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Suppression of DNA synthesis by Con A-activated human lymphocytes: role of monocytes in Con A-induced suppression

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SUMMARY

Human peripheral blood mononuclear leucocytes, activated by Con A, suppress the antigen- or mitogen-induced proliferation of autologous responder cells. Generation of suppressor cells is prevented by mitomycin treatment before Con A activation. Once cells are activated, their suppressive activity is not affected by mitomycin treatment. Monocytes are not only required for generation of suppressor cells, but are also involved in suppression as effector cells. Monocyte-mediated suppression is blocked by indomethacin, suggesting that this suppression is mediated by prostaglandins. It is concluded that monocytes are involved in Con A-induced suppression of DNA synthesis.

INTRODUCTION

Regulatory cells are considered to play an important role in the immune response. Subpopulations of lymphocytes are capable of helping, amplifying or suppressing other cell responses. Activation by concanavalin A (Con A) of peripheral blood mononuclear leucocytes (MNL) from normal human individuals results in cells which non-specifically suppress effector functions of other cells. These Con A-activated cells suppress the antigen- or mitogen-induced lymphocyte proliferation (Shou, Schwartz & Good, 1976; Hubert, Delespesse & Govaerts, 1976; de Gast *et al.*, 1977; Antel, Weinrich & Arnaton, 1978), immunoglobulin (Ig) production by B cells (Haynes & Fauci, 1977) and the generation of cytotoxic effector cells (Hunninghake & Fauci, 1978). In systemic lupus erythematosus Con A-activated peripheral blood MNL fail to suppress the polyclonal Ig production of autologous or allogeneic B cells (Fauci *et al.*, 1978), whereas in patients with active multiple sclerosis Con A-activated suppression of the proliferative response of autologous responder cells is increased (Gonzalez, Dan & Spitler, 1979). Con A-activated suppression seems an accessible way to study non-specific suppressor cell function in human peripheral blood MNL.

Whereas the occurrence of cells with suppressor function seems clearly established, the characteristics of the cells involved in Con A-induced suppression and the mechanism underlying this process are as yet not clear. Not only T cells with receptors for the Fc fragment of IgG have suppressive activity (Moretta *et al.*, 1977), but also T cells with receptors for the Fc fragment of IgM may be activated by Con A to suppress the polyclonal Ig production of B cells (Hayward *et al.*, 1978). In addition, T cell-depleted cell populations may perform this function (Haynes & Fauci, 1978). Monocytes were required for the generation of Con A-induced suppressor cells according to Raff, Cochrum & Stobo (1978), but were not necessary in other studies (Hayward *et al.*, 1978; Haynes & Fauci, 1978a). In the present report the role of monocytes in Con A-induced suppression

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of the proliferative response to antigens and mitogens is studied. We describe the involvement of monocytes in the generation of suppressor cells and in the suppressive activity itself.

MATERIALS AND METHODS

Lymphocyte isolation and separation. Peripheral blood mononuclear cells were obtained from fresh heparinized blood (20 u heparin per ml blood) drawn from healthy adult volunteers under 40 years of age by Ficoll–Isopaque density gradient centrifugation. They 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 (sp. gr. 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 a high ***g***-force (1,000–1,600 ***g***) with rapid acceleration at 20°C. The rosetting cells which appeared 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 contaminated with $<3\%$ surface Ig-positive cells and $<1\%$ monocytes (identified by morphology, latex bead phagocytosis and non-specific esterase staining). The non-T cell fraction contained 30–50% monocytes and $<3\%$ T cells. Monocyte-depleted suspensions were obtained by two methods: removal of adherent cells and carbonyl iron treatment. In order to remove adherent cells aliquots of MNL were resuspended in RPMI 1640 supplemented with 10% pooled human serum and incubated for 60 min at 37°C in flat-bottomed culture flasks (7×10^3 cells/mm^2). Non-adherent cells were removed by three washings. Adherent cells were removed from the plastic surface with a rubber policeman and resuspended. Carbonyl iron treatment was performed by incubating heparinized venous blood or cell suspensions in culture medium with carbonyl iron (GAF, NV, Delft, Holland, type SF; 2 mg/ml blood or medium) during 60 min at 37°C prior to Ficoll–Isopaque gradient centrifugation. Both procedures resulted in cell suspensions containing $<3\%$ monocytes.

Lymphocyte activation by Con A (first step). The following cell suspensions were used: unseparated cells, SRBC rosetting (T) cells, non-rosetting (non-T) cells, monocyte-depleted cells and monocyte-depleted cells supplemented with adherent cells (reconstituted cell suspension). The percentage of adherent cells in the last cell suspension was equal to the percentage of monocytes in the unseparated cell fraction. Each fraction (4×10^6 cells) was incubated with or without 25 μg/ml 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) over 24 hr at 37°C. Cells were then spun down, washed once and incubated in 1 ml RPMI containing 50 μg/ml of mitomycin (Sigma Chemical Co.) for 30 min. In some experiments cells were treated with mitomycin before activation by Con A. After mitomycin treatment cells were washed twice and incubated in medium with 0·3 M α-methylglucoside (1-0-methyl-α-D-glucopyranoside; Sigma Chemical Co.) for 20 min at 20°C and spun down. This procedure was repeated again as previously described (de Gast *et al.*, 1977). Subsequently cells were washed twice in medium and used as Con A-activated 'suppressor' cells. Control 'suppressor' cells were obtained by incubation without Con A, but were otherwise treated similarly.

Lymphocyte cultures in microtitre plates (second step). Cultures were prepared in round-bottomed plastic microtitre plates (Cooke, U-form, 220 M – 24 AR) in a final volume of 0·10 ml RPMI containing penicillin–streptomycin and 25% inactivated human serum. Each well was filled with 0·025 ml of a responder MNL suspension (containing 1×10^5 cells for stimulation by recall antigens or allogeneic cells and 3×10^4 cells for stimulation by mitogens), 0·050 ml of a Con A-suppressor or control-suppressor cell suspension (ratio suppressor : responder lymphocytes 1 : 1) and 0·025 ml of a stimulant in culture medium. This stimulant consisted, depending on the experiment, of a mitogen (Con A, 1 μg/ml), recall antigens (consisting of PPD in a final concentration of 5 μg/ml, diphtheria toxoid 10 LF/ml and tetanus toxoid 10 LF/ml) or a stimulator cell suspension Pm (this is a pool of frozen lymphocytes from ten random donors, treated with mitomycin) containing 1×10^5 lymphocytes or medium without stimulant as control. The plates were covered by a hard lid, wrapped in plastic adhesive kitchen-film and incubated at 37°C in a humidified 5% CO_2 in air atmosphere for 6

days. Sixteen hours before harvesting 0·5 μCi radioactive thymidine (Radiochemical Centre, Amersham, England) was added. A multiple cell culture harvester (Skatron, Oslo, Norway) was used. The dried (60 min at 60°C) filters were transferred to counting vials, 4 ml scintillation fluid was added and counting performed in a liquid scintillation counter. Results are expressed as disintegrations per minute (d.p.m.) per culture. The arithmetic mean and standard deviation of triplicate cultures were calculated. Apart from absolute mean d.p.m. values results were expressed as percentage suppression:

$$100 \times \left[1 - \frac{(\text{resp. cells} + \text{Con A suppr. cells}_m + \text{stimulant})^*}{(\text{resp. cells} + \text{control suppr. cells}_m + \text{stimulant})^*} - \frac{(\text{resp. cells} + \text{Con A suppr. cells}_m - \text{stimulant})^*}{(\text{resp. cells} + \text{control suppr. cells}_m - \text{stimulant})^*} \right].$$

Decreased DNA synthesis due to cytotoxicity rather than to suppression of proliferation was excluded by trypan blue exclusion tests.

RESULTS

Effect of monocyte depletion on the generation of Con A-activated suppressor cells

Unseparated normal MNL were incubated with Con A in a high concentration (25 μg/ml) for 24 hr and subsequently treated with mitomycin. These Con A-activated 'suppressor cells' were added to autologous responder cells, which were stimulated by allogeneic cells or by recall antigens. A significant decrease in DNA synthesis of these autologous responder cells was observed. Without pretreatment with Con A, addition of mitomycin-treated cells did not result in suppression of proliferation of responder cells. Subsequently monocyte-depleted cell suspensions were prepared by incubation of MNL with carbonyl iron, or by removing adherent cells, resulting in cell suspensions with less than 3% monocytes. After treatment with Con A and mitomycin these monocyte-depleted cells were used as 'suppressor cells' in the same autologous system as described above. In contrast with the afore-mentioned results no depression of DNA synthesis of autologous responder cells was observed (Fig. 1). The method of monocyte depletion did not influence the results. Reconstitution of monocyte-depleted cells with adherent cells was next performed. The resulting cell suspension was activated by Con A and used as 'suppressor cells'. The suppressive activity of this reconstituted fraction did not differ significantly from that produced by Con A-activated unseparated cells (Table 1). Results from two separate experiments are shown in Table 2.

Effect of monocyte depletion on the suppressive activity of Con A-activated suppressor cells

To evaluate whether monocytes were only necessary during the generation of suppressor cells or might themselves act as suppressor cells, the following experiment was performed. Unseparated cells were activated by Con A for 24 hr; after Con A activation part of the suppressor cell suspension was depleted of monocytes with carbonyl iron. Suppressor cell activity was studied in an assay system of autologous responder cells stimulated by mitogens and antigens. After monocyte depletion a significant decrease in suppressive capacity was observed (Fig. 2), indicating that monocytes have suppressive activity.

It has recently been shown that adherent cells suppress lymphoproliferation by prostaglandin release; this suppression is abrogated by prostaglandin synthetase inhibitors like indomethacin (Goodwin *et al.*, 1977a). In the next experiment we studied the role of the prostaglandin-producing cell in Con A-induced suppression. Con A-activated and non-activated cells were added to antigen- and mitogen-stimulated cultures with and without indomethacin (1 μg/ml). Con A-induced suppression was significantly reduced in the presence of indomethacin. When suppressor cells were depleted of monocytes after Con A activation, indomethacin did not influence the suppressive

* Expressed in mean d.p.m. of triplicate cultures with responder cells, mitomycin-treated suppressor cells ($cells_m$) with or without a stimulant.

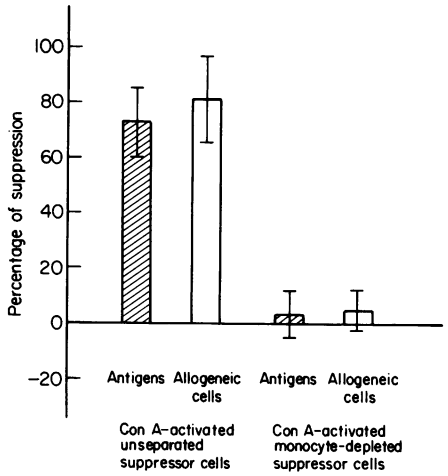

Fig. 1. Effect of monocyte depletion on the generation of Con A-activated suppressor cells. The capacity of Con A-activated cells to suppress DNA synthesis of autologous responder cells stimulated with recall antigens or with allogeneic cells is completely lost when the suppressor cell suspension is depleted of monocytes before activation by Con A. Data represent the mean (± s.e.m.) of six experiments, in which recall antigens were used and of eight experiments in which allogeneic cells were used to stimulate responder cells.

Table 1. Suppression of lymphocyte proliferation by addition of Con A-activated autologous cells. Effect of monocyte depletion on the generation of suppressor cells

Number of experiments	Stimulant	Cells added	Suppression (% ± s.e.m.)
6	Recall antigens	Unseparated	71 ± 12
6	Recall antigens	Monocyte-depleted	3 ± 8
6	Recall antigens	Reconstituted	78 ± 16
8	Allogeneic cells	Unseparated	79 ± 16
8	Allogeneic cells	Monocyte-depleted	5 ± 7
8	Allogeneic cells	Reconstituted	85 ± 20

Table 2. Suppression of antigen-induced lymphocyte proliferation by addition of Con A-activated autologous cells

Experiment	Cells added	Responder cells without addition of cells*	Addition of Con A-activated cells*	Addition of non-activated cells*	Suppression (%)
1	Unseparated	19,136	6,185	18,622	67
	Monocyte-depleted		16,349	15,812	−1
	Reconstituted		5,735	20,367	72
2	Unseparated	40,651	13,799	43,182	68
	Monocyte-depleted		42,295	45,497	7
	Reconstituted		16,537	47,876	65

* Expressed as d.p.m. per culture as measured by thymidine incorporation.

capacity (Fig. 2; Table 3). This finding indicates that the suppressive effect of Con A-activated monocytes is mediated by prostaglandins.

Con A-induced activation of isolated cell fractions

To evaluate whether isolated T cells might be activated by Con A to suppressor cells, T–non-T cell separation procedures were performed. The T cell fraction contained less than 1% monocytes. Both T and non-T fractions were treated with Con A and mitomycin and used as suppressor cells in an autologous assay system. Con A-activated T cells did not produce any significant suppression. In contrast, the non-T cell fraction, rich in monocytes, strongly inhibited DNA synthesis of autologous responder cells after Con A activation (Fig. 3; Table 4).

Effect of mitomycin treatment on the generation of Con A-activated suppressor cells

In the preceding experiments Con A-activated suppressor cells were treated with mitomycin before they were added to autologous responder cells. In this way proliferation of suppressor cells in the assay system was prevented. To evaluate the effect of mitomycin on the generation of suppressor

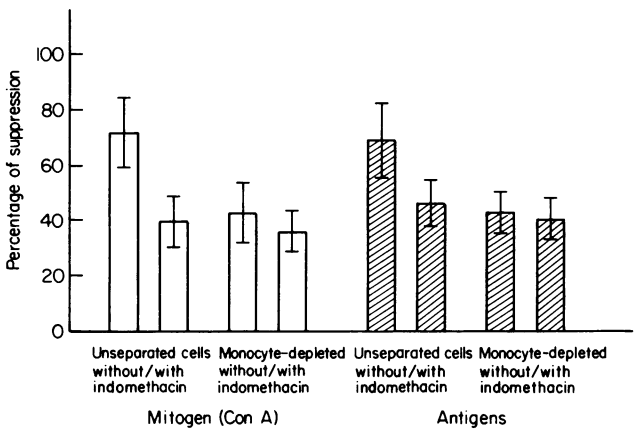

Fig. 2. Effect of monocyte depletion and indomethacin on Con A-activated suppression. Monocyte depletion after Con A activation or the presence of indomethacin in the culture medium results in decrease of suppression. Indomethacin does not influence the activity of monocyte-depleted suppressor cells. Data represent the mean (±s.e.m.) of five experiments, in which both mitogens (Con A, 1 μg/ml) and recall antigens were used to stimulate responder cells.

Table 3. Effect of monocyte depletion after Con A activation on the suppressive capacity of the resulting cell suspension and effect of indomethacin on the suppressive activity of unseparated and monocyte-depleted suppressor cells

Number of experiments	Stimulant	Cells added	Indomethacin (1 μg/ml) in culture medium	Suppression (%±s.e.m.)
5	Con A, 1 μg/ml	Unseparated	No	71±13
5	Con A, 1 μg/ml	Unseparated	Yes	39± 9
5	Con A, 1 μg/ml	Monocyte-depleted	No	42±10
5	Con A, 1 μg/ml	Monocyte-depleted	Yes	35± 7
5	Recall antigens	Unseparated	No	67±14
5	Recall antigens	Unseparated	Yes	46± 9
5	Recall antigens	Monocyte-depleted	No	42± 7
5	Recall antigens	Monocyte-depleted	Yes	39± 8

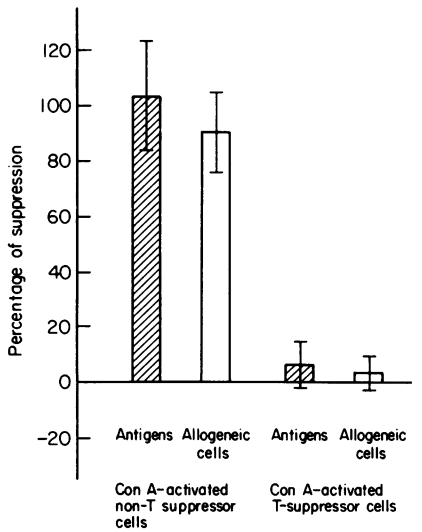

Fig.3

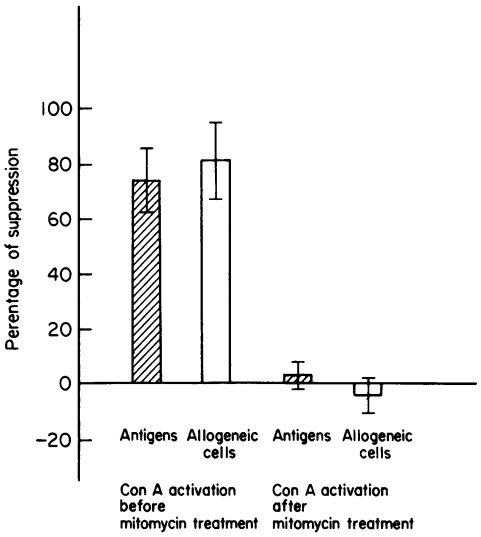

Fig.4

Fig. 3. Suppressive activity of Con A-activated T and non-T cell fractions. The figure shows the capacity of Con A-activated T and non-T cells to suppress DNA synthesis of autologous responder cells stimulated with antigens and with allogeneic cells. Data represent the mean (±s.e.m.) of six experiments.

Fig. 4. Effect of mitomycin treatment on the generation of Con A-activated suppressor cells. Mitomycin treatment before Con A activation prevents the generation of cells with the capacity to suppress DNA synthesis of autologous cells, stimulated with recall antigens or with allogeneic cells. Data represent the mean (±s.e.m.) of four experiments.

Table 4. Suppressive activity of Con A-activated T and non-T cells

Number of experiments	Stimulant	Cells added	Suppression (%±s.e.m.)
6	Recall antigens	Non-T cells	101±19
6	Recall antigens	T cells	6± 8
6	Allogeneic cells	Non-T cells	88±14
6	Allogeneic cells	T cells	3± 6

Table 5. Effect of mitomycin treatment on the generation of Con A-activated suppressor cells

Number of experiments	Stimulant	Mitomycin treatment before activation	Suppression (%±s.e.m.)
4	Recall antigens	No	72±10
4	Recall antigens	Yes	3± 5
4	Allogeneic cells	No	79±14
4	Allogeneic cells	Yes	−4± 6

cells, cells were treated with mitomycin before activation with Con A. As shown in Table 5 and Fig. 4 mitomycin prevented generation of suppressor cells. We next examined whether proliferation is involved in Con A activation. It was found that after 24 hr of activation no incorporation of ^{3}H-thymidine could be demonstrated, indicating that proliferation is not involved in activation.

DISCUSSION

Peripheral blood mononuclear cells, activated by Con A over 24 hr and subsequently treated with mitomycin, suppress the proliferation of autologous cells stimulated by mitogens, soluble antigens or allogeneic cells. The generation of suppressor cells in this assay system is independent of cell proliferation. This was shown by the fact that cells did not incorporate ^{3}H-thymidine after incubation with Con A for 24 hr, whereas after this activation period proliferation was blocked by mitomycin. This finding is in accordance with other studies in which mitomycin treatment of activated suppressor cells does not remove their suppressive action on the proliferative response of autologous cells (Shou *et al.*, 1976; Sakane & Green, 1977; Feighery *et al.*, 1978). However, Haynes & Fauci (1978b) found that the suppressive activity of Con A-activated cells, as measured in an assay system of polyclonal Ig production by PWM-stimulated B cells, is lost after mitomycin treatment. They conclude that optimal cell division is required for suppressive effector function. Another possibility seems to be that mitomycin treatment inactivates the radiosensitive suppressor T cell that is supposed to be the suppressor cell in their system (Hayward *et al.*, 1978). In our assay system treatment of cells with mitomycin before activation by Con A completely prevented their suppressive capacity. Apparently, activation of suppressor cells is mitomycin-sensitive; once activation has occurred suppressor cell function is not blocked by mitomycin and is independent of cell proliferation. As shown in a previous study (de Gast *et al.*, 1977) Con A is bound to non-T cells. The type of cell involved in Con A-induced suppressor cell generation has been further delineated in the present study. Depletion of monocytes before Con A activation resulted in a complete loss of suppressor cell activity. These data are in agreement with a recent study by Raff *et al.* (1978), demonstrating that monocytes are required for induction of Con A-activated suppressor cells. In their model, using a different assay system, Con A-induced monocyte–T cell interaction is proposed, resulting in the generation of T-suppressor cells. In our study separated T cells, activated by Con A, did not suppress the proliferation of autologous cells. One would expect this finding since T cells were completely deprived of monocytes and, as shown above, monocytes are necessary for the generation of Con A-suppressor cells. However, the non-T cell fraction, containing less than 1% E rosetting cells, demonstrated a strong suppressive activity after activation by Con A. When monocytes were removed from the Con A-activated non-T cells, the remaining cells still showed some suppressive action (unpublished data). E rosette-negative, non-adherent cells, functioning as suppressor cells after Con A activation, were also demonstrated by Haynes & Fauci (1978b). The Con A-generated suppressor cells are apparently a heterogeneous population.

As shown in this study monocytes may perform a suppressive effector function, apart from their role in the generation of suppressor cells. This effect is probably mediated by prostaglandin, as shown by the fact that it is blocked by indomethacin, a prostaglandin synthetase inhibitor. Excessive suppressive activity of monocytes, mediated by prostaglandins, is demonstrated in various pathological conditions like sarcoidosis (Goodwin *et al.*, 1979) and Hodgkin's disease (Goodwin *et al.*, 1977b). In SLE excessive monocyte suppression was also postulated as a cause of defective mitogenic responsiveness (Markenson *et al.*, 1978).

The role of monocytes in the regulation of the human immune response is as yet not completely clarified. However, there is increasing evidence for their major role in cellular interactions. The present study demonstrates that monocytes are necessary in the generation of suppressor cells by Con A and may themselves perform a suppressive effect on the antigen- and mitogen-stimulated response of autologous cells.

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