibody conjugated to Sephadex G-200 (Pharmacia Fine Chemicals) as described (17). The B lymphocytes retained on the column were eluted with 1% human immunoglobulin. Either preparation was incubated with complete culture medium containing FSA for 6 d. Where indicated, an equal number of autologous T cells preincubated for 6 d alone or with autologous B cells were added at the initiation of the culture. The anti-SRBC PFC generated were measured by the Jerne technique.

Con A. The capacity of various lymphocytes preincubated with or without Con A to suppress autologous PFC was compared (Table VI). T lymphocytes preincubated without Con A did not suppress the number of PFC. T-lymphocyte preparations incubated with Con A suppressed the PFC response by ~50%. The capacity of T-lymphocyte preparations to generate suppressor activity was dependent upon the 10–15% of adherent non-T lymphocytes. Thus, T lymphocytes that had been passed over a nylon column and contained <2% non-T cells did not generate suppressor activity in the presence of Con A. Mononuclear cells or mixtures of T and irradiated B lymphocytes generated suppressor activity when preincubated 6 d. These preparations suppressed the generation of PFC by >60%. Con A augmented the magnitude of the suppressor activity generated by these preparations. Thus, Con A activated mononuclear cells or mixtures of

T and irradiated B lymphocytes suppressed the PFC response by >90%.

## DISCUSSION

We have shown that T lymphocytes activated in the autologous MLR suppress autologous or allogeneic PFC induced by FSA. T lymphocytes incubated alone augmented or had little effect on the PFC response. Rarely, preincubated T lymphocytes suppressed the PFC response. This was probably caused by contamination of the T lymphocytes with monocytes or B lymphocytes. How T lymphocytes activated in the autologous MLR suppress the induction of PFC is not clear. Prior studies (11) had shown that no cytotoxic T lymphocytes were generated during the autologous MLR, and the present studies failed to demonstrate suppressive factors in the supernatant medium at the end of autologous mixed lymphocyte cultures. Suppressive activity appears to be cell mediated as it is eliminated by irradiation. The expression of suppressor activity did not require T lymphocytes in the lymphocyte preparation incubated with FSA. This suggested that suppressor cells were generated during the autologous MLR rather than cells that induced suppressor T cells in the responding lymphocyte preparation.

Suppressor activity has been generated in the allogeneic MLR (12) and by the preincubation of human mononuclear cells (13, 14). Lipsky and his colleagues (14) found that human mononuclear cells incubated for 7 d in the absence of mitogen suppressed the generation of PFC. A population of T lymphocytes that proliferate during incubation was shown to mediate suppressor activity. The autologous MLR is known to occur during the incubation of human mononuclear cells. Suppressor T lymphocytes generated under these conditions share several characteristics with suppressor T lymphocytes activated in the autologous MLR: (a) suppressor cells inhibit both autologous and allogeneic PFC; (b) suppressor activity is eliminated when DNA synthesis by preincubated cells is inhibited; and (c) B lymphocytes appear to be the target of suppressor T-lymphocyte activity.

Schwartz and his colleagues (13) found that mononuclear cells preincubated for 2 d inhibit immunoglobulin synthesis by autologous lymphocytes cultured with pokeweed mitogen. The inhibition of immunoglobulin synthesis generated was termed "endogenous" suppressor activity and contrasted with the augmented suppressor activity induced by Con A. We have compared suppressor activity generated when lymphocyte preparations are preincubated to that induced in these preparations by Con A. T-lymphocyte preparations containing <5% surface immunoglobulin-bearing lymphocytes and <10% adherent cells generated little or no suppressor activity in the absence of Con A.

TABLE VI  
Suppression of PFC by Lymphocyte Preparations Cultured in the Presence or Absence of Con A

Addition to culture	PFC generated by cultures (PFC/10 <sup>6</sup> cells)		
	Preincubated cells absent	Cells preincubated	
		Without Con A	With Con A
<b>Experiment 1</b>			
None	1,174		
Preincubated T cells depleted of adherent cells		1,098	1,161
Preincubated T cells		923	457
Preincubated unfractionated cells		389	48
T cells preincubated with non-T cells		122	51
<b>Experiment 2</b>			
None	406		
Preincubated T cells depleted of adherent cells		426	411
Preincubated T cells		456	261
Preincubated unfractionated cells		110	12
T cells preincubated with non-T cells		113	28

Unfractionated, T-enriched or a mixture of T- and irradiated non-T-cell preparations were preincubated for 72 h with or without 50 µg/ml Con A. All preincubation cell preparations were washed and added to autologous unfractionated mononuclear cells that were then incubated with FSA for 144 h. The anti-SRBC PFC generated were measured by the Jerne technique. Adherent cells were removed by passage over a nylon fiber column (18).

Although the suppressor cells induced by Con A are drawn from the T-lymphocyte populations (15), the induction of suppressor cells by Con A requires the presence of a small percentage of adherent non-T cells. No suppressor activity was induced in T-lymphocyte preparations that contained <2% non-T cells. Relevant to these findings is the fact that Con A-induced proliferation of immune lymphocytes is eliminated after treatment with anti-Ia serum and complement (16). As few as 4% adherent non-T cells restored the proliferative response in such preparations. As the proliferation of T lymphocytes is necessary for the induction of suppressor cells by Con A, it is not surprising that adherent non-T cells must be present in T-lymphocyte preparations for Con A to induce suppressor activity. Finally, Sakane and Green (19) have presented evidence that elimination of T cells activated in the autologous MLR with bromodeoxyuridine and light, abolishes the capacity of Con A to induce suppressor cells. Thus, Con A-induced suppressor cells appear to be drawn from the population of T lymphocytes activated in the autologous MLR.

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