Clin. exp. Immunol. (1982) **50,** 406–415.

The regulation of T cell responses by spontaneously active suppressor cells

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(Accepted for publication 4 June 1982)

SUMMARY

In studying T cell regulation, peripheral blood mononuclear cells from normal subjects were examined for 'spontaneous', rather than mitogen-induced, suppressor cell activity. Normal blood leucocytes from 30 subjects included a subpopulation of cells capable of suppressing the response of lymphocytes to the T cell mitogen phytohaemagglutinin by 21–35%. The indicator system for these studies consisted of fresh normal lymphocytes stimulated by three concentrations of PHA in the presence or absence of normal but mitomycin C treated peripheral blood lymphocytes. To measure accurately the spontaneous suppressor cell activity, additional cultures were needed to control for the suppressive effects of crowding and metabolic competition. Allogeneic, cryopreserved 'B' cell enriched populations, supplied satisfactory control cells for this purpose. While allogeneic culture systems could induce significant suppressor cell activity after 7 days of co-culture, they could not induce this activity in the 3 days required to assay spontaneous suppressor cell effects. In developing this assay we noted that (a) crowding became a factor in the cellular response to mitogens with concentrations higher than 2×10^4 cells/well, (b) spontaneous suppressor cell activity decreased rapidly once cells were placed in culture and (c) both spontaneous and concanavalin A (Con A) activated suppressor cells could significantly reduce the response to PHA even when added to cultures established with mitogens 72 hr earlier. The ability to measure spontaneous suppressor cell activity *in vitro* will allow more physiological studies of the membrane markers and functional characteristics of these cells than is possible in conventional studies utilizing Con A. In addition, this assay allows the detection of enhanced *in vivo* activity of suppressor cells not easily detected in assays relying on mitogen induction of suppression. Such increased activity is thought to be an important factor in the pathogenesis of a number of human diseases.

INTRODUCTION

It appears well established that the appropriateness of immunological effector mechanisms is controlled by specific subpopulations of mononuclear cells (Gershon, 1975; Cantor & Boyse, 1978). In humans, subpopulations of thymus derived T lymphocytes, as well as monocytes/macrophages with regulatory capacity, have been described by Sakane & Green (1977) and Rice, Laughter & Twomey (1979). Concanavalin A (Con A) will stimulate the suppressor activity of those normal human lymphocytes that regulate both T and B cell effector function (Lobo & Spencer, 1979).

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0009-9104/82/1100-0406$02.00

Failure of regulatory cells to be activated by Con A is evidence of a deficiency in T cell suppressor function (Krakauer *et al.*, 1979).

More difficult to study has been the suppressor activity of regulatory cells thought to be promoting disease through inappropriately excessive suppression of effector mechanisms. In some primary and acquired humoral immune deficient states (Broder *et al.*, 1975; Dosch & Gelfand, 1978), neoplasia (Patt *et al.*, 1978; Herr, 1979), parasitic, mycobacterial and viral infections (Colley, Lewis & Goodgame, 1978; Mehra *et al.*, 1979; Tosato *et al.*, 1979) and granulomatous conditions (Goodwin *et al.*, 1979) diminished or inadequate cell-mediated immune responses and antibody formation have been attributed to inappropriately excessive suppression of these responses by regulatory cells. If this is true, it would be expected that freshly drawn peripheral blood lymphocytes obtained from patients with active disease would possess a greater degree of 'spontaneous' suppressor cell activity than lymphocytes from healthy controls. 'Spontaneous' in this sense would be used to denote *in vivo* activation of specific and/or non-specific (Feighery *et al.*, 1978) suppressor cell activity.

Previously reported studies of *in vivo* induced suppressor cell activity in disease, (Tsoi *et al.*, 1979; Faguet, 1979; Miller & Baker, 1979) while satisfactory for demonstrating differences between groups studied, (1) provide relative rather than absolute measurements of suppressor cell activity; (2) are, because of the inherent variability of individual cultures, suitable only for group analysis unless both patient and control cells are studied repeatedly; (3) do not reveal the degree of normal spontaneous suppressor cell activity and (4) do not allow dissection of passive suppressor effects (departure from ideal cell culture conditions for the responding cells) from true active *in vivo* induced suppressor cell activity. We present here the first detailed analysis of the regulation of human T cell effector function by spontaneously active suppressor cells. We have examined the effect the cell crowding, competition for nutritional agents in culture media, and the presence of allogeneic cell combinations may have on the response of normal cells to mitogens. The assay described for the evaluation of *in vivo* suppressor cell activity utilizes control cultures containing enriched populations of 'B' lymphocytes and appears suitable for studies of abnormal enhancement of *in vivo* suppressor cell activity that may be an essential feature of the immunopathogenesis of certain diseases.

MATERIALS AND METHODS

Isolation of lymphocytes. Heparinized blood from healthy volunteers was centrifuged through Ficol-Hypaque at 400*g* for 45 min, washed and prepared for culture by standard techniques.

Mitogen stimulation of normal cells. Three doses of PHA (phytohaemagglutinin; Phaseolus spp, Wellcome Reagents, Research Triangle, North Carolina, USA) were used, the final concentration in the culture wells being 5 μg/ml (high), 1 μg/ml (medium) and 0·25 μg/ml (low). Cell cultures were performed in microtitre plates (Linbro Plastics, New Haven, Connecticut, USA). After 3 days the cultures were pulsed for 24 hr with 0·5 μCi/well of tritiated thymidine sp. act. 1·9 Ci/MM (Schwarz-Mann, Orangeburg, New York, USA), harvested and assayed by liquid scintillation. In reporting results, Δ c.p.m. values are used throughout.

In vitro *generation of suppressor cell activity* (Dwyer, Johnson & Desaules, 1979). Lymphocytes in bulk culture (2×10^6/ml) were exposed to Con A in a concentration of 100 μg/ml. After 72 hr these cells and identical control cells not exposed to Con A were incubated with mitomycin C (Sigma Chemical, St Louis, Missouri, USA) at a concentration of 100 μg/ml for 30 min at 37°C. Treated cells were washed and assessed for viability (mean 85%).

Assay for suppressor cell activity induced by concanavalin A. To measure induced suppressor activity, the ability of Con A stimulated cells to reduce the mitogenic response of normal cells to PHA was determined. Fresh responding cells were co-cultured with autologous or allogeneic 'suppressor' cells or pre-cultured, unstimulated cells, and suppressor effects were sought across a dose–response curve that utilized the three doses of PHA described above. One hundred thousand responder cells (R) were co-cultured with 10^5 unstimulated cells or 10^5 Con A treated cells. After 72

hr cultures were pulsed and terminated. Suppression is expressed as the percentage reduction in the response to PHA calculated from the following formula:

$$\left[1-\frac{\text{(c.p.m. from responder cells + suppressor cells)}}{\text{(c.p.m. from responder cells + control cells)}}\right]\times 100.$$

Preparation of 'B' Lymphocytes. Sheep red blood cells were treated with 0·14M AET (Sigma) and adjusted to 2% with Hank's balanced salt solution (HBSS). Mononuclear cells were suspended at 10×10^6/ml in RPMI 1640 + 20% FCS and an equal volume of FCS and two volumes of 2% AET SRBC added. The cells were pelleted and incubated on ice for 1 hr. Following gentle resuspension cells were spun through a Ficoll-Hypaque gradient for 20 min at 800*g*. T depleted cells were harvested from the Ficoll interface and incubated on plastic petri dishes for 1 hr at 37°C for removal of adherent cells. 'B' cell preparations contained an average of 70% surface membrane immunoglobulin positive cells. The 'B' preparations were used immediately or cryopreserved in 10% DMSO and stored for future use.

Spontaneous suppressor cell assays. To measure 'spontaneous' suppressor cell (SS) activity, the ability of fresh cells to reduce the mitogenic response of normal cells to PHA was determined. Fresh responder cells (10^5/well) were co-cultured with equal numbers of latex beads (diameter 7·6 μm) (Sigma), 'B' cells treated with m/c ($B_{m/c}$) or putative spontaneous suppressor cells treated with m/c. After 72 hr of exposure to the three doses of PHA, all cultures were pulsed and terminated as described earlier. Suppression was then expressed as the percentage reduction in the response to PHA calculated by the following formula:

$$\left[1-\frac{\text{(c.p.m. from responder cells + suppressor cells)}}{\text{(c.p.m. from responder cells + 'B' cells or latex beads)}}\right]\times 100$$

Induction of suppressor cell activity by mixed leucocyte cultures (MLC) (Tse & Dutton, 1978). Lymphocytes from a control subject (donor A) were either held in culture alone or stimulated with m/c treated allogeneic lymphocytes (from donor B) for 3, 5 and 7 days. At these time points, lymphocytes from both populations were treated with m/c and assayed for the presence of suppressor activity. One hundred thousand cells from each culture were added to 10^5 fresh allogeneic cells (from donor C) subsequently stimulated by PHA. The DNA synthesis generated in both cultures was compared after 3 days.

Kinetics of the suppression mediated by spontaneous and Con A activated suppressor cells. A PHA indicator system was again utilized and the putative suppressors, Con A or spontaneously induced, and their appropriate controls, were added at 24 hr intervals from the initiation of the culture (0 time) to the pulse (72 hr). Percentage suppression was assessed as the differential effect of controls and putative suppressors on the responder population.

Persistence of spontaneous suppressor cell activity in culture. The spontaneous suppressor cell activity of fresh cells was compared to that of the same cells pre-cultured for 24, 48 and 72 hr before their addition to an allogeneic PHA indicator system. The amount of 'spontaneous' activity at these different time points was compared with control cultures in which responder cells were co-cultured with m/c treated B cells.

RESULTS

'Spontaneous' suppressor cell activity of pre-cultured cells

Assays of Con A suppressor cell activity use control cultures containing pre-cultured cells that have not been exposed to mitogen. We noted that these m/c treated cells were able to interfere with the efficiency of the DNA response of 10^5 fresh cells exposed to PHA. After 3 days incubation, normal cells were pre-treated with m/c and 10^5 cells were added to 10^5 fresh allogeneic responder cells from normal subjects stimulated with three doses of PHA. The results were compared to the response to PHA of 10^5 responder cells cultured alone (mean c.p.m. $\pm$s.d. for high, medium and low doses of PHA being 32,579 $\pm$ 1,758; 41,851 $\pm$ 2,411 and 27,010 $\pm$ 2,136, respectively), and a marked suppression of the response of normal cells to PHA was noted (mean % suppression $\pm$s.e. for

cultures being 17±3%; 23±3% and 27±2%). This decrease in DNA synthesis could have resulted from the effects of physical crowding and/or metabolic competition as well as suppressor cell activity induced *in vivo*.

While it is unlikely that m/c treated cells could be activated to suppress by mitogens, to insure that suppression was spontaneous we performed experiments which demonstrated that equal suppression of a PHA response was obtained when the responder cells were stimulated with PHA for 24 hr, then washed well and allowed to continue in culture for 3 days with or without the putative spontaneous suppressor cells. Equivalent suppression of the responding cells' proliferation occurred whether or not the suppressor cells had been exposed to the PHA of the indicator system.

Pre-cultured human cells respond better to antigens and mitogens than fresh cells, a phenomenon widely interpreted to mean that the cells regulating T cell responses die or become inactivated quickly in tissue culture (Dwyer *et al.*, 1979; Feighery *et al.*, 1978). We therefore tested fresh cells for their 'spontaneous' suppressor cell activity.

Spontaneous suppressor activity of freshly drawn peripheral blood lymphocytes

In these experiments, lymphocytes from normal subjects were stimulated with PHA alone or in the presence of fresh or pre-cultured (72 hr) normal m/c treated allogeneic lymphocytes. Compared to the response of responder cells alone, suppression was noted when both the fresh and stored allogeneic cells were added to the culture, but the effect was more pronounced with the fresh cells (Table 1). However, control studies were needed to measure the degree of interference in the PHA indicator system caused not by spontaneous suppression but by crowding, metabolic competition from the m/c treated cells for media nutrients and the induction of suppressor cell activity from allogeneic stimulation (Fauget, 1979).

Effect on the response to PHA of increasing cell numbers (crowding) in cultures

If the DNA synthesis induced by a standard dose of PHA increased proportionally as the number of cells per well was increased, crowding would not be a potential artifact in suppressor studies. The expected response of 10^5 cells exposed to PHA in the presence of 10^5 m/c treated cells would be 50% of the response obtained by 2×10^5 responder cells, and failure to achieve this response could be attributed to suppressor cell activity.

Table 1. Reduced response to PHA with the addition of fresh or pre-cultured cells

	Δ c.p.m.* and % reduction observed after PHA stimulation					
	PHA-H†		PHA-M		PHA-L	
Cells in culture	Δ c.p.m.	% reduction	Δ c.p.m.	% reduction	Δ c.p.m.	% reduction
Responders alone	37,445§ ±2,239		40,568 ±1,827		23,356 ±1,390	
Responders and m/c treated pre-cultured cells‡ (72 hr)	31,864 ±1,716	16±3¶	32,750 ±1,477	21±4	17,180 ±1,179	24±3
Responders and m/c treated fresh cells	26,174 ±1,775	30±2	28,485 ±1,666	30±3	15,233 ±1,230	36±3

* Δ c.p.m.—increase in c.p.m. above c.p.m. of cells not exposed to PHA.

† PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).

‡ m/c—mitomycin C treated non-responding cells.

§ Mean ±s.e.m.–results reported as mean±standard error of the mean (for 10 experiments).

¶ With the exception of this result all other suppression significant at $P < 0{\cdot}05$.

Table 2. Potential control cultures for the spontaneous suppressor cell assay

	% change from the c.p.m. observed when 10^5 responder cells are cultured (alone and stimulated) with PHA					
Cell mixtures	PHA-H Δ c.p.m.*	% change	PHA-M Δ c.p.m.	% change	PHA-L Δ c.p.m.	% change
10^5 R	32,394§ ±1,543		39,516 ±1,452		22,587 ±1,137	
2×10^5 R		+31±3·9		+20·6±4·4		+14·8±4·2
10^5 R+10^5 F m/c‡		−30·5±2·0		−29·6±2·9		−35·7±3·0
10^5 R+10^5 latex beads		−7·5±2·2		−7·1±0·6		−12·9±3·2
10^5 R+10^5 'B' m/c		−15·0±2·2		−15·3±2·9		−10·0±4·7

* Δ c.p.m. — increase in c.p.m. above c.p.m. of cells not exposed to PHA.
† PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).
‡ m/c—mitomycin C treated non-responding cells.
§ Mean ± s.e.m.—results reported as mean ± standard error of the mean.

In our studies (Table 2) doubling the cell numbers in a well was associated with a stimulation of DNA synthesis 69–85% less than the 100% increase predicted if crowding were not a factor. When DNA synthesis is calculated per 10^4 cells, we found that optimal responses per single cell were obtained with very low concentrations of cells (5×10^3–2×10^4 cells/well). At the cell concentrations needed for suppressor cell studies (1×10^5–2×10^5) it was obvious that crowding was a factor that would require physical rather than mathematical controls to allow analysis of active suppressor cell function (Table 2).

Use of latex beads and purified 'B' cells to control crowding factors in spontaneous suppressor cell assays

Latex beads of approximately the same size as unstimulated lymphocytes were added to cultures to control for physical crowding in suppressor cell assays. A degree of suppression was noted (7–12%) that was significantly ($P < 0{\cdot}001$) less than that obtained with m/c treated fresh cells (30–35%) (Table 2). Therefore, it appeared that these beads would be an ideal control if crowding was entirely responsible for the decreased response. However, as m/c treated cells are metabolically active even if incapable of dividing, competition for essential nutrients may be a factor in the reduction of cell proliferation. To investigate this possibility we used 'B' cell preparations as controls for both crowding and metabolic interference. In such cultures the suppression of the response to PHA was greater than that observed with latex beads, suggesting that 'B' cells provide a more physiological control.

Mitomycin C treated 'B' cells did not respond to PHA. To examine whether B cells absorbed essential nutrients from the media in a manner similar to T cells, we examined the uptake of ^{3}H-labelled leucine by our m/c treated putative suppressor and B cell populations; no differences were noted. 'B' cells therefore provided appropriate control for crowding and metabolic competition; however, as m/c treated 'B' cells are excellent stimulators in mixed lymphocyte culture, it was necessary to determine whether these cells could induce suppressor cell activity in the responder population.

Comparison of the reduction in PHA responsiveness of cells co-cultured with allogeneic or autologous cells

We compared the reduction in the response to PHA of lymphocytes from 14 normal subjects co-cultured for 3 days with equal numbers of m/c treated fresh lymphocytes that were either autologous with, or allogeneic to, the responder cells. As spontaneous suppressor cell activity,

Table 3. Reduced responsiveness to PHA in the presence of either autologous or allogeneic cells

		c.p.m. following stimulation with PHA (mean + s.e.m.)§								
		PHA-H			PHA-M			PHA-L		
Cell Mixture	Number of Experiments	Responder cell alone Δ c.p.m.	Responder + m/c treated fresh cells‡ Δ c.p.m.	% reduction	Responder cell alone	Responder + m/c treated fresh cells	% reduction	Responder cell alone	Responder + m/c treated fresh cells	% reduction
Autologous	20¶	37,475 ± 2,239	27,537 ± 1,879	26·6 ± 2·2	40,568 ± 1,827	27,698 ± 1,951	32·2 ± 3·3	23,356 ± 1,390	16,253 ± 1,081	29·9 ± 2·6
Allogeneic	20¶	37,475 ± 2,239	26,174 ± 1,775	30·5 ± 2·0	40,568 ± 1,827	28,485 ± 1,666	29·6 ± 2·9	23,356 ± 1,390	15,233 ± 1,230	35·8 ± 3·0

* Δ c.p.m.—increase in c.p.m. above c.p.m. of cells not exposed to PHA.

† PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).

‡ m/c—mitomycin C treated non-responding cells.

§ Mean ± s.e.m.—results reported as mean ± standard error of the mean.

¶ No statistically significant difference was found in the suppression observed.

crowding, and metabolic effects would be similar, any difference would be caused by the allogeneic combination. Autologous mixed leucocyte reactions were extremely unlikely given the low number of macrophages in the Ficoll-Hypaque separated cells (Innes *et al.*, 1979). Significant suppression of the response was observed, but there was no significant difference between the cultures featuring either autologous or allogeneic combinations (Table 3).

Establishment of the time required to induce suppressor cell activity in allogeneic combinations

We held allogeneic combinations from normal individuals for 3, 5 and 7 days in bulk culture before assessing their induced suppressor cell activity (Table 4). After 3 days of co-culture, allogeneic combinations produced only slightly more suppressor cell activity than did cells from a single donor precultured for 3 days. After 5 days of co-culture some induced suppressor cell activity was definite, and by 7 days variable, but pronounced, induction of suppressor cell activity was evident. It, therefore, seemed reasonable to use allogeneic combinations essential for practical studies of human subjects, in assays of spontaneous (*in vivo*) induced suppressor cell activity that require only 3 days of culture.

Table 4. Induced suppressor activity among allogeneic cells co-cultured for 3, 5 and 7 days

Length of co-culture (days)	% increase in the suppression (mean ± s.e.m.) of the response† to PHA associated with allogeneic combinations		
	PHA-H*	PHA-M	PHA-L
3	5·4 ± 2·4‡	3·0 ± 1·5	13·6 ± 4·4
5	6·3 ± 2·3	7·6 ± 1·7	27·9 ± 7·2
7	14·5 ± 4·5	15·2 ± 3·9	57·2 ± 7·2

* PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).

† Mean ± s.e.m.—results reported as mean ± standard error of the mean.

‡ Suppression of responder cells produced by cells from an allogeneic culture compared to the suppression of the responder cells produced by cells from the same subjects precultured alone.

Table 5. Ability of Con A-induced and spontaneous suppressor cells to reduce responsiveness to PHA when added at 24 hr intervals

Hours of culture‡ when suppressor cells added	% suppression of PHA response (mean + s.e.m.)†					
	PHA-H* Suppressor cells		PHA-M Suppressor cells		PHA-L Suppressor cells	
	Induced	Spontaneous	Induced	Spontaneous	Induced	Spontaneous
0	21 ± 8	11·3 ± 2·4	26 ± 7·9	16·4 ± 2·7	49 ± 10·9	22·0 ± 3·4
24	26 ± 3·5	22·0 ± 3·1	16 ± 5·5	26·0 ± 2·3	46 ± 8·4	22·0 ± 4·3
48	36 ± 2·8	13·3 ± 3·2	27·6 ± 6·3	9·7 ± 2·2	33·6 ± 7·5	16·0 ± 3·1
72	23·5 ± 4·7	8·6 ± 2·0	26·0 ± 4·2	4·9 ± 1·8	33·8 ± 7·1	11·6 ± 2·3
96§				1 ± 0·3		3 ± 0·6

* PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).

† Mean ± s.e.m.—results reported as mean ± standard error of the mean.

‡ Control cultures contained mitomycin C but not Con A treated precultured cells.

§ All cultures were pulsed at 72 hr and terminated 24 hr later.

Suppressor cell interference with established PHA stimulation

The 'spontaneous' activity measured above assayed a suppressor cell effect over a 3 day period. The sooner after removal from the body suppressor cell activity can be measured, the more legitimate the claim of spontaneous or *in vivo* induced activity. We examined the ability of both Con A-induced and spontaneous suppressor cells or appropriate controls (m/c treated) to suppress a PHA response when added at 0, 24, 48 and 72 hr after the addition of mitogen. Con A stimulated cells were effective suppressors even when added at the time of pulsing. Spontaneous suppressor cells were most effective when added before the last 24 hr of the culture (Table 5).

Spontaneous suppressor cell activity decreases with pre-culture

If spontaneous suppressor cell activity decreases when normal cells are held in undisturbed culture conditions, then their activity is likely to have been induced *in vivo*. B cells, or freshly isolated cells, from normal individuals were added to a PHA indicator system after they had been held in culture for 24, 48 and 72 hr. Spontaneous suppressor cell activity fell rapidly with pre-culture, suggesting that all spontaneous suppressor cell activity had been induced *in vivo* (Table 6).

Table 6. Spontaneous suppressor cell activity of fresh and pre-cultured cells

	% suppression of the PHA response (mean + s.e.m.)†		
	PHA-H*	PHA-M	PHA-L
0	18·8 ± 2·5	30·6 ± 2·3	31·9 ± 3·2
24	10·2 ± 2·6	13·8 ± 2·2	28·6 ± 3·6
48	4·3 ± 2·8	9·8 ± 3·2	18·1 ± 3·8
72	1·3 ± 0·7	6·3 ± 4·0	16·3 ± 6·9

* PHA-H,-M,-L—three doses of PHA used; high (H), middle (M) and low (L).

† Mean ± s.e.m.—results reported as mean ± standard error of the mean.

Note that denominator for calculated SSC activity here consists of c.p.m. from stimulated responder cells in the presence of m/c treated 'B' cells whereas in Table 3 reductions are calculated from responder cells cultured alone.

DISCUSSION

Most of the studies of human suppressor cells have looked at the mechanisms by which B cells are regulated (Saxon & Stevens, 1978; UytdeHaag, Heynen & Ballieux, 1978) and relied largely on Con A activated cells which can suppress antibody secretion and T cell responses (Tardieu & Dupuy, 1978). We have concentrated on the immunoregulation of T cells and have examined, in normal subjects, suppressor cell function activated *in vivo* and therefore physiologically.

The suppression reported in Con A-induced suppressor studies represents the degree of suppression by which the Con A activated cells exceeds that of the non-Con A stimulated cells. In studying this system, we found that compared to the response of cells cultured alone, the response of cells to which had been added normal cells pre-cultured for 3 days (the control cells for the Con A assay) was significantly decreased. The finding that freshly drawn cells produced significantly more suppression of the PHA response did suggest that a degree of true suppressor cell activity was present and likely to have been induced *in vivo*. This hypothesis was supported by previous indirect evidence suggesting that suppressor cell activity diminishes rapidly in cultured but unstimulated cells (Dwyer *et al.*, 1979; Stobo *et al.*, 1976).

The cell concentrations associated with the most efficient response to PHA were surprisingly low (Farrant & Knight, 1979). Optimal responses occurring at a concentration of 3×10^3–2×10^4 cells/well. With increased cell numbers per well, efficiency was lost and crowding phenomena certainly require control cultures to allow active suppression to be discriminated. 'B' cells produced only slightly more suppression of the response to PHA than did latex beads. These cells can be cryopreserved in large numbers to supply a standard reagent for many studies. 'B' cells as noted in the results, are satisfactory control cells, for we could not reproduce previous findings that they exert strong immunoregulatory activity on delayed type hypersensitivity *in vivo* (Noble *et al.*, 1977; Gill & Liew, 1979).

Studies of mixed lymphocyte reactions in animals have demonstrated the induction of suppressor cell activity during the course of the reaction (Tse & Dutton, 1978), and we confirmed this in humans, however, significant suppression was not induced by 3 days of allogeneic co-culture. This was expected as our cultures showed no more suppression of the PHA response associated with the use of m/c treated allogeneic, rather than autologous, fresh cells.

It was of interest to note that with both Con A and spontaneously activated suppressor cells, suppression could be measured even when the cells were added to cultures established 72 hr earlier. If this is true for blastogenic responses to antigens the problems of allogeneic activation of suppressor cells could be avoided. Antigen specific suppressor cells would be added during the final 3 days of the culture.

The mechanisms by which suppressor cells non-specifically diminish T cell response to mitogens, is poorly understood. The mechanism is different from that operative in suppression of antibody secretion *in vitro* where it is essential to have the suppressor cells present from the initiation of the culture (Haynes & Fauci, 1978) and the cells responsible become more potent with a few days in resting culture (Lipsky *et al.*, 1978). Although spontaneous suppressor cell activity decreased rapidly among cells in culture, significant activity was still present after 24–48 hr. Those laboratories which use 24–48 hr of incubation for Con A suppressor cell studies will have significant suppressor cell activity in their control cultures. In such assays, failure to achieve good suppressor cell function in Con A stimulated populations may reflect maintained spontaneous suppressor cell activity rather than a defective response to Con A.

As a number of diseases are suspected to be caused by inappropriately excessive suppressor cell activity, the ability to document this factor is important. The assay for spontaneous suppressor cell activity may well supply the investigational tool needed for this purpose.

This was financially supported by National Institutes of Health grant No. 11785.

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