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Suppression of DNA synthesis by Con A-activated human lymphocytes

STIMULATION BY CON A BOUND TO NON-T CELLS UNLESS REMOVED AFTER ACTIVATION

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SUMMARY

The capacity of human peripheral blood lymphocytes to suppress DNA synthesis of other lymphocytes was studied in an assay consisting of two steps: firstly, activation by Con A during 24 hr followed by α-methylglucoside and mitomycin treatment; secondly, incubation of these Con A-activated 'suppressor' cells with autologous responder cells and stimulants, or incubation with allogeneic responder cells. The results were compared with cells similarly treated but not incubated with Con A.

If α-methylglucoside treatment is omitted, stimulation of T and non-T cells occurs by Con A bound to the Con A-activated cells. Con A is especially bound to non-T lymphocytes and even gives a T cell-independent proliferation of non-T cells without differentiation to plasma cells. With α-methylglucoside treatment, 'suppressor' cells, activated by high Con A concentrations, are able to suppress DNA synthesis of autologous lymphocytes stimulated by allogeneic cells or soluble antigens to about 50%.

In a one-way MLC, in which the cell suspension containing the suppressor cells is also used as a stimulator cell suspension, a similar suppression was observed. Suppression of DNA synthesis was correlated with suppression of proliferation without evidence of cytotoxicity.

INTRODUCTION

In animals, the cellular regulations of both the humoral (Gershon, 1974; Baker, 1975) and cell-mediated immune response (Zembala & Asherson, 1973; Polak & Geleick, 1975) is now considered an important aspect of immunity. *In vitro* mouse spleen cells also are able, spontaneously or after activation by concanavalin A (Con A), to suppress antibody synthesis (Dutton, 1972; Rich & Pierce, 1973), lymphocyte proliferation induced by mitogens (Folch & Waksman, 1973) or allogeneic cells (Folch & Waksman, 1974; Rich & Rich, 1975), and can suppress the generation of cytotoxic lymphocytes (Peavy & Pierce, 1974).

In man, suppressor cells seem to be present in the spleen of uraemic patients (Kauffman, 1975) and in the peripheral blood from some patients with Hodgkin's disease (Twomey *et al.*, 1975), hypogammaglobulinaemia (Waldmann *et al.*, 1974), multiple myeloma (Broder *et al.*, 1975) and aplastic anaemia (Hoffman *et al.*, 1977). In two reports (Shou, Schwartz & Good, 1976; Hubert, Delespesse & Govaerts, 1976) the possibility of activation of suppressor cells from the peripheral blood of normal donors by incubation with Con A was described.

The present investigation aimed to extend these studies by looking for the optimal conditions for Con A-activated suppressor cell activity. We found that after Con A activation and mitomycin treatment of peripheral blood lymphocytes, stimulation of other lymphocytes occurs by Con A bound to non-T

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cells, thus disturbing the evaluation of Con A-induced suppression. In this report we describe this problem of Con A binding to cells, and the conditions for a proper assessment of the capacity of normal peripheral blood lymphocytes to suppress DNA synthesis of autologous and allogeneic lymphocytes.

MATERIALS AND METHODS

Lymphocyte isolation and separation. Peripheral blood mononuclear cells were obtained by Ficoll–Isopague gradient centrifugation and are referred to as unseparated cells. T and non-T cell populations were obtained by separating E rosettes on Ficoll–Isopaque of high specific gravity (1·090), as described by Parish (1975).

Briefly, unseparated cells were mixed with SRBC and layered at once on Ficoll–Isopaque (sp. gr. 1·090) and centrifuged at 1000–1600 *g*, with rapid acceleration at 20°C. The rosetting cells which appear in the red cell pellet were freed from erythrocytes by lysis with 0·91% ammonium chloride for 5 min at 0°C. For effectiveness, the separation procedure was repeated for the non-T cells. The T-cell population was usually contaminated by < 3% surface Ig-positive cells and < 1% monocytes. The non-T cell fraction contained 30–40% monocytes (identified by latex bead phagocytosis) and < 3% T cells.

Monocyte-depleted suspensions were obtained by incubation of the heparinized venous blood with carbonyl iron (GAF N.V., Delft, Holland, type SF; 2 mg/ml blood) for 60 min at 37°C prior to Ficoll–Isopaque gradient centrifugation. The lymphocyte suspension usually contained 1–4% monocytes after this procedure.

Lymphocyte activation by Con A (first step). Mononuclear cells (4×10^6) were incubated with or without 25 μg/ml of Con A (Calbiochem) in 1 ml RPMI 1640 with HEPES buffer (Gibco Biocult), containing 10% heat-inactivated (30 min at 56°C) pooled human serum of male donors, penicillin 100 u/ml and streptomycin 100 μg/ml, in polystyrene tubes (Flow Laboratories, 12 × 75 mm) during 24 hr at 37°C. The cells were then spun down, washed once and incubated in 1 ml RPMI containing 50 μg/ml of mitomycin (Sigma Chemical Co.) during 30 min.

After mitomycin treatment the cells were washed twice and incubated in medium with 0·3 M α-methylglucoside (1-methyl-α-D-glucopyranoside, Sigma Chemical Co.) for 20 min at 20°C, then they were spun down and again incubated in medium with methylglucoside, 0·3 M, for 20 min. Subsequently the cells were washed twice in medium and then used as Con A-activated 'suppressor' cells in lymphocyte cultures in microtitre plates. Control 'suppressor' cells were obtained by incubation without Con A, but were further treated similarly. Several conditions of this activation stage were studied. In the first experiments, Con A was not removed from the cells and the cells responsible for binding Con A and the subsequent stimulation of autologous cells were studied.

Lymphocyte cultures in microtitre plates (second step). Unseparated cells or purified lymphocytes were cultured in round-bottom microtitre plates (Cooke, U-form, 220M–24AR) in a final volume of 0·10 ml RPMI containing penicillin–streptomycin and 25% inactivated human serum as previously described (de Gast *et al.*, 1976).

Generally each well was filled as follows: (a) 25 μl of a responder mononuclear cell suspension containing 10^5 lymphocytes for stimulation by an antigen cocktail or by allogeneic lymphocytes, or containing 3×10^4 lymphocytes for stimulation by PHA (further referred to as responder cells); (b) 50 μl of Con A 'suppressor' or control 'suppressor' lymphocyte suspension (ratio suppressor:responder lymphocytes 1:1); (c) 25 μl of the antigen cocktail solution in medium (containing PPD in a final concentration of 5 μg/ml, diptheria toxoid 10 LF/ml, and tetanus toxoid 10 LF/ml); or (d) 25 μl/ml of PHA (Wellcome, stock solution consisted of 1 ampoule PHA dissolved in 5 ml distilled water (final concentration 1 μl of stock solution per ml), or 25 μl of a stimulator cell suspension P_m (this is a pool of frozen lymphocytes from ten random donors, treated with mitomycin, containing 10^5 lymphocytes).

The final serum concentration was 25%.

All cultures were carried out in triplicate. Control cultures without stimulant and control cultures without 'suppressor' cells were included. The plates were covered by a hard lid and wrapped in plastic adhesive kitchen film. They were incubated at 37°C in a humidified 5% CO_2 in air atmosphere for 5 days (PHA stimulation) or for 6 days (stimulation by the antigen cocktail or by allogeneic lymphocytes).

0·5 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, England) was added 16 hr before harvesting. Harvesting was performed with a multiple cell culture harvester (Skatron, Oslo, Norway) using glass fiber filters. The dried (60 min at 60°C) filters were transferred to counting vials, 4 ml of scintillation fluid were added and counting was performed in a liquid scintillation counter. The results are expressed as disintegrations per min (d/min) per well. The arithmetic mean and standard deviation of triplicate cultures were calculated. Apart from absolute mean d/min values of triplicate cultures, the results were expressed as a percentage suppression:

$$100\left[\frac{(\text{responder cells}+\text{Con A suppressor cells}_m+\text{stimulant})^* - (\text{responder cells}+\text{Con A suppressor cells}_m-\text{stimulant})^*}{(\text{responder cells}+\text{control suppressor cells}_m+\text{stimulant})^* - (\text{responder cells}+\text{control suppressor cells}_m-\text{stimulant})^*}\right].$$

Attention was paid to the question whether decreased DNA synthesis was due to cytotoxicity rather than suppression of proliferation.

*Expressed in mean d/min of triplicate cultures with responder cells, mitomycin-treated 'suppressor cells' ($cells_m$) and stimulants.

Identification of T cells, B cells and monocytes. T cells were identified by their capacity to spontaneously form rosettes with SRBC (Jondal, Holm & Wigzell, 1972). For making E rosettes, 2 vol. of 2% SRBC were centrifuged with 1 vol. of lymphocytes (2×10^6/ml) and 1 vol. of absorbed foetal calf serum at 150 *g* for 5 min at room temperature and then kept at 0°C for 1 hr. After resuspending carefully, only lymphocytes with more than three SRBC were counted positive.

B cells were identified by direct immunofluorescence using polyvalent anti-human immunoglobulin.

Monocytes were identified by morphology and ability to phagocytose latex particles.

RESULTS

Lymphocytes previously incubated in medium with high concentrations of Con A (25 or 50 μg/ml) during 24 hr, and subsequently treated with mitomycin, depressed DNA synthesis of autologous cells stimulated by allogeneic lymphocytes or by an antigen cocktail (Fig. 1), whereas the influence on stimulation by PHA was small. In the cultures without stimulants, however, increased DNA synthesis was noted if lymphocytes pre-incubated in medium with 12·5, 25 or 50 μg Con A per ml were used, and the degree of stimulation seemed to be related to the Con A concentration used during pre-incubation (Fig. 1). Mitomycin treatment of the Con A-incubated cells was adequate, as DNA synthesis of these cells cultured alone was consistently below 200 d/min per culture. Depletion of monocytes by carbonyl iron prior to incubation with Con A, 25 μg/ml, had no effect on this increased DNA synthesis in cultures without stimulants: DNA synthesis in lymphocyte–monocyte suspensions (14% monocytes) was 10,223 ±3991 d/min per culture, in monocyte-depleted suspensions (2% monocytes) it was 10,870±5201 (mean±s.d. of four experiments).

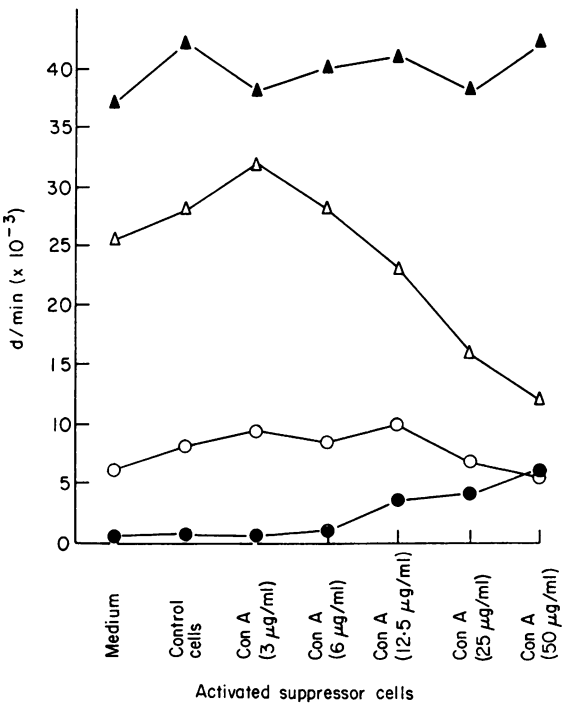

FIG. 1. Influence of mitomycin-treated 'suppressor' cells after pre-incubation with various concentrations of Con A on DNA synthesis of autologous responder cells. Expressed as mean thymidine incorporation of lymphocytes from two persons. On the horizontal axis the conditions of the pre-incubation step (24 hr) are represented: medium alone, control 'suppressor' cells and 'suppressor' cells activated by various concentrations of Con A. Points representing the d/min values in cultures with the same stimulant are connected. Cells pre-incubated with high Con A concentrations depress DNA synthesis of autologous cells stimulated by allogeneic lymphocytes (▲, PHA; △, MLC) or by antigen cocktail (○), but DNA synthesis in cultures without stimulant is increased (●).

When Con A-incubated cells were treated with α-methylglucoside after incubation with mitomycin, their stimulation of autologous cells was completely abolished: without methylglucoside treatment DNA synthesis was 15,969±5638 d/min, after methylglucoside treatment it was 1610±1550 d/min, whereas control cell suspensions not incubated with Con A, but treated similarly with mitomycin and α-methylglucoside had a thymidine incorporation of 975±533 d/min (mean±s.d. of four experiments). To study whether Con A is removed or covered by α-methylglucoside, lymphocytes were incubated during 2 hr in medium with Con A, 25 μg/ml, prior to either washing (three times) or treatment with α-methylglucoside and subsequently cultured during 5 days. It was assumed that Con A covered with α-methylglucoside will still trigger the cell to begin DNA synthesis. As shown in Table 1, DNA synthesis was much lower in the cultures of cells treated with α-methylglucoside (mean±s.d.: 2226±212 d/min) than in the cultures of cells only washed (three times) after incubation with Con A (6366±3255 d/min). Control cultures, not incubated with Con A, but treated similarly, showed a thymidine incorporation of 355±288 d/min.

Which subpopulation of lymphocytes binds Con A and are T or non-T lymphocytes stimulated by cell-bound Con A?

In experiments with separated T and non-T cells, the binding of Con A was studied. As shown in Table 2(a) and (b), a high degree of stimulation of autologous responder cells was found when non-T cells incubated with Con A were added. Even non-T responder cells, cultured together with non-T cells

TABLE 1 The removal of covering of Con A by α-methylglucoside treatment

Experiment	Control without Con A incubation	Washed in medium (three times) after 2 hr Con A incubation	Treated with α-methyl-glucoside after 2 hr Con A incubation
(1)	160±45	4176±568	2361±373
(2)	327±24	3033±636	2281±465
(3)	771±254	9694±1425	1912±414
(4)	161±6	8559±830	2348±560
Total mean±s.d.	355±288	6366±3255	2226±212

* Lymphocytes were incubated with Con A, 25 μg/ml, during 2 hr prior to either washing or methylglucoside treatment (no mitomycin incubation). Subsequently cells (3×10^4) were incubated in RPMI with 20% heat-inactivated human serum for 5 days and thymidine incorporation determined.

TABLE 2 (a) Lymphocyte stimulation by mitomycin-treated autologous T and non-T cells previously incubated with Con A

Responder cells	Autologous cells incubated for 24 hr with Con A and mitomycin-treated	Thymidine incorporation (d/min)			
		A*	B*	C*	D*
Unseparated	T_m	2894	4306 (2551)†	4968 (1209)†	1234 (898)
T	T_m	n.d.	n.d.	666	360
Non-T	T_m	365	221	4490	542
Unseparated	Non-T_m	15598	19928 (16014)	16163 (7861)	10593 (6372)
T cell	Non-T_m	20229	8133	12035	2518
Non-T cell	Non-T_m	19935	7198	13461	21788

* Donors.

† Values in parentheses are of experiments in which heated (10 min 56°C) cells, previously incubated with Con A, are used.

‡ n.d. = Not done.

TABLE 2. (b) Effectiveness of separation and heating tested in MLC donor A–B, donor C–D

Responder cells	Homologous cells not incubated with Con A but mitomycin-treated	Thymidine incorporation (d/min)			
		A*	B*	C*	D*
Unseparated	T_m	950	905	3468 (856)†	2385 (915)
Unseparated	non-T_m	15670	8926	33236 (1300)	22318 (833)
T	non-T_m	18237	9964	n.d.	n.d.
Non-T	non-T_m	442	397	n.d.	n.d.

* Donors.

† Values in parentheses are of experiments in which heated (10 min, 56°C) cells, previously incubated with Con A, are used.

incubated with Con A and mitomycin-treated, showed increased DNA synthesis. No differentiation to plasma cells occured, as demonstrated by the absence of cytoplasmic Ig-containing cells after 7 days of culturing. T cells incubated with Con A and mitomycin-treated (T_m) gave only a low degree of stimulation of unseparated autologous responder cells.

After heating (10 min at 56°C), both T cells and non-T cells previously incubated with Con A and mitomycin-treated induced lower, but still evident, DNA synthesis of autologous lymphocytes. The effectiveness of the separation and heating (for killing) was checked in a MLC. In this experiment, T and non-T cells, not incubated with Con A, were used as stimulator or responder cells. As shown in Table 2(b), only non-T cells were good stimulator cells, whereas only T cells were good responder cells. Heated non-T cells lost their capacity to stimulate in the MLC.

Con A-induced suppression of DNA synthesis

After establishing the necessity to remove Con A from Con A-activated lymphocytes for a proper evaluation of suppressor cell activity, several conditions were studied. The influence of various Con A concentrations in the first activation step on the capacity of these cells to suppress DNA synthesis of autologous lymphocytes, stimulated by PHA, an antigen cocktail or allogeneic lymphocytes, is depicted in Fig. 2 (mean of three experiments ±s.e.). Lymphocytes from healthy persons, activated by high Con A concentrations (12·5, 25 and 50 μg/ml), suppressed 50 to 60% of DNA synthesis of autologous lymphocytes stimulated by an antigen cocktail or by allogeneic lymphocytes, whereas lymphocyte

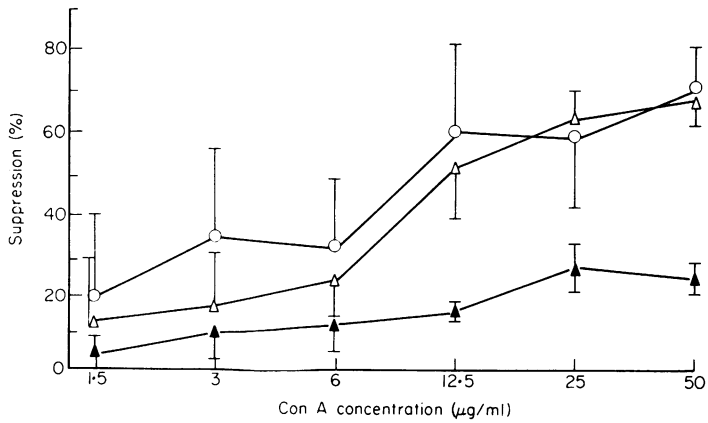

FIG. 2. Influence of mitomycin and α-methylglucoside-treated suppressor cells, previously activated by various concentrations of Con A, on DNA synthesis of autologous responder cells. Expressed as mean percentage suppression compared to control 'suppressor' cells of peripheral blood lymphocytes from four donors (± s.e.). Stimulation of autologous responder cells by an antigen cocktail (○) or allogeneic lymphocytes (MLC, △) is evidently suppressed, whereas little suppression of PHA stimulation (▲) is noted.

stimulation by PHA is only slightly suppressed (10 to 20%). Activation during 8, 24 or 48 hr with Con A, 25 μg/ml, gave the same degree of suppressor cell activity.

When Con A-activated suppressor cells were added to autologous lymphocytes at several times, from 24 hr before until 24 hr after addition of stimulants (Ag cocktail or allogeneic lymphocytes), no significant differences in the degree of suppression of DNA synthesis was noted (Table 3).

Relation between suppression of DNA synthesis and suppression of proliferation

Studying suppression of autologous responder cells (Table 4), a rough correlation was found between suppression of DNA synthesis and suppression of proliferation as determined by cell counts after 6 days of culture. When suppression is studied in a homologous system, in which the cell suspension containing the mitomycin-treated Con A suppressor cells or control suppressor cells is also used as stimulator cell suspension in a one-way MLC, the same rough correlation was found (Table 5). This was not due to cytotoxicity of the Con A-activated cells, because the number of dead cells, detected by trypan blue exclusion, was not increased. When the results tabulated in Tables 4 and 5 are compared, Con A-activated suppressor cells appear to have suppressor cell activity against autologous responder cells, as well as against homologous responder cells.

TABLE 3. The influence of the moment of addition of autologous suppressor cells

Time (hr) of addition of suppressor cells*	Suppression of Ag cocktail stimulation†	Suppression of MLC (P_m)†
−24	38±14	50±14
−4	21±17	53±6
0	22±18	37±18
+4	40±11	39±19
+24	27±19	56±14

* Suppressor cells were added to autologous lymphocytes from 24 hr before until 24 hr after addition of stimulants (Ag cocktail) or allogeneic lymphocytes (P_m).

† Percentage suppression (mean±s.d. of four experiments).

TABLE 4. Relation between suppression of DNA synthesis and suppression of proliferation in an autologous system

Experiment	Stimulant	Cultures with control suppressor cells		Cultures with Con A suppressor cells			
		Counts*	DNA synthesis†	Counts	Suppression (%)‡	DNA synthesis	Suppression (%)‡
(1)	None	1·1	553	1·2		903	
	Ag cocktail	2·1	3221	2·0	20	2865	26
	Allogeneic lymphocytes (P_m)	4·0	13363	2·4	59	6894	53
(2)	None	1·0	890	1·1		1351	
	Ag cocktail	3·2	33218	3·0	14	23233	32
	Allogeneic lymphocytes (P_m)	4·1	41206	2·1	68	21487	50

* Counts in cells × 10^{-6} per ml after 6 days of culture.

† DNA synthesis in d/min per culture as measured by thymidine incorporation.

‡ Suppression (%) is given by the equation in the Materials and Methods section.

TABLE 5. Relation between suppression of DNA synthesis and suppression of proliferation in a homologous system: suppressor cell suspension is used at the same time as stimulator cell suspension in a one-way MLC

Experiment	Cells used in MLC	Cultures with control suppressor cells		Cultures with Con A suppressor cells			
		Counts*	DNA synthesis	Counts*	Suppression (%)	DNA synthesis	Suppression (%)
(1)	$A+A_m$	1·0	890	1·1		1351	
	$A+B_m$	2·0	25384	1·3	80	11459	59
	$A+B_m+P_m$	2·5	43206	2·0	40	23654	47
(2)	$B+B_m$	1·1	553	1·2		903	
	$B+A_m$	1·8	17571	1·6	43	14747	19
	$B+A_m+P_m$	2·4	35762	2·2	23	28864	21
(3)	$C+C_m$	1·1	724	1·1		1206	
	$C+D_m$	3·1	31293	2·0	55	14482	57
	$D+C_m$	2·4	6894	1·8	46	3221	67

See Table 4 for explanatory footnotes.

DISCUSSION

Lymphocytes, preincubated with relatively high concentrations of Con A (12·5–100 μg/ml) during 24 hr and subsequently treated with mitomycin, were able to suppress the stimulation of autologous cells by soluble antigens and allogeneic cells. However, a high degree of DNA synthesis was observed in the control cultures without stimulants, which was correlated with the Con A concentration used during the preincubation step. Inefficient mitomycin treatment of the Con A-incubated cells was excluded. The stimulation was not due to Con A taken up or bound by monocytes, as their removal had no influence on the degree of stimulation. The cause of this phenomenon appeared to be Con A-binding by the Con A-incubated cells and stimulation of autologous responder cells by cell-bound Con A, because the stimulation disappeared completely after treatment of the Con A-incubated cells with α-methylglucoside. This excluded other possible causes of stimulation of DNA synthesis, such as lymphoblast antigens (Weksler, 1976) or mitogenic factor production by Con A.

Cell separation experiments showed that especially non-T cells preincubated with Con A and then mitomycin-treated caused a high degree of DNA synthesis of autologous responder cells. To exclude the possibility that the T-cell suspension showed a lower stimulation after Con A activation because of the presence of activated suppressor T cells at the same time, the Con A-pre-incubated T and non-T cell suspensions were heated to abolish cell function and then added to autologous cells. Even after this procedure the non-T cell suspension caused much higher stimulation, which indicated that more Con A was bound to non-T cells than to T cells. This is not in keeping with other studies, in which it was found that mouse T and B cells bind both Con A and PHA equally well (Greaves, Bauminger & Janossy, 1972; Möller *et al.*, 1973).

It is of interest that even non-T cells were stimulated by Con A bound to non-T cells. The T–non-T cell separation in this experiment was good, as the T cells had no stimulator capacity and the non-T cells had no responder capacity in the MLC.

Despite the T cell-independent non-T cell proliferation by cell-bound Con A, no differentiation of immunoglobulin-containing cells occurred. Cell-bound Con A can probably be compared with insolubilized Con A or PHA, as these are also able to cause a T-independent B-cell proliferation (Greaves & Bauminger, 1972; Andersson *et al.*, 1972).

Methylglucoside treatment of the Con A-activated cells seems to remove Con A rather than cover it, because thus-treated cells show less DNA synthesis than cells only washed after 2 hr incubation with Con A.

Removal of Con A after activation was not performed in two previous studies of Con A-activated

suppressor cell activity of normal human lymphocytes (Shou *et al.*, 1976; Hubert *et al.*, 1976). After this necessary methylglucoside treatment, Con A-activated cells still have the capacity to suppress DNA synthesis of autologous lymphocytes stimulated by soluble antigens or allogeneic lymphocytes.

Thus after activation of suppressor cells by Con A, Con A is not required for the expression of suppressor cell activity, whereas it is necessary in Con A-induced cytotoxicity tests (Asherson, Ferluga & Janossy, 1973). The suppression of DNA synthesis correlated with the suppression of proliferation as determined by cell counts at the end of the culture period, without evidence of cytotoxicity. When the Con A-activated, or the control, suppressor cell suspension was used as a stimulator cell suspension in a MLC with allogeneic responder cells, about the same degree of suppression (50%) was observed as in the autologous situation. In this situation, cytotoxicity was also not involved. The moment of addition of suppressor cells to the autologous responder cells was not important within the first 24 hr of stimulation, indicating that the suppressor cells are not acting during the activation stage.

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