Clin. exp. Immunol. (1979) **38**, 499–513.

Behaviour of human immunoregulatory cells in culture

I. VARIABLES REQUIRING CONSIDERATION FOR CLINICAL STUDIES

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(*Accepted for publication* 18 *May* 1979)

SUMMARY

The suppressor function of lymphocytes stimulated with concanavalin A (Con A) provides a potential method for examining disorders of immunoregulation. Clinical application, however, requires definition of the culture conditions that influence the expression of normal suppressor cell activity. In the present studies culture conditions were modified until a sensitive assay for non-specific suppressor cell function was reproducible utilizing the response to varying doses of phytohaemagglutinin (PHA) as an indicator system. Practical conclusions included (1) that sensitivity was not lost if the suppressor cells and responder cells were allogenic; (2) that fresh responder cells were as sensitive as precultured responder cells; (3) that a wide range of Con A concentrations could induce suppressor activity; and (4) that the sensitivity of the assay was much enhanced by using suboptimal mitogen doses of PHA. Twelve percent of normal subjects gave false negative results but these could be avoided by studying cells at more than one time point after stimulation with Con A.

Cells resting in culture for 7 days could be induced to suppress after stimulation with Con A and these suppressor cells were very sensitive to pharmacological doses of dexamethasone. Studies utilizing different times of cell pre-incubation before Con A stimulation and different periods of exposure to Con A revealed fluctuation in the induction of suppression that may represent alternating periods of suppression and amplifying activity among stimulated cells *in vitro*. Such variations will need to be taken into account in the application of this type of assay to clinical studies seeking disordered immunoregulation.

INTRODUCTION

It is now well established that at least one T cell subpopulation plays an active immunoregulatory role in both humoral and cell mediated immune responses (Gershon, 1975). 'Suppressor cell' activity has now been observed in many experimental situations and is important in the development and maintenance of immunological tolerance (Benjamin, 1975), allotype suppression (Jacobsen *et al.*, 1972) and some cases of H-2 linked antigen specific unresponsiveness (Gershon, Maurer & Merryman, 1973).

In man, strong circumstantial evidence suggests that adequate performance of this T cell population may be influenced by alleles closely associated with the D locus of the sixth chromosome (Vladutin & Rose, 1974). The association of the gene products of this locus and those of the nearby B locus with certain diseases that feature immunopathogeneic mechanisms strengthens this hypothesis (Ritzman, 1976). Attempts to establish *in vitro* assays that assess the immunoregulatory performance of T cells have met with considerable success and conditions of both excessive and inadequate suppressor cell activity

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have been implicated in disease processes. Excessive suppressor activity may account for reduced immunoglobulin production in many cases of common variable hypogammaglobulinaemia (Waldmann *et al.*, 1974), multiple myeloma (Broder *et al.*, 1975) and in certain individuals, a deficient cell mediated response to fungal infections (Stobo *et al.*, 1976). Conversely, inadequate suppressor cell activity has been noted in patients with systemic lupus erythematosus (Bresnihan & Jasin, 1977) and the NZB mouse which develops a similar illness (Gerber *et al.*, 1974).

Most assays for suppressor cell activity in man have used human blood lymphocytes and depend on the observation first made in animals (Dutton, 1972; Redelman *et al.*, 1976) and later demonstrated in humans (Shou, Schwarz & Good, 1976) that the mitogen Con A, while inducing DNA synthesis, also stimulates at least some cells to express suppressor cell activity.

In our laboratory we initially experienced difficulty in standardizing this assay so that the performance of suppressor T cells from individual subjects rather than grouped subjects could be reliably and reproducibly analysed. In defining the conditions needed to produce a more reliable test we have (1) found a number of practical, methodological steps that appear to improve the assay for clinical application, and (2) have learned much about the behaviour of human lymphocytes, particularly suppressor lymphocytes in culture. We here report these studies which provided as major conclusions (1) the abolition of false negative responses when the time of exposure of patients cells to Con A is broadened, and (2) a fluctuating responsiveness of suppressor cells to this mitogen in culture that suggested the presence of naturally occurring but alternating periods of suppressor and amplifier activity amongst cells in culture. These variations in the response to mitogens will need to be considered in seeking pathological disturbances of immunoregulation in mitogen-dependent assays.

MATERIALS AND METHODS

Isolation of Lymphocytes. Heparinized blood from healthy volunteers was centrifuged through Ficoll-Hypaque at 400 *g* for 45 min. Lymphocytes were removed from the gradient interface and washed three times in Hanks' balanced salt solution (HBSS) (Flow Laboratories, Rockville, Maryland). The HBSS contained 100 units of Penicillin and 100 mcg of streptomycin (Microbiological Associates, Bethesda, Maryland) per 1 ml. For tissue culture the final cell suspension was made in RPMI 1640 (Gibco, Grand Island, New York) containing the antibiotics, 2 mmol-glutamine (Microbiological Associates) and 15% pooled human serum. Cell counts were made with a Coulter counter and viability assessed by eosin exclusion. Cell counts mentioned herein refer to viable cells.

Detection of SMIg. Cells with readily detectable membrane-bound immunoglobulin were detected using a method previously described in detail (Kincade, Lawton & Cooper, 1971). Polyvant anti-human immunoglobulin sera (Meloy Labs, Springfield, Virginia) were used throughout. After incubation, cells were washed twice, resuspended in FCS and a drop was placed on each of two slides, one for staining with Wright's stain for a differential analysis, the other for examination with fluorescent microscopy. Alternate phase contrast and fluorescent microscopy was used to determine the integrity of fluorescent cells.

Detection of cells bearing receptors for complement components (EAC). A 5% solution of SRBC in RPMI 1640 media was prepared and to 4 ml of this reagent was added 4 ml of IgM anti-SRBC antiserum (Cordis Labs, Miami, Florida) previously diluted 1:200 with normal saline. The mixture was held at 37°C for 30 min, washed three times and finally resuspended in 8 ml of RPMI 1640 (EAb). Eight ml of a human complement source (human serum diluted 1:50 with phosphate buffered saline) was added to the EAb preparation (EAC). From that point a routine technique was used (Mendes *et al.*, 1973).

Detection of cells rosetting with sheep erythrocytes (E). Washed SRBCs (0·5% concentration) were added to equal volumes of lymphocyte preparations containing 2×10^6 cells/ml. The suspension was incubated at room temperature for 30 min, pelleted in a refrigerated centrifuge and stored at 4°C overnight. Following gentle resuspension in FCS, drops of suspension were placed on two glass slides, one for staining and differential analysis, the other for counting of rosette-forming cells. Lymphocytes with three or more SRBCs attached were considered to be T lymphocytes.

Mitogen stimulation of normal cells for surface marker studies. PHA (Wellcome Reagents, Research Triangle, North California) was added in a dose known to be optimally mitogenic for normal cells. Similar cultures utilizing PWM (Gibco, Grand Island, New York) as the mitogen were also prepared (Mangi *et al.*, 1974). Cell cultures were performed in microtitre plates (Linbro Plastics, New Haven, Connecticut) with wells being set aside for viability and cell survival studies. After 3 days of stimulation the cultures were pulsed for 4 hr with tritiated thymidine, terminated, harvested and prepared for assay using liquid scintillation by a multiple automated sample harvester. Stimulation indices were obtained by dividing the counts per minute (c.p.m.) obtained from cultures containing PHA or PWM by the mean c.p.m. obtained from triplicate control cultures.

Preculture of lymphocytes before stimulation with mitogens. Lymphocytes were stored in bulk cultures in which the cells were suspended at a concentration of 2×10^6 cells/ml in the culture media described above without mitogen. When removed from

culture, cell samples were centrifuged and resuspended in fresh culture medium. After viability counts, cell concentration was adjusted to 1×10^6/ml. Samples were stimulated with PHA on the day of removal from the donor and at 24-hr intervals for the next 5 days. 0·1 ml of the cell suspension was added to the wells of microculture plates (Linbro) containing 0·1 ml of tissue culture media to which had been added one of three concentrations of PHA or Con A (Calbiochem, La Jolla, California) used. The plates were then held in a humidified 5% CO_2 incubator for either 24, 48, 72 or 96 hr before pulsing and termination. At this time, 0·25 μCi of ^{3}H-thymidine (Schwarz-Mann, Orangeburg, New York) was added to each of the wells. After 24 hr samples were harvested with a multiple automated sample harvester placed in a scintillation cocktail of toluene and liquifluor (Beckman, Mountainside, New Jersey) and ^{3}H-thymidine incorporation measured in a liquid scintillation counter (Beckman, Fullerton, California). Results were expressed as the number of counts per minute (c.p.m.) obtained from cells stimulated with mitogens minus the c.p.m. obtained from unstimulated cultures (Δ c.p.m.).

Generation of suppressor cell activity. Typically, cells in bulk culture were exposed to Con A at a concentration of either 1, 5, 10, 25, 50, 100, or 200 μg/ml. After 3 days, cells from these cultures were removed, pelleted and resuspended in fresh medium. After a viability count (usually 85% viable), cells were resuspended at a concentration of 4×10^6/ml and incubated with mitomycin C (Sigma Chemicals, St Louis, Missouri) at a concentration of 100 μg/ml for 30 min at 37°C. Treated cells were washed four times with HBSS and a further count of cell numbers and viability performed. These cells were then used as suppressor cells.

Other experiments were designed to study the effect on suppressor-cell induction of varying the time of exposure of potential suppressor cells to Con A. Cells were treated as above but removed from culture with Con A at 24, 48, 72 and 96 hr and after a similar preparation to that described above, assayed for suppressor ability.

In another variation designed to examine the ability of Con A to induce suppressor activity amongst precultured cells, cells precultured for 24, 48 and 72 hr were then stimulated with Con A as above and the suppressor ability so induced compared to that induced in similarly stimulated fresh cells.

Assay for suppressor cell induction by Con A. To measure induced suppressor activity, the ability of Con A stimulated cells to reduce a normal mitogenic response to PHA was determined. Either fresh or stored cells to be stimulated with PHA were co-cultured with autologous or allogenic 'suppressor' cells. As suppression may be more difficult to quantitate amongst cells stimulated with optimal doses of PHA, suppressor effects were sought across a dose response curve that utilized either 10, 2 or 0·5 μg/ml of PHA.

Cell cultures were performed in microculture plates whose wells contained a total of 2×10^5 cells in 0·2 ml. For control cultures, 1×10^5 responder cells were mixed with 1×10^5 autologous or allogenic mitomycin treated unstimulated cells. In experimental cultures 1×10^5 responder cells were mixed with 1×10^5 cells that had been stimulated by Con A. The variations used in the induction of the suppressor cells have been described above.

After 72 hr of exposure to PHA with or without the presence of suppressor cells, all cultures were pulsed and terminated as described earlier. Suppression is expressed throughout as the percentage reduction in the response to PHA calculated from the following formula:

$$1 - \frac{\text{c.p.m. from responder cells and suppressor cells}}{\text{c.p.m. from responder cells and control cells}} \times 100$$

Effect of dexamethasone on suppressor cells induced by Con A. The effect of dexamethasone sodium phosphate (Decadron) on control cells and cells stimulated by 100 mg of Con A for 3 days was examined. When equated with the corticosteroid effect of naturally occurring steroids a dexamethasone concentration of 1×10^{-6} molar would be physiological. A pharmacological level of 1×10^{-3} molar was used in these experiments. A molar solution of dexamethasone containing 392 g/litre, thus fluid concentration of 0·4 mg/ml provided the desired molarity. In nine experiments half of the control lymphocytes and half of the Con A activated lymphocytes were incubated with the pharmacological concentration of dexamethasone. All groups were treated with mitomycin C and added to allogenic lymphocytes. These latter responder cells had been cultured for 3 days in media without mitogen and were then stimulated with PHA at the time the suppressor cells were added.

RESULTS

Survival rate and changes in lymphocyte membrane markers on normal cells cultured for 2 weeks

Suppressor cell assays require complicated co-culture conditions in which cells are held in culture either stimulated or unstimulated for 6 or more days. We have assessed the T and B cell population changes that occur during this period. The results of these experiments are shown in Table 1. It is obvious that many lymphocytes die during their first 24 hr in culture. These cells presumably lyse as the viability of the cells remaining is excellent through 10 days of culture. After the first 24 hr the remaining cells are lost more slowly. Cells with readily detectable SMIg (B cells) disappeared most rapidly from these cultures. Thus after three days in culture normal cells which might be used as responder cells in a suppressor assay, control cells for Con A stimulated suppressor cells or for studies of mitogen enhancement with precultured cells, contain only 30% of the T cells present at the initiation of the culture.

TABLE 1. Survival rate and changes in lymphocyte membrane markers on normal cells cultured for 2 weeks

Day	Viability*	Original cells remaining (%)	E†	T cell loss (%)	SMIg§	B cell loss (%)	EAC**
0	100	100	71±16††	—	21±8	—	14±6
1	96±1	57±6	75±11	45	4±1	90	10±3
2	97±1	47±11	57±1	63	9±3	81	8±2
3	89±2	38±4	61±7	70	4±2	89	11±4
4	90±4	41±3	55±8	73	9±4	80	15±6
8	87±4	22±6	61±7	83	4±1	95	14±6
10	80±4	12±4	41±8	94	2±1	99	6±1
12	29±8	12±4	31±12	99	2±1	99	4±3
14	11±7	7±6	14±4	99	2±1	99	4±1

* Trypan blue.
† E rosette = T cell.
‡ Percentage of original T cell numbers lost in culture.
§ SMIg = surface membrane immunoglobulin (B cells).
¶ Percentage of original B cell numbers lost in culture (data derived from SMIg studies).
** EAC = cells binding complement coated red cells; mainly B cells.
†† Mean %±1 s.d. of twenty experiments throughout.

TABLE 2. Effect of mitogen stimulation on survival and lymphocyte membrane characteristics of normal cells in culture

Mitogen*	Days in culture	Viability†	Original cell count remaining (%)	E‡	SMIg§	EAC¶	S.I.**
	0	100	100	70	14	13	0
PHA	1	93±6	38±11	66	19	29	10±4
	2	97±4	42±8	55	12	15	26±12
	3	61±11	61±13	54	10	8	40±13
	0	100	100	70	14	13	0
PWM	1	97±4	34±6	56	9	21	5±2
	2	96±3	58±20	46	13	9	12±6
	3	95±12	64±18	46	8	5	21±7

* Optimal mitogenic doses in culture, results with Con A were similar so data is not shown.
† Trypan blue exclusion.
‡ E rosetting T cells.
§ % of surface membrane immunoglobulin-positive B cells (SMIg).
¶ Cells binding complement-coated sheep erythrocytes.
** Stimulation index (tritium incorporation; c.p.m. of cells with PHA/c.p.m. of cells without PHA).

Effect of mitogen on survival and lymphocyte membrane characteristics of normal lymphocytes in culture

The addition of mitogens to normal lymphocytes did not prevent the rapid loss of cells that occurs in the first 24 hr of culture (Table 2). However, from 48 to 72 hours there is, not surprisingly, some division occurring as the cell numbers increase on the second and third days of culture. PHA and Con A gave similar results. The lymphoblastoid state produced by mitogens does not appear to affect surface membrane markers. SMIg positive cells remain positive longer in cultures containing mitogen. Thus the induction of suppressor cells by mitogen is associated with a loss of 60% of the initially present cell population and the relatively greater survival of T lymphocytes.

TABLE 3. Effect of preculture on response of lymphocytes to phytohaemagglutinin (PHA)

		Days in culture before stimulation					
Result	PHA (μg/ml)	0	1	2	3	4	5
Mean± 1 s.d.	Control	320*	365	756	457	943	669
		168	164	442	179	1,018	181
Mean± 1 s.d.	0·5	19,846†	14,530	28,692	22,645	34,666	31,250
		4,478	14,581	10,483	17,235	13,140	11,528
Mean± 1 s.d.	2	28,538	24,376	32,615	34,111	45,714	49,750
		14,997	24,779	9691	14,913	3251	3863
Mean± 1 s.d.	10	27,115	22,069	32,307	31,363	44,250	37,666
		15,267	18,840	15,658	13,162	12,872	7094

* Counts per minute (c.p.m.) obtained from incorporated tritiated thymidine.

† Increase in cpm above counts obtained from unstimulated cultures (Δ c.p.m.) 3 days after the addition of PHA. Mean± 1 s.d. of data from thirteen subjects is reported.

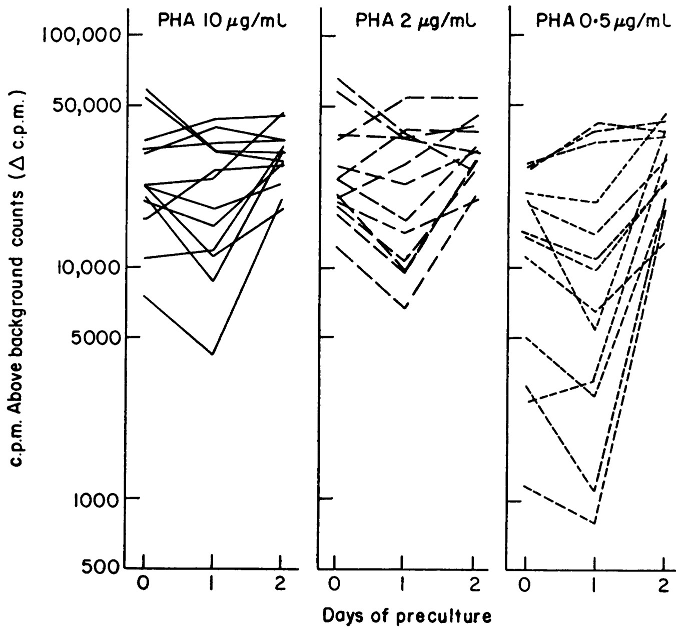

FIG. 1. Lymphocytes from thirteen subjects were stimulated with PHA when fresh (0 time) or after 1 or 2 days in tissue culture. At the end of 3 days of exposure to PHA a consistent decrease in the responsiveness of cells precultured for 24 hr was noted in most subjects. Recovery of responsiveness to levels exceeding that of fresh cells was frequently seen. These effects are most marked when the cells are stimulated with the lowest concentration of PHA.

Effect of preculturing lymphocytes on their response to PHA

There has been considerable interest in the concept that suppressor cells die rapidly in culture. Thus responder cells held in culture for 3 days before the addition of the putative suppressor cells may be a functionally different population from autologous but fresh cells. Lymphocytes from 13 normal subjects were stimulated with each of three doses of PHA on the day of sampling and on each of the following 5 days. During the pre-stimulation period the cells to be studied rested in bulk culture. Table 3 details

the results of the experiments. In all cases the response to PHA of cells precultured for 5 days was significantly greater than the identical cells stimulated when fresh ($P<0.001$). There was a consistent decrease in the mean response of cells cultured for one day before stimulation when compared to the response of fresh cells. This mean decrease seen on group analysis does not reflect the marked fall seen in many individuals (Fig. 1) but did reach statistical significance ($P<0.05$).

The number of cells stimulated with PHA in each culture was held constant and thus it appeared that certain cells, or the population as a whole, were becoming increasingly responsive with time to PHA, perhaps due to release from an inhibitory mechanism initially present. The results with Con A were similar in that four or five days of preculture increased the sensitivity of cells to stimulation with Con A, however, the decrease in responsiveness to PHA noted at 24 hr was less frequent with Con A stimulation.

Rapidity of DNA synthesis in precultured lymphocytes

Lymphocytes from two subjects were stimulated with 10, 2 and 0·5 μg/ml concentrations of PHA after varying periods in culture. Instead of pulsing and terminating the cultures 3 days after the addition of PHA as was customary, stimulated cultures were pulsed and terminated 1, 2, 3 and 4 days after the addition of PHA. In this manner the increasing sensitivity of precultured cells to PHA could be examined in kinetic terms. The most striking results were seen with the lowest dose of PHA (Table 4). Results were similar with PHA at 2 and 10 μg/ml, mean c.p.m. increasing from 2289 to 16,417 and 1274 to 18,776, respectively, when fresh cells and cells precultured for 4 days were compared for responses to an exposure to PHA of 24 hr.

Seen throughout, but most marked with terminations at 24 hr is the marked increase in the rapidity of the DNA response within precultured cells responding to mitogen stimulation. The daily increases in DNA synthesis measured at the end of 24 hr of exposure to PHA were statistically significant for each of the 4 days of preculture ($P<0.001$). These data seem compatible with those presented above and further suggest escape from an inhibitory mechanism normally operative upon freshly withdrawn peripheral blood lymphocytes.

Suppression of the response to PHA of precultured cells simultaneously exposed to autologous cells precultured with concanavalin A

To examine a number of variables in the induction and expression of suppressor activity among cells from normal peripheral blood, cells were exposed to various concentrations of Con A for 3 days, mitomycin treated and added to autologous cells precultured for 3 days. This cell mixture was then stimulated with various doses of PHA. Appropriate controls included the comparison of the response to PHA when Con A treated cells or an equal number of mitomycin treated but unstimulated autologous cells were

TABLE 4. Effect of preculture on the rapidity of the DNA synthetic response to PHA stimulation*

Days of stimulation with PHA (0·5 μg/ml)	Sample	Days in culture before stimulation				
		0	1	2	3	4
1	A	194	1464	†	6874	8471
	B	155	656	†	7166	12,564
2	A	288	13,674	15,558	13,281	9626
	B	467	3674	21,670	16,470	15,086
3	A	367	34,914	29,691	27,649	15,816
	B	2971	24,871	33 807	32,800	23,410
4	A	18,494	39,526	41,584	30,039	n.t.
	B	12,646	29,694	49,716	31,674	n.t.

* Mean increase in c.p.m. above counts obtained from unstimulated culture (Δ c.p.m.).
† Technical failure.
n.t. Not tested.

TABLE 5. Suppression of the response to PHA of precultured cells exposed to autologous cells precultured with concanavalin A

Unstimulated control cells (c.p.m.)*	Dose of PHA (μg/ml)	Response to PHA (c.p.m.)†‡	Dose of Con A inducing suppressor cells (μg/ml)	Unstimulated control cells with mitomycin treated Con A stimulated cells added	c.p.m. induced by PHA in cultures containing suppressor cells	Suppression induced (%)	Number of experiments
2146	10	36,527	200	2460	25,147	30±6	8
1028	10	26,931	100	511	18,094	33±8	8
953	10	30,366	50	561	20,166	38±3	8
1028	10	26,931	25	249	22,199	18±2	8
953	10	30,366	10	329	25,372	16±1	8
356	10	27,285	5	327	19,853	27±4	6
356	10	27,285	1	278	27,865	0	4
2146	2	43,377	200	2460	15,241	65±11	8
1028	2	26,931	100	511	10,469	61±6	8
053	2	30,366	50	432	15,086	50±11	8
1928	2	26,931	25	314	18,320	32±4	8
953	2	30,366	10	329	22,405	22±5	8
356	2	25,125	5	326	19,740	21±6	6
356	2	25,125	1	278	24,789	1±3	4
2146	0.5	23,137	200	2460	1780	92±8	8
1028	0·5	16,718	100	511	4338	74±5	8
953	0·5	19,620	50	400	5676	71±12	8
1028	0·5	16,718	25	314	4960	69±8	8
953	0·5	19,620	10	329	8445	57±9	8
356	0·5	17,861	5	326	12,151	32±6	6
356	0·5	17,861	1	278	15,257	14±2	4

* Precultured for 3 days before PHA added.

† These cultures contained mitomycin treated autologous cells to mimic conditions of cultures containing Con A induced suppressor cells (see Methods).

‡ Increase in counts above c.p.m. from unstimulated cultures (Δ c.p.m.).

TABLE 6. Suppression of the response to PHA of precultured cells exposed to allogeneic cells precultured with concanavalin A

Unstimulated control cells (c.p.m.)*	Dose of PHA (μg/ml)	Response to PHA (c.p.m.)†‡	Dose of Con A inducing suppressor cells (μg/ml)	Unstimulated control cells with mitomycin treated Con A stimulated cells added	c.p.m. induced by PHA in cultures containing suppressor cells	Percentage suppression induced	Number of experiments
481	10	23,190	200	7809	20,224	13 ± 5	6
579	10	24,511	100	2315	17,446	29 ± 8	6
579	10	24,511	50	867	19,089	23 ± 4	6
439	10	23,840	25	515	16,854	29 ± 4	6
439	10	23,840	10	556	26,533	-10 ± 51	6
997	10	26,526	5	958	26,419	0 ± 4	4
481	2	25,606	200	7809	23,294	10 ± 4	6
579	2	25,099	100	1157	15,427	39 ± 6	5
579	2	25,099	50	867	16,561	34 ± 6	6
439	2	26,445	25	515	16,648	37 ± 9	6
439	2	23,610	10	556	16,437	31 ± 7	6
481	0·5	7537	200	7809	1984	74 ± 16	6
579	0·5	13,502	100	1157	4948	64 ± 11	5
579	0·5	13,502	50	867	4372	68 ± 5	6
419	0·5	12,091	25	515	4816	61 ± 9	6
419	0·5	12,091	10	556	5204	57 ± 9	6
997	0·5	17,737	5	958	14,649	17 ± 4	4

* Precultured for 3 days before PHA added.

† These cultures contained mitomycin treated allogenic cells to mimic conditions of cultures containing Con A induced suppressor cells (see Methods).

‡ Increase in counts above c.p.m. from unstimulated cultures (Δ c.p.m.).

present. Table 5 details the results of these experiments and demonstrates that a wide range of doses of Con A can induce suppressor activity. The latter is more readily demonstrated with the lowest dose of PHA used.

Suppression of the response to PHA of precultured cells simultaneously exposed to allogenic cells precultured with concanavalin A

The above experiments were repeated with one change. The cells cultured with Con A and subsequently mitomycin treated were allogenic to the responder cells. The results are shown in Table 6. To control these experiments allogenic mitomycin treated but not Con A stimulated cells were added to the culture stimulated with PHA to allow subsequent comparison of cultures stimulated with PHA in the presence of Con A treated allogenic cells.

Con A stimulation of allogenic cells induced in the responding population suppressor capacities that were equal to that seen with autologous suppressor cells. The dose-dependent response to Con A and the easier suppression of cells stimulated with low doses of PHA were similar to the experiments using autologous cells. No allogenic barrier appears to exist for non-specific human suppressor cells.

Suppression of the response to PHA of fresh cells simultaneously exposed to allogenic cells precultured with concanavalin A

If preculturing cells allows them to escape from suppressor influences, it might be expected that the re-establishment of this influence by the addition of cells with suppressor capacity might more readily demonstrate suppressor cell activity than when such cells are added to fresh responder cells. To test this we repeated the experiments described above but used fresh responder cells.

Fresh responder cells were stimulated with PHA in the presence of Con A stimulated, mitomycin treated allogenic cells or autologous mitomycin treated cells. The results are detailed in Table 7. The suppression observed was equally as marked as that seen in experiments using precultured responder cells.

An important observation was made in the three experimental protocols described above. Twelve percent of normal subjects whose cells were assayed for suppressor capacity after stimulation for 3 days with Con A in any of the above systems, had suppressor indices 2 s.d. below the mean for the group. Such subjects in a clinical study would have been regarded as abnormal. Six subjects whose suppressor cell functions seemed abnormally low when assayed with each of the three doses of PHA used in the indicator system were reassessed with their cells stimulated for 4 instead of 3 days with Con A. Cells from all six subjects then exhibited suppressor function within the normal range. Conversely, many subjects whose cells were suppressing well after three days stimulation with Con A suppressed less well after four days stimulation. These observations resulted in the following experiments.

Effect of preculturing normal cells before attempting to induce suppressor cell activity and effect of varying the exposure time to concanavalin A

Peripheral blood lymphocytes were stimulated with Con A upon separation or placed in a bulk culture. Some of the stored but unstimulated cells were removed from the bulk culture each 24 hr for 7 days and stimulated with Con A. Suppressor activity of cells so stimulated was compared to the suppressor activity of autologous cells that were stimulated with Con A when fresh. Lymphocytes from seven subjects were studied, suppressor cell activity being induced with both 50 and 100 μg of Con A/ml and the cells added to allogenic cultures stimulated with 0·5 μg of PHA.

Suppressor activity could be induced in cells stored in culture media for 7 days but otherwise neglected. Daily analysis of the responses to Con A of cells stored in culture revealed marked day-to-day fluctuation in inducible suppressor activity. Frequently cells precultured for 24 or 48 hr were then less responsive to Con A as a stimulator of suppressor function than fresh cells but often by the third day of preculture cells were capable of responding better to Con A than fresh cells. The fluctuating nature of individual responses is clearly demonstrated in Fig. 2, which for the sake of clarity, includes only five subjects, however, the curves are typical. As the timing of the fluctuating responsiveness observed in different

TABLE 7. Suppression of the response of fresh cells to PHA by allogenic cells cultured with concanavalin A

Unstimulated control cells (c.p.m.)*	Dose of PHA (μg/ml)	Response to PHA (cpm)†‡	Dose of Con A inducing suppressor cells (μg/ml)	Unstimulated control cells with mitomycin treated Con A stimulated cells added	c.p.m. induced by PHA in cultures containing suppressor cells	Percentage suppression induced	Number of experiments
1927	10	33,683	200	1457	24,864	26±8	6
1380	10	29,316	100	663	21,126	28±4	6
1380	10	29,316	50	477	23,377	20±3	6
1380	10	29,316	25	447	20,569	29±3	6
1603	10	30,602	10	436	27,114	21±7	4
1927	2	29,138	200	1457	18,016	39±5	6
1380	2	27,927	100	663	17,290	38±11	6
1380	2	27,927	50	477	19,965	30±10	6
1380	2	27,927	25	447	20,416	27±4	6
1603	2	27,768	10	436	23,904	14±5	4
3197	0·5	6356	200	1613	948	85±17	6
1380	0·5	11,941	100	845	4940	59±17	5
1380	0·5	11,941	50	477	3087	74±11	6
1380	0·5	11,941	25	447	6622	45±16	6
806	0·5	5110	10	509	1704	65±17	3

* Precultured for 3 days before PHA added.

† These cultures contained mitomycin treated allogenic cells to mimic conditions of cultures containing Con A induced suppressor cells (see Methods).

‡ Increase in counts above c.p.m. from unstimulated cultures (Δ c.p.m.).

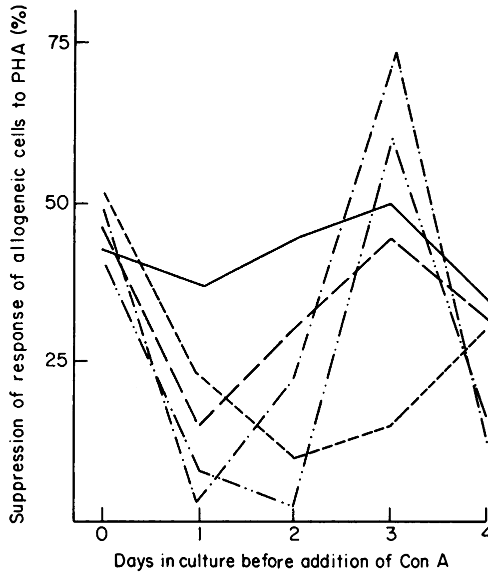

FIG. 2. Lymphocytes from five normal subjects were preincubated from 0 to 4 days before Con A (100 μg/ml) was added in an attempt to induce 'suppressor' cells. The Con A effect was assayed by addition of the 'suppressor' population to allogenic cells stimulated with PHA. The effectiveness of the Con A treated cells as suppressor cells fluctuated markedly with the time of preculture. Successful induction of suppressor activity seemed phasic, one cell population responding very differently within 24 hr periods. Clearly, study at any one time point alone may give misleading information re the potential for the regulatory activity of a given population.

individuals was often out of phase, summing the data does not reflect the magnitude of the variation. Similar fluctuations were noted by varying the length of time suppressor cells were incubated with Con A. Fresh cells were cultured with various doses of Con A in a bulk culture. Each day, commencing 24 hr after the addition of Con A, cells were removed and assayed for suppressor ability. While most cells were maximally suppressive after 72 hr with Con A, daily fluctuations occurred suggesting some internal

TABLE 8. Effect of dexamethasone on suppressor cells

	PHA used to stimulate responder cells (μg/ml)		
Cells compared	10	5	2
Con A* induced suppressor cells (n = 9)‡	27±4·9†	33±3·2	56±6·6
Con A induced suppressor cells with dexamethasone (n = 9)	10±5·6	11±3·2	19±7·5

* Suppressor cells induced by stimulation for three days with concanavalin A.

† Mean±1 s.d. of % suppression obtained.

‡ Number of experiments pooled to give data.

feedback system controlling the balance of the suppressor-nonsuppressor response to Con A. Maximum suppressor activity always occurred after either 3 or 4 days exposure to Con A. Poor suppression in 3 day cultures was always associated with good suppression in 4-day cultures. Good suppression could be obtained even with cells exposed to Con A for 7 days.

Effect of dexamethasone on suppressor cells

Treatment with 10^{-3} M dexamethasone significantly reduced ($P<0.001$) the ability of suppressor cells to influence responder cells (Table 8). The effect was most noticeable when responder cells were stimulated with suboptimal doses of PHA. Dexamethasone- and mitomycin-treated control cells neither enhanced nor suppressed the ability of responder cells to react to the PHA.

DISCUSSION

The present study gives rise to practical information and theoretical considerations that can be applied to the study of suppressor cell activity in humans. For clinical studies of immunoregulation the induction of suppressor cell function by antigens rather than mitogens may be more physiological. The use of antigen stimulation is, however, more difficult as the normal response to antigens is more variable than to mitogens and the longer time necessary for the responder cells to be in contact with antigens compared to mitogens raises further difficulties. The use of allogenic responder cells with antigens runs the risks of activating suppressor cells in the latter stages of the mixed lymphocyte reaction, while the use of autologous responder cells requires that either a subject returns for a second bleeding or that the responder cells are kept in culture for a number of days while suppressor cells are being generated.

From our studies it would appear that non-specific assays for suppressor cells can be made reliable and sensitive using the familiar response to PHA as an indicator system. The practical points to be noted are (1) allogenic responder cells are equally as sensitive as autologous responder cells; (2) there is no advantage gained by using precultured as opposed to fresh responder cells; (3) examining the effects of suppressor cells on a dose response curve constructed from the responses to three concentrations of PHA greatly increases the sensitivity of the assay; (4) to avoid false negative results suppressor cells should be generated with Con A for both 3 and 4 days; (5) the effects of steroids on suppressor cells can be significant and long lasting, a factor requiring careful consideration in studies of patients treated with steroids and probably other immunosuppressive agents.

While many studies of the effects of corticosteroids on human lymphocytes have been reported most have examined the effect of steroids added with mitogen or antigen to lymphocyte cultures. Clinically relevant studies examining the effect of steroids on committed human lymphocytes have been few and a recent study showed little effect of steroids on the response of lymphocytes stimulated for 48 hr with mitogen (Ramer & Yu, 1978). In contrast committed suppressor cells are very sensitive to the effects of dexamethasone used as described here. This finding is compatible with observations in animals that suppressor T cells are more sensitive than effector cells to alkylating agents and radiation (Thestrup-Pedersen, Dwyer & Askenase, 1977).

With suboptimal mitogen concentrations of PHA suppression in excess of 60% is reproducible in normal subjects. Such significant suppression with cells from normal subjects increases the chances of recognizing abnormalities in disease states. However, many clinical studies will be needed to determine the ability of this type of assay to reflect disturbed *in vivo* immunoregulation.

Apart from the practical considerations outlined above we believe our observations are of theoretical interest. Many biological systems are regulated on the 'tug of war' principle: two opposing forces supplying a mean effect that will vary with increases or decreases in the influence of either component. Such a system offers a mechanism for very sensitive regulation and may be utilized in immunoregulation.

Cell-to-cell interaction is a well known phenomenon in immunology and while such interactions may be mandatory the effect in many cases may be permissive rather than regulatory (Cantor & Asofsky, 1970). Thus, the required T–T interaction demonstrated for the generation of the graft versus host response (Wagner, 1973), helper cell response (Feldmann *et al.*, 1975) and delayed hypersensitivity

(Bullock, Katz & Benacerraf, 1975), may be examples of obligatory enhancing phenomena and only represent control mechanisms in that failure of this cell cooperation to occur will prevent an immunological response.

Suppressor cell activity may be much more subtle, however, and more closely mimic the 'tug of war' idea suggested above. The suppressor cell response appears to be dependent on T–T interaction (Feldmann & Kontiainen, 1976), but evidence is appearing which suggests that in cell populations that contain suppressor cells there may exist T amplifier cells.

In the immunoregulation of the humoral response to the thymus-independent antigen, type III pneumococcal polysaccharide, the lack of a requirement for conventional helper cells allowed both suppressor and amplifier T cells to be demonstrated (Baker *et al.*, 1974). A recent analysis in the mouse of Ly alloantigens and Ia antigens on T cell subpopulations suggested that an amplifier cell with an Ly-$1^+2^+3^+$ phenotype may, following antigenic stimulation, amplify either suppressor or effector responses (Feldmann *et al.*, 1977). The activation of helper or suppressor mechanisms depends on the degree of sensitization of the responding cell (Eardley, Staskowicz & Gershon, 1976). Poorly sensitized cells responding minimally will activate helper mechanisms, strongly sensitized cells responding accordingly will initiate suppressor mechanisms. The generation of two functionally distinct types of thymic derived regulator cells having opposing functions seems likely.

In the present studies the maintenance of human lymphocytes in culture was associated with a number of continually changing phenomena. The generally better survival of T cells is important and further analysis is needed to determine whether helper or suppressor T cells are dying disproportionately.

Fresh cells in culture may be capable of a degree of immediate responsiveness to PHA that is determined by opposing stimulation from suppressor and amplifying forces. Our data from studies of precultured cells shows a fluctuating but time-dependent increase in the magnitude of cellular DNA synthesis following stimulation with PHA. Similar observations have already been reported by Bresnihan & Jasin (1977) and Feighery *et al.* (1978). However, in our studies the rapidity of DNA synthesis as well as the magnitude of DNA synthesis in precultured cells was also noted to increase. It should be noted, however, that in most samples studied, 24 hr of preculture was frequently associated with decreased responsiveness of cells to PHA compared to these cells stimulated when fresh. Only after 24 hr of culture was the situation consistently reversed. Diminished responsiveness could result from the death of a large number of PHA-responsive cells but the later enhanced responses makes this unlikely. The first 24 hr of culture may be associated with the death of large numbers of suppressor cells that release suppressor lymphokines that inhibit PHA responses. While this may be partially true, subsequent experiments in which we noted the induction by Con A of suppressor cells from cells precultured for 4 days and later, proves that many potential suppressor cells survive.

Thus, many normal suppressor cells may be active in the first 24 hr of culture until a feedback loop temporarily suspends such activity. Certainly after a few days of culture without stimulation, inhibitory forces though still present and inducible, become less influential and cells released from such influences respond better to PHA. *In vivo* it is likely that periods without stimulation are rare and the continuation of active feedback cycles (suppression/amplification) may be antigen dependent. Spontaneously generated suppressor activity was occasionally observed among our precultured normal cells and such spontaneously induced suppression has been previously observed in animals (Dorr Burns *et al.*, 1975).

Our observation of fluctuating responsiveness when attempts were made to study the daily susceptibility of suppressor cells to stimulation by Con A supports this concept. Similarly, cells cultured for 24, 48, 72 and 96 hr with Con A demonstrated a fluctuating rather than linear increase in suppressor function over this period. As responses amongst individuals simultaneously studied were not always in phase, lack of good suppressor activity induced amongst given cells at a specific time point may not signify any abnormality.

A previous study of Con A induced suppressor cells amongst human peripheral blood lymphocytes found normal subjects in whom Con A did not induce suppressor cells (Shou *et al.*, 1976). We also have seen this phenomenon but all normal subjects studied serially did produce suppressor activity. We have reported, however, a consistent failure to induce suppressor cell activity with cells from patients with

multiple endocrinopathies associated with IgA deficiency and chronic mucocutaneous candidiasis (Dwyer, 1977). Clearly, the easiest and most reproducible assay of suppressor activity involved the suppression of cells stimulated with suboptimal doses of mitogen.

It seems clear that studies of immunoregulatory disorders will help clarify many immunopathogenetic mechanisms, but immunoregulation appears increasingly complicated. For example, attention to the possibility that there is in humans a phenotypically and functionally distinct group of amplifier cells seems appropriate. Disordered immunoregulation resulting in clinical disease may result from abnormal activity of this cell as well as the distinctive suppressor cell.

Studies of normal control mechanisms are essential for understanding physiological responses that can then in turn be compared to those suspected of being pathological.

J.M. Dwyer is an Investigator for the Howard Hughes Medical Institute with support from U.S. Public Health Service Grant AI 11785.

REFERENCES

BAKER, P.J., STASHAK, P.W., AMSBOUGH, D.F. & PRESCOTT, B. (1974) Regulation of the antibody response to type III pneumococcal polysaccharide. II. Mode of action of thymic-derived suppressor cells. *J. Immunol.* **112**, 404.

BENJAMIN, D.C. (1975) Evidence for specific suppression in the maintenance of immunological tolerance. *J. exp. Med.* **141**, 635.

BRESNIHAN, B. & JASIN, H.E. (1977) Suppressor function of peripheral blood mononuclear cells in normal individuals and in patients with systemic lupus erythematosus. *J. Clin. Inv.* **59**, 106.

BRODER, S., HUMPHREY, R., DRUM, M., BLACKMAN, M., MEADE, B., GOLDMAN, C., STROBER, W. & WALDMANN, T. (1975) Impaired synthesis of polyclonal (non-paraprotein) immunoglobulins by circulating lymphocytes from patients with multiple myeloma. Role of suppressor cells. *New Engl. J. Med.* **293**, 887.

BULLOCK, W.W., KATZ, D.H. & BENACERRAF, B. (1975) Induction of T lymphocyte responses to a small molecular weight antigen. III. T-T interaction to determinants linked together: suppression *vs* enhancement. *J. Exp. Med.* **142**, 273.

CANTOR, H. & ASOFSKY, R. (1970) Synergy among lymphoid cells mediating the graft *vs* host reaction produced by BALB/c lymphoid cells of different anatomic origins. *J. Exp. Med.* **131**, 253.

DORR BURNS, F., MARRACK, P.C., KOFFLER, J.W. & JANEWAY, C.A. JR (1975) Functional heterogeneity among T-derived lymphocytes of the mouse. IV. Nature of spontaneously induced suppressor cells. *J. Immunol.* **114**, 1345.

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

DWYER, J.M. (1977) Failure of Concanavalin A stimulated lymphocytes to suppress responsiveness to PHA and allogeneic cells as a marker of disordered immunoregulation. In: *Proceedings of the Eleventh Leucocyte Culture Conference*. D. O. Lucas edn, pp. 773–775. Academic Press, New York.

EARDLEY, D.D., STASKOWICZ, M.O. & GERSHON, R.K. (1976) Suppressor cells: Dependence on assay conditions for functional activity. *J. exp. Med.* **143**, 1211.

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.

FELDMANN, M., BEVERLY, P.C.L., WOODY, J. & MCKENZIE, I. (1977) T-T interactions in the induction of suppressor and helper T cells: analysis of membrane phenotype of precursor and amplifier cells. *J. exp. Med.* **145**, 793.

FELDMANN, M., ERB, P., KONTIAINEN, S. & DUNKLEY, M. (1975) The mechanism of induction of helper cells and suppressor cells *in vitro*. In: *Membrane Receptors of Lymphocytes* (ed. by M. Seligmann, J. L. Preud'houme & F. M. Kouritsky). North Holland Publishing Co., Amsterdam.

FELDMANN, M. & KONTIAINEN, S. (1976) Suppressor cell induction *in vitro*. II. Cellular requirements of suppressor cell induction. *Eur. J. Immunol.* **6**, 302.

GERBER, N.L., HARDIN, J.A. CHUSED, T.M. & STEINBERG, A.D. (1974) Loss with age in NZB/W mice of thymic suppressor cells in the graft vs host reaction. *J. Immunol.* **113**, 1618.

GERSHON, R.K. (1975) A disquisition on suppressor T cells. *Transplant. Rev.* **26**, 170.

GERSHON, R.K., MAURER, P.H. & MERRYMAN, C.F. (1973) A cellular basis for genetically controlled immunologic unresponsiveness in mice: tolerance induction in T cells. *Proc. natn. Acad. Sci. (Wash.)* **70**, 250.

JACOBSEN, F.B., HERZENBERG, L.A., RIBLET, R. & HERZENBERG, L.A. (1972) Active suppression of immunoglobulin allotype synthesis. *J. exp. Med.* **135**, 1163.

KINCADE, P.A., LAWTON, A. & COOPER, M.D. (1971) Restriction of surface immunoglobulin determinants to lymphocytes of the plasma cell line. *J. Immunol.* **106**, 1421.

MANGI, R.J., DWYER, J.M., GEE, B. & KANTOR, F.S. (1974) Immunological competence of subjects with sarcoidosis. *Clin. exp. Immunol.* **18**, 505.

MENDES, N.F., TOLNAI, M.E., SILVEIRA, N.P., GILBERTSON, R.B. & METZGAR, R.S. (1973) Technical aspects of the rosette tests used to detect human complement receptor (B) and sheep erythrocyte-binding (T) lymphocytes. *J. Immunol.* **111**, 860.

RAMER, S.J. & YU, D.T.Y. (1978) Effect of corticosteroids on committed lymphocytes. *Clin. exp. Immunol.* **32**, 545.

REDELMAN, D., SCOTT, C.B., SHEPPHARD, H.W. & SELL, S. (1976) *In vitro* studies of the rabbit immune system. V. Suppressor T cells activated by Concanavalin A block the proliferation, not the induction of antierythrocyte plaque-forming cells. *J. exp. Med.* **143**, 919.

RITZMAN, S.E. (1976) HLA patterning and disease associations. *J. Am. Med. Ass.* **236**, 2305.

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.

STOBO, J.D., PAUL, S., VAN SCOY, R.E. & HERMANS, P.E. (1976) Suppressor thymus-derived lymphocytes in fungal infection. *J. Clin. Inv.* **57**, 319.

THESTRUP-PEDERSEN, K., DWYER, J.M. & ASKENASE, P.W. (1977) Studies on the role of the thymus and T cells in the *in vivo* suppression of delayed hypersensitivity (desensitization): Radiosensitivity of the mechanism inducing non-specific anergy. *J. Immunol.* **118**, 1665.

VLADUTIN, A.O. & ROSE, N.R. (1974) HL-A antigens: Association with disease—a review. *Immunogenetics*, **1**, 305.

WAGNER, H. (1973) Synergy during *in vitro* cytotoxic allograft responses. Evidence for cell interaction between thymocytes and peripheral T cells. *J. exp. Med.* **138**, 1397.

WALDMANN, T.A., BRODER, S., BLAESE, R.M., DURM, M., BLACKMAN, M. & STROBER, W. (1974) Role of suppressor T cells in pathogenesis of common variable hypogammaglobulinemia. *Lancet*, **2**, 609.