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Relative ability to provide help: an explanation for Con A-induced suppression

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SUMMARY

Concanavalin A (Con A) induced suppression of human peripheral lymphocytes has been studied *in vitro* at different cell concentrations using a 20 μl inverted Terasaki plate culture system in which the uptake of ^{3}H-thymidine was measured. Conventional assessment of suppression relates the response of cells to Con A in the presence of Con A-pretreated autologous cells, with the response in the presence of cells preincubated without Con A (control cells). The level of suppression so calculated was affected both by serum-related high background counts and by autologous stimulation of responding cells by mitomycin-treated Con A-pretreated cells. The high background was largely removed by the use of serum-free Iscove's medium. The data involving different cell concentrations and periods of cultivation indicate that the assessment of suppression is inadequate. Both Con A-pretreated and control cells are shown to 'help' the response of autologous responder cells to added soluble Con A. When the help by Con A-treated cells is less marked than that by control cells, apparent suppression is seen since the control cells provide the conventional baseline from which suppression is judged. After longer periods of cultivation the Con A-pretreated cells increased responses more than did the control cells leading to apparent enhancement.

INTRODUCTION

Suppression of DNA synthesis in autologous cultures by concanavalin A (Con A) treated lymphocytes has been described both in mouse (Dutton, 1972) and man (Shou, Schwartz & Good, 1976). Conventionally, the measure of the extent of suppression has been the response above background (or stimulation index) of the culture in the presence of Con A-treated cells compared with the response of an identical culture to which autologous cells not treated with Con A ('control' cells) have been added (Shou *et al.*, 1976).

Experimental variables have been shown to affect the extent of suppression measured in this way. These factors include mitomycin treatment of the Con A-treated cells (Fineman, Mudawwar & Geha, 1979), the time of treatment with Con A (Hallgren & Yunis, 1977), the concentration of Con A (Rich & Pierce, 1973) and the ratio of 'suppressor' cells to responder cells (Reinherz & Schlossman, 1979). Data from cell-mixing experiments using monoclonal lymphoid cell line cells suggest that suppression need not necessarily be the intrinsic property of a different subpopulation but can be expressed within a single cell type under appropriate conditions (Farrant & Knight, 1979).

Doubts have recently been expressed concerning the existence of 'Con A suppressor cells'

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(Fernandez & MacSween, 1980). We have therefore done a multifactor study of Con A-induced suppression in man using a 20 μl Terasaki plate culture system to see how experimental ('environmental') variables affect the presence and extent of suppression of DNA synthesis. The data show that the system is largely controlled by the interactions between different variables and also that the simple assessment of suppression which compares the effect of Con A-treated cells with control cells, is inadequate. Our data show that the calculated suppression is affected both by high background counts due to serum in the medium and by autologous stimulation by the Con A-treated cells. Using responding cells alone as the reference point, both Con A-treated and control cells 'help' the response.

METHODS

Cells and media. Peripheral human blood was freshly drawn from normal volunteers and heparinized to a final concentration of 20 units/ml (preservative-free heparin, Paines & Byrne). The blood was mixed with half its volume of L15 medium (Flow) and the mononuclear cells separated by centrifugation at 400 ***g*** for 30 min on a Ficoll gradient (Ficoll–Paque, Pharmacia). The cells were washed (400 ***g***, 10 min) once in L15 medium, once in culture medium and readjusted to the appropriate concentration of lymphocytes in culture medium. This culture medium was either RPMI 1640 (bicarbonate) (Flow) to which 10% heat-inactivated fetal calf serum (FCS, GIBCO) had been added, or more usually serum-free Iscove's medium (Flow) (Iscove & Melchers, 1978) with the following additions: L-glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml).

Induction cultures. Cells were cultured in 1-ml aliquots at 1×10^6 lymphocytes/ml in culture medium. Except for the data reported in Fig. 1, this culture medium was serum-free Iscove's Medium. The cultures were done in round-bottomed tubes (Falcon) at 37°C in a humidified incubator maintained with CO_2 5% and air 95%. To half of the tubes a final concentration of 10 μg/ml concanavalin A (Con A, Pharmacia) was added. After 2 days, 50 μl of the Con A antagonist α-methyl-D-pyranoside (2 M) in sterile saline was added to each 1-ml tube culture. After 30 min at 37°C the cells from each treatment (with or without Con A) were consolidated and washed. The cells were then treated for 25 min at 37°C with mitomycin C (Sigma) (50 μg/ml) in culture medium with α-methyl-D-pyranoside (0·1 M). The cells were then washed twice with L15 medium and once with the appropriate culture medium. Cells treated with Con A, cultured for 2 days, washed with α-methyl-D-pyranoside and treated with mitomycin were called *Con A-treated cells.* Similar cells without the Con A treatment were called *control cells.*

Assay cultures. On the same day that the Con A-treated and control cells were prepared (that is, after they had been in culture for 2 days), the same donor was re-bled and as before mononuclear cells were separated on Ficoll, washed and the lymphocytes counted. The cell culture method used was that of 20 μl hanging drop cultures in inverted Terasaki plates (O'Brien *et al.*, 1979; Farrant *et al.*, 1980). Essentially, 10-μl aliquots of twice the final desired concentration of responder cells were pipetted into wells of Terasaki plates (NUNC). Twice the final concentration of either control or Con A-treated cells was then added in a further 10-μl aliquot. Where appropriate a further 1 μl of Con A (20 μg/ml) in sterile saline was added. These pipettings were done with repeating syringes (Hamilton). The Terasaki plates were inverted and placed (without their lids) on petri dishes in closed plastic sandwich boxes containing, at the bottom, sterile saline to provide humidity. During the culture period these sandwich boxes were kept at 37°C in the 5% CO_2 humidified incubator.

Harvesting and counting. On the appropriate day of harvest the cultures were pulsed for 2 hr with ^{3}H-thymidine (^{3}H-TdR) (2 Ci/mmol) with a final concentration of total thymidine in the culture of 1·0 μg/ml (0·16 μCi/well) (Farrant *et al.*, 1980). This thymidine was added to the Terasaki plates (still in the inverted position) in a 1-μl volume (Hamilton syringe). After the 2-hr pulse period in the incubator the cell pellets on the meniscus of the hanging drops were harvested using the harvester already described (O'Brien *et al.*, 1979). Filters (Titertek, Flow, Cat. No. 78-105-05), pre-cut on the harvester by a dry Terasaki plate, absorbed the cell pellet by suction when the culture plate was pressed onto the harvester. After discarding the culture plate the cells on the filter were washed with (per plate) 10 ml each of saline, 5% w/v trichloroacetic acid and methanol. Each filter was then

picked out of the harvester and counted in 0·5 ml of scintillant (NE 260, Nuclear Enterprises) in plastic inserts (Minivials Cat. No. 881, Zinsser) using an LKB Wallach scintillation counter.

Analysis of data. No quench corrections were done since the residual material on the filters was insoluble. Because there is a more normal distribution of ^{3}H-TdR uptake data using $\log_{10}$ c.p.m. rather than arithmetic c.p.m. (Dei & Urbano, 1977; Farrant *et al.*, 1980), log-transformed data were used throughout. As previously described, an analysis of variance was performed on the log-transformed data from the symmetrically designed experiments so that an overall error variance could be obtained (Farrant *et al.*, 1980). This error variance was used in a *t*-test to calculate any differences between treatment means that were statistically different at a given *P* value (see figure legends).

When a conventional calculation for per cent suppression was done for comparative purposes (Shou *et al.*, 1976), arithmetic data were used. The equation used for this was:

$$\% \text{ Suppression} = 100 - \frac{\text{Response to Con A (1 } \mu\text{g/ml) of responder cells in presence of Con A-treated cells}}{\text{Response to Con A (1 } \mu\text{g/ml) of responder cells in presence of control cells}} \times 100,$$

where 'response' means that the background caused by the same cell combination but without the Con A (1 μg/ml) has been subtracted.

Fluorescein isothiocyanate-labelled Con A (FITC-Con A). Some induction cultures (48 hr) were set up using FITC-Con A (Miles–Yeda, 10 μg/ml) and after the standard washing procedures that included treatment with both α-methyl-D-pyranoside and mitomycin C the cells were examined both by fluorescence microscopy and the fluorescence-activated cell sorter (FACS).

RESULTS

Apparent suppression of different concentrations of responding cells by a single concentration of Con A-treated cells: problem of high backgrounds

Traditionally, Con A-induced suppression is measured by comparing the responses of lymphocytes in the presence of autologous cells previously incubated with Con A (Con A cells) to the responses of lymphocytes cultivated with autologous cells previously incubated without Con A (control cells). Fig. 1 shows the ^{3}H-TdR uptake of responding cells over a range of initial cell concentrations (0·125–2 × 10^6 cells/ml) after 4 days of cultivation with or without Con A (1 μg/ml). In Fig. 1a the

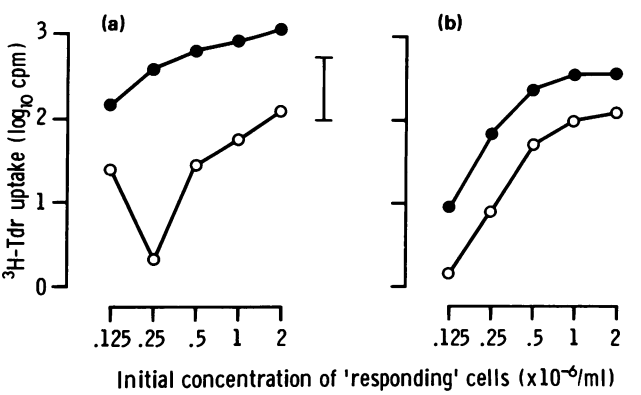

Fig. 1. Uptake of ^{3}H-TdR ($\log_{10}$ c.p.m.) by human peripheral lymphocytes as a function of initial cell concentration in the presence (•) or absence (o) of Con A (1 μg/ml) after 4 days of cultivation. Mitomycin-treated autologous cells from a previous 48-hr culture in the absence (a) or presence (b) of Con A (10 μg/ml) were present at a concentration of 0·5 × 10^6 cells/ml. The culture medium was RPMI 1640 with FCS (10% v/v). The bar line represents the difference between any two means for them to differ significantly at $P = 0{\cdot}05$.

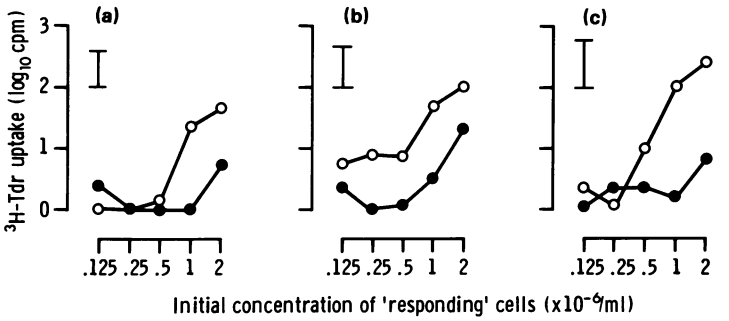

Fig. 2. Uptake of ^{3}H-TdR ($\log_{10}$ c.p.m.) by human peripheral lymphocytes as a function of initial cell concentration after 4 (a), 5 (b) or 6 (c) days of cultivation in either RPMI 1640 with FCS (10% v/v) (o) or Iscove's serum-free medium (•). No mitogens were added. The bar lines indicate the difference between any two points for them to differ significantly at the $P = 0{\cdot}05$ level.

cultures were done in the presence of $0{\cdot}5 \times 10^6$ cells/ml of control cells. Fig. 1b shows the corresponding effects when $0{\cdot}5 \times 10^6$ cells/ml of Con A cells were present. Fig. 1 shows that DNA synthesis of all five responder cell concentrations was apparently suppressed by the presence of Con A cells as compared with control cells. These cultures were done in RPMI 1640 medium with FCS (10%) and there was an appreciable background uptake of ^{3}H-TdR even when no Con A was present in the second culture.

In order to observe responses with minimal background activity a study was made of the background uptake of ^{3}H-TdR by different cell concentrations in serum-free Iscove's medium (Iscove & Melchers, 1978) compared with that in RPMI 1640 medium with FCS (10%). Fig. 2 shows the results after 4, 5 or 6 days of cultivation of the same five responder cell concentrations in the absence of Con A or any mitomycin-treated cells. There is a marked reduction in background ^{3}H-TdR uptake with the serum-free Iscove's medium. The 'mitogenic activity' of the RPMI 1640 medium containing FCS (10%) was greater at the higher responder cell concentrations. The remaining experiments reported in this paper were therefore done using Iscove's serum-free medium throughout.

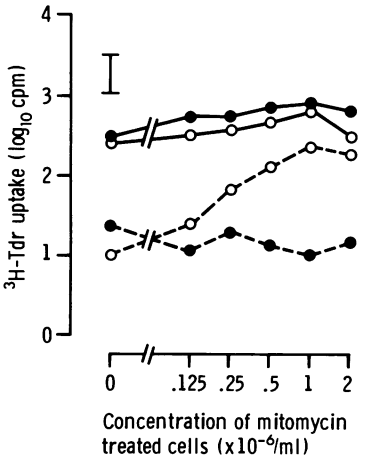

Fig. 3. Effect of concentration of autologous mitomycin-treated cells on the uptake of ^{3}H-TdR ($\log_{10}$ c.p.m.) by peripheral lymphocytes (1×10^6/ml, initial cell concentration), in the presence (——) or absence (– – –) of Con A (1 μg/ml) during 3 days of cultivation in Iscove's medium. The autologous mitomycin-treated cells were from a previous 48-hr culture with (o) or without (•) Con A (10 μg/ml).

Apparent suppression of a single concentration of responding cells by different concentrations of Con A cells: autologous stimulation by Con A cells

Fig. 3 shows the response to Con A (1 μg/ml) of 1×10^6 responding cells/ml on day 3 in the presence of either Con A cells or control cells. The Con A-treated cells alone appear to act as autologous stimulators of the responding cells even in the absence of soluble Con A (1 μg/ml). Control cells did not act as autologous stimulators. Parallel cultures containing mitomycin-treated cells alone suggested that this autologous stimulation was a response of the 1×10^6 cells/ml responding cells rather than a 'breakthrough' of the mitomycin blockade.

Fig. 4 illustrates the kinetics of this autologous stimulation by different concentrations of Con A-treated mitomycin-treated cells acting on different concentrations of responder cells with the ^{3}H-TdR uptake measured on days 3 and 4. In general, the autologous stimulation was higher at the higher responder cell concentrations and was greater after 4 days than 3. In addition, at the higher responder cell concentrations the response increased with an increase in the concentration of stimulating Con A-treated cells to a maximum level and then decreased.

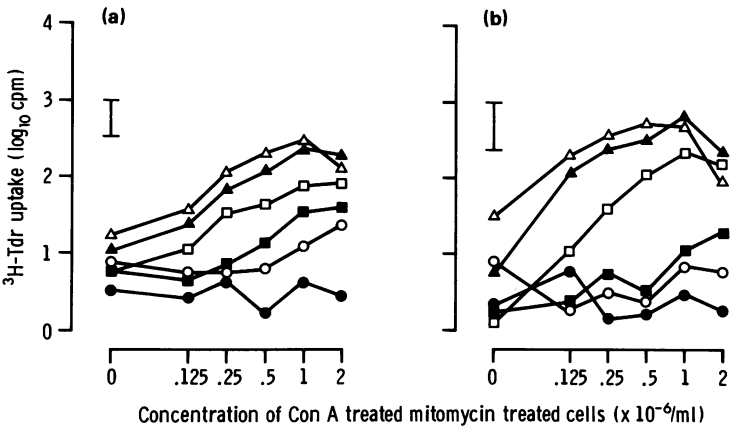

Fig. 4. Autologous stimulation by different concentrations of Con A-treated mitomycin-treated cells measured by the uptake of ^{3}H-TdR ($\log_{10}$ c.p.m.). Initial concentrations of responding cells were zero (●), 0·125 (○), 0·25 (■), 0·5 (□), 1·0 (▲) or 2·0 (△) $\times 10^6$/ml. The period of cultivation in serum-free Iscove's medium was for 3 (a) or 4 days (b). The error bars show the differences between any two points for them to differ significantly at $P = 0{\cdot}05$.

Suppression revealed as inferior help by Con A-treated cells in comparison with control cells

The data already given in Fig. 3 show that when mitomycin-treated cells, whether pretreated with Con A or not, are added to a single concentration of responding cells, the ^{3}H-TdR uptake of the responding cells is not suppressed. Further, multivariable studies including several periods of cultivation and several concentrations of both responding and mitomycin-treated cells revealed that the DNA synthesis of the responding cells was 'helped' by the addition of either Con A or control cells. The extent of this help varied with all these factors. Examples of this from a single experiment are given in Fig. 5. This data shows that at some cell concentrations (e.g. $0{\cdot}5 \times 10^6$ responder cells/ml; Fig. 5b & e) the addition of previously cultured mitomycin-treated cells increased the ^{3}H-TdR uptake markedly. In Fig. 5b (on day 4 of cultivation) the control cells 'helped' the response to a greater extent than did the Con A-treated cells. Since suppression by Con A-treated cells is conventionally calculated in comparison with control cells, this inferior help by the Con A-treated cells leads to high estimates of the per cent suppression when the commonly used formula (see Methods) is used. Thus, for Fig. 5b the calculated values were 72, 77, 79, 80 and 94% suppression for the additions of 0·125, 0·25, 0·5, 1 and 2×10^6 mitomycin-treated cells/ml respectively. In dramatic contrast with this, Fig. 5e shows, again with $0{\cdot}5 \times 10^6$ responder cells/ml but with the period of cultivation extended to 5 days, that the 'help' given by the Con A-treated cells now exceeds that given by the control cells. This means that the conventional calculations for suppression by Con A-treated cells will now show 'enhancement' rather than 'suppression'. At

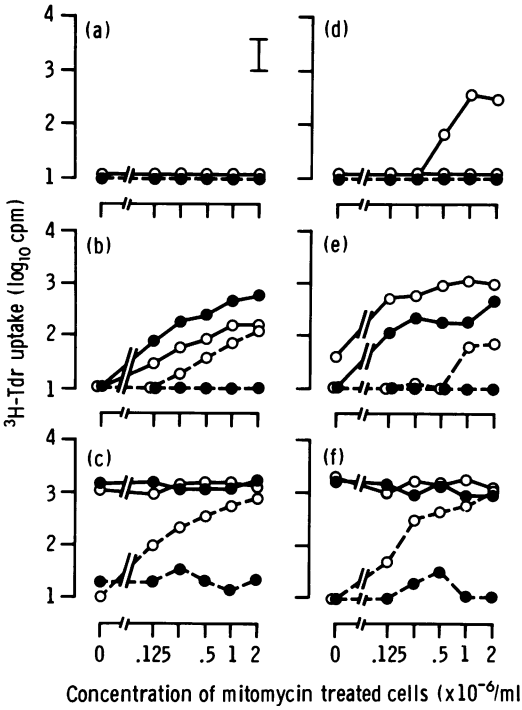

Fig. 5. Effect of increasing concentrations of autologous mitomycin-treated cells on the uptake of ^{3}H-TdR ($\log_{10}$ c.p.m.) by peripheral lymphocytes at different initial cell concentrations: $0{\cdot}25 \times 10^6$ cells/ml (a & d), $0{\cdot}5 \times 10^6$ cells/ml (b & e) and 2×10^6 cells/ml (c & f). Cultures were done in the presence (——) or absence (– – –) of Con A (1 μg/ml) during either 4 (a, b & c) or 5 (d, e & f) days of cultivation. The autologous mitomycin-treated cells were from a previous 48-hr culture with (○) or without (●) Con A (10 μg/ml). In the absence of responder cells the maximum ^{3}H-TdR uptake of any concentration of mitomycin cells on either day was less than $0{\cdot}8$ ($\log_{10}$ c.p.m.). The bar line in (a) indicates the difference between any two points throughout the figure for them to differ significantly at the $P = 0{\cdot}05$ level.

lower responder cell concentrations in this individual (e.g. $0{\cdot}25 \times 10^6$ cells/ml; Fig. 5a & d) responses are at background levels except that when 'help' did occur on day 5 (Fig. 5d), it was the Con A-treated cells that allowed responses above background. Conversely, when the responder cell concentration is high, with responses to the soluble Con A (1 μg/ml) perhaps even maximal in the absence of any mitomycin-treated cells (e.g. 2×10^6 responder cells/ml; Fig. 5c & f), then no further help was seen by adding increasing concentrations of mitomycin-treated cells whether they had been treated with Con A or not. However, in the absence of added soluble Con A these high concentrations of responder cells would still respond to stimulation by Con A-treated cells ('autologous stimulation') but not to control cells (Fig. 5c & f).

Similar results to those of Fig. 5 were seen in experiments with cells from four different individuals. An example of the raw data obtained in these experiments is given in Table 1. This shows the ^{3}H-TdR uptake from individual wells using $0{\cdot}5 \times 10^6$/ml responding cells from three different individuals all harvested on day 3. The effect of adding increasing concentrations of either control or Con A-treated mitomycin-treated cells is shown in the presence or absence of Con A (1 μg/ml) in the assay culture. The 'help' by both the control and Con A-treated cells to the subsequent Con A response is clearly seen. Table 1 shows only a sample of the data from these experiments. Five responding cell concentrations and three harvest days were done in all experiments. Thus under most circumstances the addition of mitomycin-treated cells helped autologous responder cells to incorporate more ^{3}H-TdR in response to added soluble Con A. However, only Con A-treated, mitomycin-treated cells acted as stimulators in the absence of added soluble Con A. With different individuals the extent and pattern of help varied under comparative conditions. For example, with

Table 1. ^{3}H-thymidine uptake (log c.p.m.) of individual Terasaki wells containing $0{\cdot}5 \times 10^6$ responding cells/ml from three different individuals

Individual	Pretreatment of mitomycin cells added	Con A (1 μg/ml) in assay culture	Concentration of added mitomycin-treated cells ($\times 10^{-6}$ cells/ml)					
			0	0·125	0·25	0·5	1	2
J.T.	Control	−	0·85	1·23	1·00	0·90	1·15	0·85
			0·90	1·11	0·78	1·15	0·48	0·70
	Con A	−	1·04	1·20	1·67	1·80	2·00	1·84
			0·70	0·95	1·53	1·69	1·94	2·02
	Control	+	1·58	2·09	2·15	2·29	2·39	2·34
			1·90	2·28	2·38	2·37	2·54	2·49
	Con A	+	1·65	2·07	2·48	2·38	2·51	2·31
			1·80	2·28	2·21	2·28	2·46	2·35
P.C.	Control	−	0·78	0·78	1·18	1·26	0·60	0·00
			0·48	1·00	0·60	0·78	1·32	0·00
	Con A	−	0·00	0·78	1·20	1·04	1·28	0·85
			0·85	0·70	0·90	1·11	1·11	0·90
	Control	+	1·40	1·68	1·75	1·68	1·98	2·05
			1·30	1·51	1·92	1·78	1·98	1·93
	Con A	+	0·70	1·28	1·23	1·72	1·95	1·32
			1·15	1·54	1·66	1·52	1·49	1·45
B.C.	Control	−	0·00	0·30	0·00	0·00	0·00	1·18
			0·00	0·48	0·00	0·00	0·00	0·48
	Con A	−	0·00	0·30	0·00	0·70	1·63	1·85
			0·00	1·11	0·48	1·26	1·63	1·81
	Control	+	0·00	0·85	0·90	1·18	1·95	2·08
			0·48	1·36	1·56	1·81	1·78	2·11
	Con A	+	1·20	1·67	1·81	2·15	1·71	2·39
			1·40	1·92	1·99	2·26	2·32	2·34

Responses (in duplicate) were obtained with or without Con A (1 μg/ml) in the presence of different concentrations of control or Con A-treated mitomycin-treated cells. All wells were harvested on day 3.

an intermediate concentration of responder cells, the switch from inferior help by Con A-treated cells in comparison with control cells to the opposite sometimes occurred between days 3 and 4 and sometimes between days 4 and 5 as in the example given in Fig. 5b & e. The level of ^{3}H-TdR uptake by responder cell concentrations alone was always lower than when mitomycin-treated cells were added (i.e. 'absolute suppression' was never seen) provided that the concentration of responder cells alone was less than that at which a maximal ^{3}H-TdR uptake was observed. Some absolute reduction of ^{3}H-TdR uptake with high concentrations of responder cells (e.g. 2×10^6 cells/ml; Fig. 5f) did, however, occur when high concentrations of mitomycin-treated cells were added.

DISCUSSION

These results attempt to study the effect of some of the 'environmental' factors (e.g. responder cell concentration, putative 'suppressor cell' concentration, period of cultivation, etc.) on the

interaction by cells cultivated with Con A for 48 hr on autologous cultures stimulated by Con A. Using the almost universal convention of calculating suppression in terms of the ^{3}H-TdR uptake of responders in the presence of Con A-pretreated cells compared with that in the presence of control cells (e.g. Shou *et al.*, 1976) apparent suppression was found. The value of suppression so calculated is affected by background levels. Our data show high background levels of ^{3}H-TdR incorporation which could be attributed to the use of a culture medium containing FCS.

The use of FCS is often avoided in clinical studies (Shou *et al.*, 1976) or a batch of FCS is selected with low mitogenic activity (Feighery *et al.*, 1978). However, any cause of high backgrounds will affect conventional calculations of apparent suppression. One possible way of minimizing background levels is to use the recently developed serum-free medium of Iscove & Melchers (1978). This medium gave exceedingly low background levels of ^{3}H-TdR uptake (Fig. 2) and yet allowed the cells to respond well on stimulation.

In addition, our data show that the Con A-pretreated cells act as stimulators of autologous responders even after the Con A-pretreated cells had been washed and incubated with the specific Con A antagonist (α-methyl-D-pyranoside). It is not clear from our data whether this autologous stimulation is due to residual Con A on the surface of mitomycin-treated cells or to a Con A-induced change in the membrane properties leading to autologous mixed lymphocyte reactions. However, parallel studies with FITC-Con A (10 μg/ml) indicated the retention of some Con A on the cell surface despite several washes and treatment with α-methyl-D-pyranoside. Previously, this autologous stimulation has only rarely been reported (e.g. de Gast *et al.*, 1977) even though several authors (Shou *et al.*, 1976; Hubert, Delespesse & Govaerts, 1976; Sakane & Green, 1977) did not use the Con A antagonist during the washing procedures. One reason why this point has not been discussed more fully may be that the response in the second culture is frequently recorded only after the backgrounds have been subtracted (Shou *et al.*, 1976; Hubert *et al.*, 1976; Sakane & Green, 1977; Kallenberg, de Gast & The, 1980; Fernandez & MacSween, 1980). In addition, it is possible that we have observed autologous stimulation more easily because we have used a wider range of concentrations of Con A-treated cells than in previous reports and have reduced backgrounds by the use of serum-free medium. The autologous stimulation was clearly dependent on the cell concentration of both responders and Con A-pretreated cells and by the period of cultivation (Fig. 4). Consideration of the two problems of high backgrounds and autologous stimulation did not provide a complete explanation for apparent suppression, they merely affected its calculated value.

The most important finding from this study came from investigating the interactions between concentrations of responding cells, control cells and Con A-pretreated cells together with the period of cultivation. This was done using the response of the responding cells alone as a reference point.

Especially with intermediate concentrations of responding cells (e.g. $0{\cdot}5 \times 10^6$ cells/ml) the addition of increasing concentrations of both control cells and Con A-pretreated cells increased the ^{3}H-TdR uptake of a constant concentration of the responding cells in response to the added soluble Con A (1 μg/ml). This increase of 'help' in the ^{3}H-TdR response was not due to a breakdown in the blockade by mitomycin and therefore was a response of the 'responding' cells. Under some circumstances—as with $0{\cdot}5 \times 10^6$ responding cells/ml on day 5 (see Fig. 5b)—the 'help' given to the responding cells by the Con A-pretreated cells was less effective than the 'help' given by control cells. Since the response in the presence of control cells is the baseline from which suppression has always been calculated this difference in the extent of 'help' leads to an apparent suppression. Under different circumstances, e.g. when $0{\cdot}5 \times 10^6$ responding cells/ml are harvested on day 5 (see Fig. 5e), the extent of help is reversed and the Con A-treated cells enhanced the response to a greater extent than the control cells.

At lower responder cell concentrations this help may also be seen (Fig. 5d) and again it is affected by whether the 'helping' cells have been pretreated with Con A or not. At higher responder cell concentrations where the responding cells alone are giving maximal responses to the added soluble Con A (Fig. 5c & f), then it is clear that the added mitomycin-treated cells cannot increase the responses further. All these changes to the response to Con A when mitomycin-treated cells are added can only be seen clearly in context against the backdrop of the cell concentration dependence of the response to Con A of the responding cells alone. This curve exhibits a low response at low cell concentrations increasing to a maximal response at higher cell concentrations. This can be observed

in Fig. 5 from the responses in the absence of any mitomycin-treated cells. When mitomycin-treated cells are added to low concentrations of responder cells, it is therefore possible for the response to Con A to be increased. If, however, the response of higher concentrations of responding cells alone is already maximal, the addition of mitomycin-treated cells cannot increase the response further and may even decrease it by pushing the cellular environment into conditions of supramaximal cell concentrations. Our data (e.g. Fig. 5b, d & e) suggest, therefore, that the different help we see when either control or Con A cells are added to low or intermediate concentrations of responding cells is the phenomenon previously described as Con A-induced suppression. However, the lack of help (seen in Fig. 5c & f) when mitomycin cells are added is due to already maximally responding concentrations of responders. With yet higher cell concentrations the addition of mitomycin-treated cells may even lead to a decrease in the response whether the added cells have been treated with Con A or not. However, our data do not extend into this region of supramaximal cell concentration effects.

There has been a previous report that the extent of 'suppression' depends on the ratio of 'suppressor' cells to responder cells (Reinherz & Schlossman, 1979). The reasons for the different kinetics of 'help' by the Con A-treated cells compared with control cells are not clear. Previous reports suggest that the generation of 'Con A suppressor cells' (that is cells that differ from control cells in their effect on autologous cells) does not depend on cell proliferation in that it can still occur if drugs such as mitomycin C are applied before the first culture (Kallenberg *et al.*, 1980; Shand, Orme & Ivanyi, 1980). Higher responses in the presence of Con A-treated cells when compared with control cells have also been reported at particular combinations of the doses of Con A in the induction and responding cultures (Hallgren & Yunis, 1977).

Thus 'help' and 'suppression' clearly depend to a great extent on experimental variables. In addition, our data emphasize the importance of multivariable studies in interpreting lymphocyte functions *in vitro* and support the view that helper and suppressor effects can be produced by 'environmental' factors such as cell concentration and period of cultivation even when only one cell population is present (Farrant & Knight, 1979). Although other workers, notably Dwyer, Johnson & Desaules (1979), have also performed multifactor studies of the Con A 'suppressor' system in man, we have concentrated our experiments on the interaction between variables in the second assay culture. For the first culture we have used conditions (Con A 10 μg/ml, 48-hr culture) within the range most often used by other workers. It is clear from the work of Dwyer *et al.* (1979) that changing the conditions of Con A dose and time in the induction culture will greatly affect the quantitative levels of data obtained. Our results do not therefore represent a complete description of the system. However, we feel that our data does demonstrate the principle that previous interpretations of suppression reflect instead quantitative changes in the extent of 'help'.

Recently, Fernandez & MacSween (1980) have cast doubt on the existence of Con A 'suppressor' cells based on their finding that the addition of control cells increases the response to Con A over and above the response observed with responding cells alone. Our data confirm their finding and since the culture conditions of Fernandez & MacSween (1980) were those of the standard 0·2 ml microtitre system, their results suggest that our data is not an artifact of the recently developed 20 μl hanging drop Terasaki system that we have used but is of more general significance.

Finally, the role of 'Con A suppressor cells', especially in diseases (e.g. multiple sclerosis) (Antel, Weinrich & Arnason, 1978; Arnason & Antel, 1978), may need to be reconsidered.

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REFERENCES

ANTEL, J.P., WEINRICH, M. & ARNASON, B.G.W. (1978) Mitogen responsiveness and suppressor cell function in multiple sclerosis. *Neurology*, **28**, 99.

ARNASON, B.G.W. & ANTEL, J. (1978) Suppressor cell function in multiple sclerosis. *Ann. Immunol. (Inst. Pasteur)*, **129C**, 159.

DE GAST, G.C., THE, T.H., PONDS, E. & KALLENBERG, C. (1977) Suppression of DNA synthesis by Con A activated human lymphocytes. Stimulation by Con A bound to non-T cells unless removed after activation. *Clin. exp. Immunol.* **30**, 457.

DEI, R. & URBANO, P. (1977) Biometrical implications

of factorial experiments for the study of lymphocyte mitogenic response. *J. Immunol. Methods*, **15,** 169.

DUTTON, R.W. (1972) Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigens. I. Characterization of the inhibitory cell activity. *J. exp. Med.* **136,** 1445.

DWYER, J.M., JOHNSON, C. & DESAULES, M. (1979) Behaviour of human immunoregulatory cells in culture. I. Variables requiring consideration for clinical studies. *Clin. exp. Immunol.* **38,** 499.

FARRANT, J., CLARK, J.C., LEE, H., KNIGHT, S.C. & O'BRIEN, J. (1980) Conditions for measuring DNA synthesis in PHA stimulated human lymphocytes in 20 μl hanging drops with various cell concentrations and periods of culture. *J. Immunol. Methods*, **33,** 301.

FARRANT, J. & KNIGHT, S.C. (1979) Help and suppression by lymphoid cells as a function of cellular concentration. *Proc. Natl. Acad. Sci. USA*, **76,** 3507.

FEIGHERY, C., WHELAN, C.A., WEIR, D.G. & GREALLY, J.F. (1978) *In vitro* studies of suppressor cell function in human peripheral blood mononuclear cells. *Clin. exp. Immunol.* **32,** 459.

FERNANDEZ, L.A. & MACSWEEN, J.M. (1980) Generation of suppressor cells by concanavalin A: a new perspective. *J. Immunol.* **125,** 267.

FINEMAN, S.M., MUDAWWAR, F.B. & GEHA, R.S. (1979) Characteristics and mechanisms of action of the concanavalin A activated suppressor cell in man. *Cell. Immunol.* **45,** 120.

HALLGREN, H.M. & YUNIS, E.J. (1977) Suppressor lymphocytes in young and aged humans. *J. Immunol.* **118,** 2004.

HUBERT, C., DELESPESSE, G. & GOVAERTS, A. (1976) Concanavalin A-activated suppressor cells in normal human peripheral blood lymphocytes. *Clin. exp. Immunol.* **26,** 95.

ISCOVE, N.N. & MELCHERS, F. (1978) Complete replacement of serum by albumin, transferrin and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J. exp. Med.* **147,** 923.

KALLENBERG, C.G.M., DE GAST, G.C. & THE, T.H. (1980) Suppression of DNA synthesis by Con A-activated human lymphocytes: role of monocytes in Con A-induced suppression. *Clin. exp. Immunol.* **41,** 583.

O'BRIEN, J., KNIGHT, S.C., QUICK, N.A., MOORE, E.H. & PLATT, A.S. (1979) A simple technique for harvesting lymphocytes cultured in Terasaki plates. *J. Immunol. Methods*, **27,** 219.

REINHERZ, E.L. & SCHLOSSMAN, S.F. (1979) Con A inducible suppression of MLC: evidence for mediation by the TH_2+ subset in man. *J. Immunol.* **122,** 1335.

RICH, R.R. & PIERCE, C.W. (1973) Biological expression of lymphocyte activation. II. Generation of a population of thymus derived suppressor lymphocytes. *J. exp. Med.* **137,** 649.

SAKANE, T. & GREEN, I. (1977) Human suppressor T cells induced by concanavalin A: suppressor T cells belong to distinctive T cell sub-classes. *J. Immunol.* **119,** 1169.

SHAND, F.L., ORME, I.M. & IVANYI, J. (1980) The induction of suppressor T cells by concanavalin A is independent of cellular proliferation and protein synthesis. *Scand. J. Immunol.* **12,** 223.

SHOU, L., SCHWARTZ, S.A. & GOOD, R.A. (1976) Suppressor cell activity after concanavalin A treatment of lymphocytes from normal donors. *J. exp. Med.* **143,** 1100.