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ORIGINAL

STUDIES ON THE SUPPRESSION OF LYMPHOCYTE PROLIFERATION BY CONCANAVALIN-A ACTIVATED MONONUCLEAR CELLS IN NORMAL INDIVIDUALS

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SUMMARY

Suppressor lymphocytes are known to play an important role in the control of immune responses. The property of the lectin concanavalin-A (Con-A) to induce suppressor cell activity (SCA) has been used as the basis of a method to assess SCA in the peripheral blood of patients. In this study we confirm previous data on the standard double culture assay for Con-A induced SCA and we analyse the possibility to demonstrate SCA in a single culture assay. Although suppression could be demonstrated with this new assay, we discuss the reasons that can represent a major limitation to its use in clinical studies.

RESUMO

Supressão da proliferação linfocitária por células mononucleares activadas pela concanavalina-A: Estudo em indivíduos normais.

O controlo das respostas imunológicas depende pelo menos em parte de linfocitos supressores. A propriedade da concanavalina-A de induzir actividade supressora, tem sido usada como a base de um método para avaliar a actividade supressora de células mononucleares do sangue periférico de doentes. Neste trabalho, depois de confirmar resultados da literatura usando o modelo convencional da supressão induzida pela concanavalina-A constando de dois sistemas de cultura, analisou-se a possibilidade de demonstrar a actividade supressora num sistema de cultura único. Apesar da demonstração da actividade supressora neste novo modelo, apresentam-se argumentos contra a sua aplicabilidade em estudos clínicos.

INTRODUCTION

The lectin concanavalin-A (Con-A) in addition to its ability to induce lymphocyte transformation [1] and *lectin dependent* lymphocyte cytotoxicity [2] also stimulates a lymphocyte subpopulation to generate suppressor activity.[3-5] This property has been used as the basis of a method to assess suppressor cell activity in clinical immunology as part of studies attempting to assess the possibility of suppressor cell disturbances having a role in immunologically mediated diseases. The technique previously used consists of two culture systems: in the first, peripheral blood mononuclear cells (PBMC) are exposed to Con-A for 48 hours. Control cells are cultured for the same period of time in the absence of Con-A. Both control cells and Con-A exposed cells (putative suppressor cells), are then treated with mitomycin-C to prevent subsequent proliferation and washed with αmethyl-D-mannoside to displace cell bound Con-A. Suppressor activity in the Con-A exposed cells has been shown to affect both autologous and allogeneic cells. This is shown by co-culturing fresh autologous (or allogenic cells) with equal numbers of the Con-A exposed cells or the control cells, and measuring the responses of the fresh cells to mitogens, in the presence of the cells added. In normal individuals, the responses of fresh cells co-cultured with Con-A exposed cells are consistently decreased as compared to the responses of fresh cells co-cultured with control cells. This inhibition is interpreted as Con-A generated suppressor activity.[3, 4, 6, 7]

The study reported here is an attempt to demonstrate the phenomenon of Con-A induced suppression in a single culture system. The rationale for this is as follows: firstly, relatively low doses of Con-A can induce suppressor cell activity;[8] secondly, several authors [9, 10] have demonstrated that cell proliferation was not a prerequisite for the generation of Con-A induced suppression. Therefore, it could theoretically be anticipated that a low dose of Con-A could be identified, low enough not to cause detectable cell proliferation, but still capable of inducing suppressor cells. If such a dose could be selected, this would constitute the basis of a single culture model for demonstrating Con-A induced suppression. Thus, peripheral blood mononuclear cells (PBMC) were firstly exposed to Con-A in non-mitogenic doses (not inducing proliferation) but possibly capable of inducing suppressor activity in a particular subset of T cells. In a second stage in the same culture system after 24 hours, a stimulating dose of mitogen was added to induce proliferation. The proliferative responses of PBMC pre-exposed to the non-mitogenic dose of Con-A were compared to control cells, initially incubated without Con-A, but then challenged with an identical stimulatory dose. If exposure to low dose Con-A induced suppressor cells, one would expect the proliferative responses of this group of cells to be lower.

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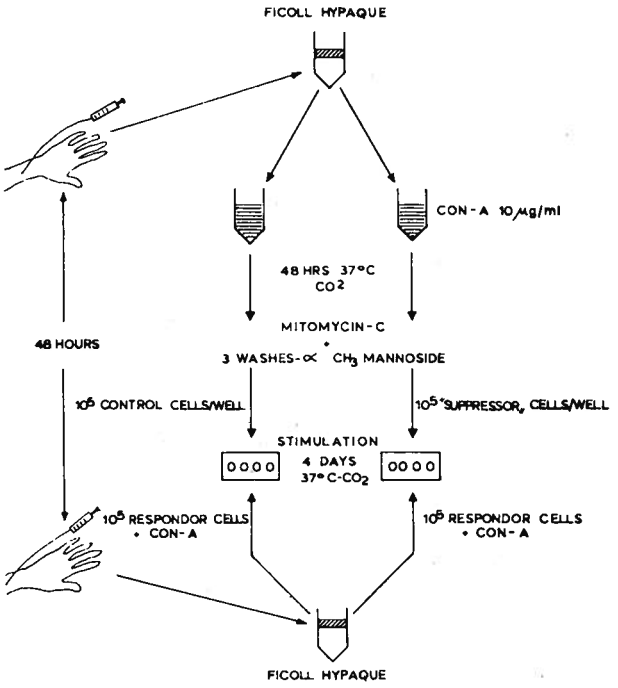

Figure 1: Protocol followed in the double culture model for Con-A induced suppression. Con-A 10µg/ml represents the final concentration in the culture tube.

MATERIAL AND METHODS

Mononuclear cells were isolated from peripheral blood of normal subjects by a Ficoll-Hypaque gradient [11] and used for the functional assays at a concentration of 1×10^6/ml in RPMI 1640 culture medium containing 15 % heat inactivated foetal calf serum (FCS), 4 mM glutamine, 100 units/ml of Penicilin and 100 ug/ml of Streptomycin (complete medium).

a) Double culture assay for suppressor cell activity

Con-A induced suppressor cell activity was assayed in PBMC according to the method described by Shou et al [3] with minor modifications according to Hodgson et al.[8] After isolatiom, PBMC were cultured either in the presence ($PBMC_s$) or absence ($PBMC_c$) of Con-A for 48 hours. The final concentration of Con-A (Sigma) used for induction of suppression was usually 10 µg/ml, but in some experiments the concentration varied as defined in results. Cultures were done in universal tubes (Sterilin Ltd. UK.). The two cell suspensions were then treated with mitomycin-C (Sigma) 50 µg/ml for 35 minutes at 37° and washed three times with 30 mM ∝methyl mannoside to remove Con-A. Finally, cells were washed once more in Hank's solution and resuspended in complete medium. They were then mixed with fresh cells as detailed below.

The second stage of the assay commenced with the preparation of fresh PBMC from a normal individual. These were used as responder cells (R), stimulated with Con-A and co-cultured with either PBMC previously exposed to Con-A ($R + PBMC_s + Con\text{-}A$), or with those not exposed to Con-A ($R + PBMC_c + Con\text{-}A$). Cultures were done in quadriplicate by adding 1×10^5 of each type of cells in 100 µl of complete medium and 20 µl of a solution with 200 µg/ml of Con-A in RPMI medium. Total volume per well of the microtitre plates was therefore 220 µl and cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 for 90 hours. Lymphocyte proliferation was assessed by adding 1 µCi ^{3}H-Thymidine (specific activity 20-25 Ci/mM — Amersham) for the last 18 hours after what cells were harvested with an automatic harvester (Dynatech) into glass-fiber filter papers which were transfered to scintillation vials for determination of the amount radioactive thymidine incorporation into cells using a liquid scintillation counter.

The following cultures were set up:

1 — $R + PBMC_s + Con\text{-}A$
2 — $R + PBMC_c + Con\text{-}A$
3 — $R + PBMC_s$
4 — $R + PBMC_c$

The degree of suppression was calculated by the following formula:

$$\text{Percent. of suppression} = \left(1 - \frac{(R + PBMC_s + Con\text{-}A) - (R + PBMC_s)}{(R + PBMC_c + Con\text{-}A) - (R + PBMC_c)}\right) \times 100$$

The expression in brakets represent ^{3}H Thymidine incorporation in cpm cultures. Subtraction of the cpm of cultures 3 and 4, corrects for ^{3}H Thymidine incorporation due to the mixed lymphocyte reaction. In all experiments cultures were also set up to confirm that mitomycin-C had adequately suppressed ^{3}H Thymidine incorporation in the preincubated cultures.

The diagramatic representation of this model in standard conditions is shown in Figure 1.

TABLE 1 **Representative examples of the double culture assay for concanavalin-A induced suppressor cell activity in healthy subjects.**

Individuals Studied	Control Cultures $(R + PBMC_c + Con\text{-}A) - (R + PBMC_c)$	«Suppressor Cultures» $(R + PBMC_s + Con\text{-}A) - (R + PBMC_s)$	% Suppression $= (1 - \frac{\text{Suppressor Cultures}}{\text{Control Cultures}}) \times 100$
OB	101,429 − 6,795 = 94,634	59,307 − 3,841 = 55,466	$(1 - \frac{55,466}{94,634}) \times 100 = 41$ %
ZT	118,082 − 8,893 = 109,189	95,211 − 7,073 = 88,138	$(1 - \frac{88,138}{109,189}) \times 100 = 19.5$ %
JD	106,682 − 6,728 = 99,954	96,234 − 6,211 = 90,023	$(1 - \frac{90,023}{99,954}) \times 100 = 9.4$ %

Numbers shown represent mean counts per minute of the cultures performed in quadriplicate.
Details in methods. Standard errors of means were in all cases less than 10 % of the mean.

b) Experiments in a single culture model

PBMC were isolated as before. In this model there were also two stages: a preincubation stage and a stimulation stage but these were however performed in the same culture system.

Preincubation stage: aliquots of 100 μl of PBMC at a concentration of 1×10^6 were cultured for 24 hours at 37 °C in a humidified atmosphere with 5 % CO_2 in microtitre plates either with or without Con-A. The dose of Con-A added was 20 μl of a solution of Con-A 1.0 μg/ml in RPMI medium. Cells cultured without Con-A received 20 μl of RPMI medium as a control. This dose was selected by previous experiments in normal individuals showing that such a dose did not produce lymphocyte proliferation.

In some experiments as indicated in results a range of higher and lower doses were used.

Stimulation stage: after 24 hours, both cells preincubated with or without Con-A were then stimulated with Con-A in doses capable of producing lymphocyte proliferation. These consisted of 20 μl of 25 or 50 μg/ml Con-A solutions. As control for the non-mitogenicity of the preincubation doses, some cells of both groups were left without receiving the Con-A stimulation. Each group of cells was cultured in triplicate for 90 hours. Lymphocyte proliferation in response to Con-A and in controls was assessed as described before.

In experiments designed to determine the possible role of the Con-A left bound to the lymphocyte's Con-A receptors after the first incubation, cells were cultured in the preincubation stage as described above; then they were washed three times with 30 mM αmethyl mannoside in RPMI 1640 medium and resuspended in complete medium. Both control cells and cells exposed to Con-A in the preincubation stage were washed. They were then stimulated with different doses of various mitogens.

Expression of results: the inhibitory effect occuring in cells explosed to non-mitogenic doses of Con-A was expressed as a percentage of inhibition calculated by the following formula:

$$\text{Percentage of inhibition} = \left(1 - \frac{A - a}{B - b}\right) \times 100, \text{ where}$$

A = counts per minute of cultures stimulated after 24 hours preincubation with Con-A; a = counts per minute of cultures preincubated with Con-A not receiving stimulation; B = counts per minute of cultures stimulated after 24 hours preincubation without Con-A; b = counts per minute of cultures neither princubated with Con-A nor stimulated.

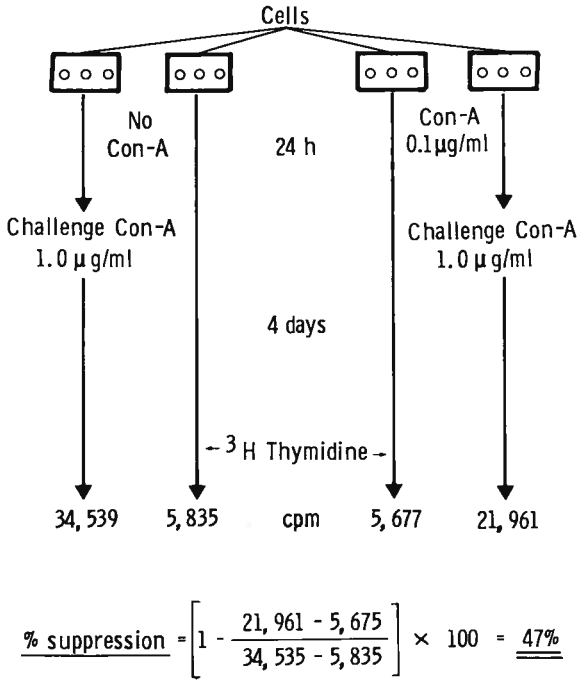

Figure 2: Diagram of a representative experiment with the single culture model for Con-A induced suppresion. The Con-A concentrations indicated, refer to concentrations in the solutions added to culture wells (see methods).

In experiments where no suppression was found but the opposite phenomenon of enchancement, this is shown by the sign +.

RESULTS AND COMMENTS

a) Double culture model for Con-A induced suppression

Table 1 shows three representative examples of the results obtained with the double culture model for Con-A induced suppression.

The effects of varying the dose of Con-A used for inducing suppressor cells are shown in Table 2. It is clear that as the dose is increased, increasing levels of suppression were obtained, although with very high doses results were variable. It is worth noting that with relatively low doses of Con-A that induce minimal cell proliferation, a certain degree of suppression was already obtained.

TABLE 2 **Concanavalin-A induced suppressor cell assay (double culture): effect of varying the dose of concanavalin-A in the first culture system.**

Final Concentration of Concanavalin-A used in the first System	^{3}H Thymidine incorporation in cpm in cultures consisting of R + PBMC + Con-A	% Suppression
0 µg/ml	72,102	—
1.0 µg/ml	62,931	12.7
2.5 µg/ml	64,737	10.2
5.0 µg/ml	59,311	17.7
10 µg/ml	52,827	26.7
25 µg/ml	51,102	29.1
50 µg/ml	54,811	24.0

- Values shown are the means of counts per minute of quadriplicate cultures after subtraction of the counts per minute of cultures consisting of R + PBMC without Con-A.
- Concentration of Con-A employed in second system was constant (200 µg/ml – 20 µl per well).

TABLE 3 **«Con-A induced suppression» in a single culture system.**

Subjects	Unstimulated cultures A Control (RPMI) B Con-A Activated	Incorporation of ^{3}H Thymidine Con-A stimulated cultures Con-A 25 μg/ml	% Suppression	Con-A 50 μg/ml	% Suppression
1	A 5,259 ± 451 B 6,465 ± 579	34,539 ± 2,359 21,961 ± 683	47.1 %	64,226 ± 3,544 50,088 ± 2,110	26.0 %
2	A 5,835 ± 706 B 5,697 ± 752	39,878 ± 5,890 23,136 ± 1,959	48.8 %	83,983 ± 2,614 84,965 ± 2,864	1.4 %
3	A 4,749 ± 659 B 6,123 ± 379	22,094 ± 1,536 16,999 ± 981	37.3 %	64,805 ± 580 50,826 ± 1,742	25.5 %
4	A 5,165 ± 607 B 3,912 ± 427	38,955 ± 3,227 27,668 ± 1,962	29.7 %	65,680 ± 3,427 64,649 ± 3,301	0.4 %
5	A 7,895 ± 1,226 B 6,541 ± 673	58,278 ± 3,054 50,309 ± 3,525	13.1 %	86,010 ± 326 80,778 ± 3,020	4.9 %
6	A 3,675 ± 370 B 4,883 ± 719	43,081 ± 3,127 40,454 ± 4,692	9.3 %	87,420 ± 6,008 88,037 ± 5,312	0.7 %
7	A 3,621 ± 251 B 3,570 ± 199	41,460 ± 3,248 41,319 ± 1,197	0 %	88,351 ± 5,210 93,334 ± 7,113	+5.9 %
8	A 4,933 ± 451 B 5,314 ± 396	28,351 ± 1,921 21,833 ± 981	29 %	78,198 ± 3,937 60,145 ± 2,229	25.2 %
MEAN + SEM	A 5,142 ± 477 B 5,314 ± 396	38,312 ± 3,802 30,460 ± 4,228	26.8 + 6.3	77,334 ± 3,798 71,602 ± 6,094	9.4 + 4.8

Values shown for individual subjects are the means ± SEM of cpm of cultures performed in triplicate. Con-A stimulation was done by adding 20 μl of the solutions indicated in the Table, to 100 μl of PBMC 1×10^6/ml.

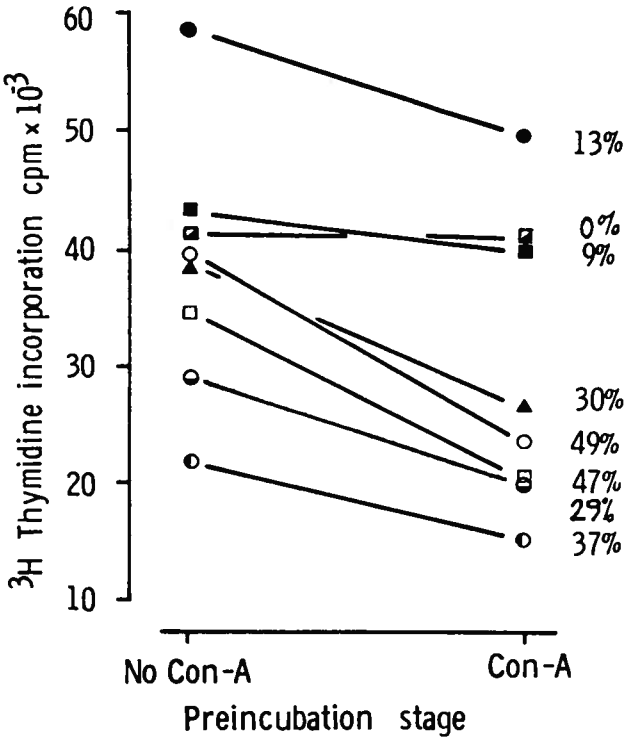

Figure 3: Experiments where mononuclear cells pre-exposed or not to non mitogenic doses of Con-A, were not washed with αmethyl-mannoside, and stimulated with Con-A (25μg/ml). Inhibition of the proliferative responses is seen in the group of cells pre-exposed to Con-A and the percentage of inhibition is shown for each individual experiment.

b) Experiments in a single culture system

A diagramatic representation of one experiment is shown in Figure 2. It is shown in Table 3 and Figure 3 that cells exposed for 24 hours to non-mitogenic doses of Con-A (20 ul of 1.0 ug/ml for 200 μl of cells 1×10^6/ml) had decreased proliferative responses to the subsequent stimulatory dose of Con-A (20 μl of 25 μg/ml). This occurred in all subjects but one. Comparison by the paired t test of paired values of ^{3}H Thymidine incorporation in stimulated cultures pre-exposed or not to Con-A, revealed this suppression to be significant ($t = 4.059$ $p < 0.01$). The levels of inhibition varied between 9.3 % and 48.8 % and the mean of the percentage of inhibition was 26.8 % ± 6.3 SEM, $n = 8$ (Table 3). The second column of Table 3 shows that when the dose of Con-A used to produce proliferation was increased to 20 μl of 50 μg/ml Con-A, the levels of inhibition of the proliferative response were considerably reduced, with a mean percentage of inhibition of 9.4 % ± 4.8 SEM ($n = 8$).

One possible explanation for the inhibition was that Con-A exerted a toxic effect during the preincubation stage. This was checked by incubating PBMC with or without Con-A in the doses used in preincubation and leaving them for 90 hours. The percentage of dead cells assessed by trypan blue exclusion did not differ between the two groups. Similarly no differences were found between viability of cells that were stimulated with Con-A after having been submitted to the preincubation stage with or without Con-A .

Figure 4 shows the results of experiments in which PBMC, after being exposed to non-mitogenic doses of Con-A, were washed with αmethyl-mannoside prior to stimulation by mitogens. In these experiments stimulation was done with three different mitogens: Pokeweed mitogen PWM

TABLE 4 **Variation of doses of Con-A used during the preincubation stage of the single culture model for Con-A induced suppression.**

Subjects Studied	Concentration of Con-A used in pre-stimulation period (μg/ml)	cpm of unstimulated cultures	cpm in stimulated cultures	Percentage of suppression
Subject 1	0	5,259	34,359	—
	0.5	6,329	24,321	38.2
	1.0	6,465	21,961	47.1
	2.0	8,238	30,233	24.5
Subject 3	0	4,749	22,094	—
	0.5	6,906	18,325	28.9
	1.0	6,123	16,999	37.3
	2.0	7,521	19,094	33.3
Subject 4	0	5,165	38,955	—
	0.5	4,383	24,833	39.5
	1.0	3,912	27,668	29.7
	2.0	6,989	30,223	31.3
Subject 7	0	3,621	41,322	—
	0.5	3,323	40,382	0
	1.0	3,570	41,319	0
	2.0	4,899	34,821	+1.6

- Values shown are means of counts per minute of cultures done in triplicate.
- Stimulation in second stage was performed with a constant dose of Con-A (20 μl of 25 μg of Con-A per ml for 100 μl of PBMC 1×10^6/ml).

4 μg/ml, Con-A 25 μg/ml and PHA 50 μg/ml. There was again inhibition of ^{3}H Thymidine incorporation in cells preexposed to Con-A, suggesting that the inhibitory phenomenon was not a consequence of blocking of Con-A receptors in the surface of lymphocytes since Con-A had presumably been removed by methyl mannoside washing. Moreover the results suggest that the inhibitory phenomenon was not dependent on soluble factors liberated during the preincubation stage as the cultures during the stimulatory stage were done in freshly prepared medium.

Finally, in Table 4, results of experiments where different preincubation doses of Con-A were used, are shown. It can be seen that no simple linear or logarithmic dose-response curves in regard to levels of inhibition could be obtained.

CONCLUSIONS

The results with the double culture model showing that peripheral blood mononuclear cells exposed to concanavalin-A were capable of suppressing the lymphocyte proliferation of allogeneic fresh mononuclear cells are in agreement with data from other authors.[3, 12, 13, 14]

Previous work indicated that the Con-A inducible suppressor cell is a T cell[5, 6, 10] and probably this cell does not require cell proliferation for expression of suppressor properties.[9, 10, 15] It was shown here that normal peripheral blood mononuclear cells exposed for 24 hours to concanavalin-A in doses that were incapable of inducing cell proliferation (non-mitogenic), had decreased proliferative responses when subsequently stimulated with mitogenic doses of the mitogen. In contrast to the standard technique for concanavalin-A inducible suppression where two distinct culture systems are used, the inhibitory phenomenon recorded in these experiments only required one single culture system.

One interpretation is that the Con-A in low doses used in the first incubation stage, although incapable of inducing

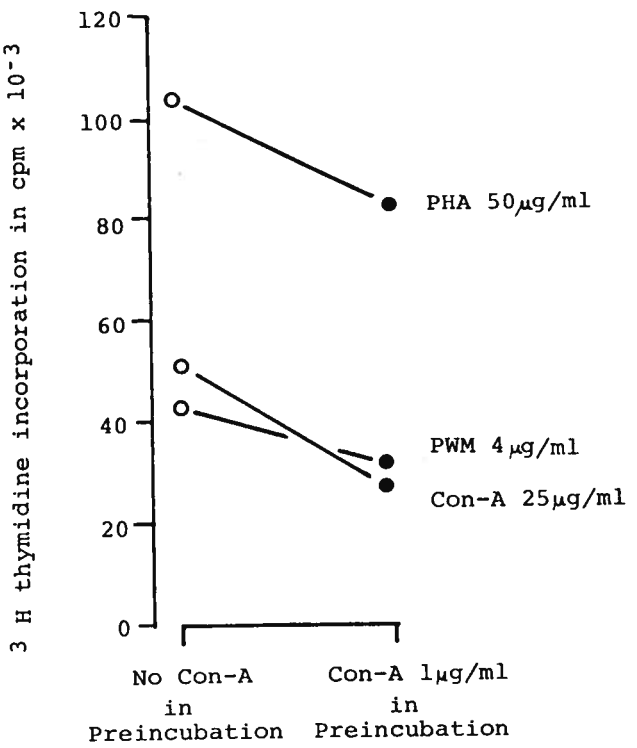

Figure 4: Lymphocyte proliferation in response to mitogens in cultures preincubated (•) or not pre-incubated (○) with non mitogenic doses of concanavalin-A and washed with αmethyl mannoside before being stimulated. Circles represent the means of an experiment done in triplicate. SEM >8% in all cultures. Mitogen stimulation was done by adding 20μl of the mitogen solutions indicated above, to 100 μl of PBMC 1×10^6/ml (see methods).

detectable cell proliferation, is still capable of inducing suppressor cells that inhibit the proliferative responses of cells present in the same culture system. This could form the basis of a single culture method for detection of inducible suppressor activity. However, no simple dose-response relationship emerged between the dose of Con-A used to *induce suppressor cells* in the preincubation stage and the amount of suppression of the subsequent mitogen-induced proliferation. The lack of a simple relationship probably reflects the complex events induced by Con-A, which in different lymphocyte subpopulations can induce suppression, or help or cytotoxicity, as well as proliferation.

In conclusion, although our results in a single culture system supported the concept that such a system could be used to record suppression, the wide variation in the suppression induced and the lack of a clear dose-response curve appear to form a major limitation to its use in clinical studies.

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