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Ovine concanavalin A-induced suppressor cells: generation, assay, age-related effects and re-evaluation of mechanism of suppression

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Summary. Preactivation of ovine peripheral blood and lymph node mononuclear cells with mitogenic doses of concanavalin A (Con A) induced cells which suppressed mitogen-stimulated proliferative responses of untreated autologous and allogeneic responder cells. The degree of suppression varied with preactivating doses of Con A, length of preactivation time, and ratio of preactivated to responder cells. The role of macrophages in generation of suppressor cells was not evaluated. However, macrophages were not required to mediate suppression in cocultures, as lymphoblasts depleted of macrophage by plastic adherence and nylon wool columns mediated equal, and often greater, suppression than unseparated, preactivated cells. Suppressor cell activity in peripheral blood increased from the neonatal period to adulthood. Supernatants from Con A preactivated cell cultures with detectable interleukin-2 activity abrogated suppression when added at 0 and 24 hr of the 72 hr coculture period, suggesting that Con A-induced suppressor cells exert their function by decreasing available levels of IL-2 in the cocultures.

INTRODUCTION

The role of cells in the suppression of immune responses was first established by Gershon & Kondo (1970) in mice. Subsequently, non-specific suppressor cells have been induced in humans (Shou, Schwartz & Good, 1976), cattle (Smith *et al.*, 1981) and cats (Langweiler & Cockerell, 1982) by *in vitro* cultivation of mononuclear leucocytes with concanavalin A (Con A) and numerous other activators (Ellner, 1981). Similarly, *in vitro* incubation of lymphocytes in the absence of extraneous activators can result in the generation of suppressor cells (Lederman, Ellner & Rodman, 1981). Suppressor cells induced in these systems non-specifically inhibit a variety of both humoral and cellular responses, as demonstrable by several assays (Ellner, 1981; Smith *et al.*, 1981; Langweiler & Cockerell, 1982). Apparent phenotypic differences in suppressor cell subsets and variation in assay parameters have led to conflicting results regarding age-related levels of Con A suppressor cell activity (Miyawaki *et al.*, 1981; Kay & Makinodan, 1981).

While studies investigating the mechanisms of suppression observed in these *in vitro* systems have generally identified soluble factors produced by T lymphocytes as mediators (Rich & Pierce, 1974; Greene, Fleisher & Waldmann, 1981; Lederman *et al.*, 1981), the mechanisms and validity of the Con A-induced suppressor cell assay have been questioned on several accounts. Exaggerated helper effect in control cultures (Fernandez & McSween, 1980; Farrant & Newton, 1981), essential nutrient depletion (Maca, Bonnard & Herberman, 1979), interleukin-2 depletion (Palacion & Möller, 1981) and cytotoxicity arising in prolonged cell cultures (Fineman, Medawar & Geha, 1979) have all been posited as mechanisms that could mimic active suppression by soluble factors. Resolution of these issues is central to the interpreta-

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tion of the assay as a basis for the assessment of lymphocyte subset function.

The proliferative response of ovine peripheral blood lymphocytes to mitogen stimulation is well characterized (Burrells & Wells, 1977; Fiscus, DeMartini & Pearson, 1982). To date, there have been no reports detailing the regulatory functions of ovine T cells. The following study was undertaken to assess the applicability of the Con A-induced suppressor cell assay to the ovine system as a basis for the study of immunoregulatory function in sheep.

MATERIALS AND METHODS

Animals

Thirty-six clinically normal cross-bred sheep of both sexes and known ages were used in this study.

Isolation of mononuclear leucocytes from peripheral blood

Mononuclear leucocytes were isolated from heparinized blood (10 U/ml blood) using Ficoll-Hypaque (FH) specific gravity 1·077; Sigma, Winthrop Labs, New York, NY) density centrifugation (Fiscus *et al.*, 1982) and suspended in RPMI 1640 culture medium (Grand Island Biological Co., Grand Island, NY) supplemented with L-glutamine (2 mM/ml), gentamicin (100 μg/ml) and 10% fetal bovine serum (FBS) (referred to below as supplemented RPMI-1640). This method of cell separation consistently yielded more than 95% mononuclear cells with more than 98% viability, as judged by trypan blue dye exclusion.

Isolation of mononuclear cells from lymph nodes

Fresh, aseptically-collected lymph nodes were placed in cold Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS), trimmed, minced and passed through a 60-mesh stainless steel sieve. The resulting suspension was diluted with HBSS, layered over FH and centrifuged for 30 min at 200 *g* at 20°. The interface mononuclear cell layer was washed and resuspended in supplemented RPMI-1640. This method effectively removed dead cells and debris.

Isolation of surface immunoglobulin-negative (sIg−) cells

SIg− cells were isolated by passage of mononuclear cell suspensions through nylon wool columns (Cahill *et al.*, 1978). Cells in the effluent population were consistently greater than 90% sIg−.

Identification of surface immunoglobulin-positive (sIg+)) lymphocytes

SIg^+ cells were labelled with fluorescein isothiocyanate (FITC)-conjugated anti-sheep IgG antisera (Miles-Yeda, Rehovot, Israel) using a modification of a previously described technique (Mishell & Shiigi, 1980) and examined for surface fluorescence using a microscope equipped with epifluorescence, counting a minimum of 200 cells.

Identification of macrophages with neutral red

Macrophages were identified using a previously described method (Smith *et al.*, 1981). Briefly, 20 μl of cell suspension (6×10^6 cells/ml) were added to microslides coated with neutral red, cover slips were applied, and the slides were incubated for 15 min in a humidified 37° incubator. Macrophages were identified by light microscopy as mononuclear cells containing numerous red-brown cytoplasmic granules. A minimum of 200 total cells were counted to determine percentages.

Con A activation of lymphocytes

Lymphocyte suspensions (4×10^6 cells/ml) were added to 25 cm^2 flasks (Corning, Corning, NY) with variable doses of Con A (Sigma Chemical Co., St Louis, MO). Control lymphocytes (media preactivated) similarly prepared without Con A. After 48 hr at 37° and 4% CO_2, lymphocytes were washed twice in HBSS containing α-methyl-D-pyranoside (HBSS-Me) (20 mg/ml) (Sigma Chemical Co.). Pellets were then resuspended in 1 ml of HBSS-Me with mitomycin C (25 μg/ml) (Sigma Cemical Co.). After 1 hr incubation 37° in 4% CO_2, lymphocytes were washed three times in HBSS and resuspended at 4×10^6 cells/ml in supplemented media. This procedure consistently yielded viable lymphoblasts (>95% excluded trypan blue) at a recovery rate of 20–30% of the original cultured population. Supernatants from Con A-stimulated cultures were filter sterilized (0·2 μm filter) and stored at −70° until further evaluated for interleukin-2 (IL-2) activity (Ellis & DeMartini, 1984).

Mitogenic assay

Fresh responder lymphocytes were separated from peripheral blood as described above and resuspended in supplemented RPMI (2×10^6 cells/ml). Varying concentrations of suppressor cells or control cells were added to equal volumes of responders, and 200 μl of the mixed population were dispensed into 96-well flat-bottomed plates (Falcon Microtest II, Oxnard,

CA). Con A (10 μg/ml), PHA-P (5 μg/ml). PWM (25 ug/ml) (Grand Island Biological Co.) and LPS (20 μg/ml) Difco Lab, Detroit, MI) were delivered in 20-μl volumes to triplicate wells. Supplemented RMPI-1640, 20 μl, was added to wells containing no mitogen for the assessment of background proliferation. Cultures were incubated for 3 days at 37° in 4% CO_2. Eighteen hours before harvesting, 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA) (6·7 Ci/mM) was added to each well. The cells were harvested onto glass fibre filter paper (Grade 934 AH, Whatman, Clifton, NJ) with a semiautomatic cell harvester (Otto Hiller Co., Madison, WI). The dry filter discs were counted in 2 ml of toluene-based scintillation fluid (4 g/l PPO, 50 mg/l POPOP).

The amount of suppression was determined using the means of triplicate cultures and the following formula:

$$S = 1 - \frac{(cmCa - CCA)}{(Cm - C)} \times 100$$

Where CmCA = mean counts per minute (c.p.m.) of responders with Con A-activated cells and mitogens; CCA = mean of responders with Con A-activated cells and no mitogen; Cm = mean c.p.m. of responders with control cells and mitogen; C = mean c.p.m. of responders with control cells and no mitogen (Smith *et al.*, 1981).

Statistical methods

One-way analysis of variance was used to determine if significant differences existed among levels of suppression among various age-based populations of animals, doses of Con A used in suppressor cell generation, and preactivation times. Results within these groups were compared using a two-tailed Student's *t*-test.

RESULTS

Suppression of the proliferative responses of autologous and allogeneic cells by Con A-preactivated cells

Peripheral blood mononuclear leucocytes (PBML) which had been preactivated with a mitogenic concentration of Con A (50 μg/ml) for 48 hr and treated with mitomycin C were cocultured with autologous freshly harvested PBML at a ratio of 2:1. There was marked suppression of proliferative responses to Con A, PHA and PWM in cocultures containing Con A-preactivated cells, compared to cocultures of responders and media-preactivated (without Con A) cells (Table 1). Proliferative responses to LPS were enhanced (Table 2). Suppression of Con A, PHA and PWM stimulated responses was also observed in allogeneic suppressor/-responder cocultures from animals shown to be histo-incompatible by a positive-mixed lymphocyte reaction (MLR) (Table 1). Con A-preactivated cells irradiated with 2000 rads were capable of mediating suppression to levels correspondent with mitomycin C-treated cells (data not shown).

Reproducibility of suppression of mitogenic response in autologous and allogeneic combinations was also demonstrated (Table 3).

Con A-preactivated cells from lymph node mononuclear leucocytes (LNML) mediated similar levels of suppression of mitogen-induced responses in allogeneic PBL (data not shown). All blastogenic activity in cocultures was attributable to responder lymphocytes, since mitogen stimulation of mitomycin C-treated Con A or media-preactivated cells resulted in background (< 2000 c.p.m.) levels of [^{3}H]thymidine incorporation (data not shown). Addition of either autologous or allogeneic media-preactivated, mitomycin C-treated control cells to responders usually resulted in enhanced [^{3}H]thymidine incorporation by responders when compared to responders alone. However, [^{3}H]thymidine uptake in cocultures containing suppressors was consistently lower than either cocultures with responders and control cells, or responders alone.

Conditions for optimal generation and assay of Con A-induced suppressor cells

There were significant differences ($P < 0{\cdot}001$) in levels of suppression obtained by varying the dose of Con A added to 4×10^6 cells/ml at the beginning of a constant 48 hr preactivation (Fig. 1). The differences between 5 and 10 μg/ml was not statistically significant ($P < 0{\cdot}19$). Significant increases in suppression were achieved by increasing the preactivating dose from 10 μg/ml to 20 μg/ml ($P < 0{\cdot}03$), from 20 μg/ml ($P < 0{\cdot}003$) and from 50 μg/ml to 100 μg/ml ($P < 0{\cdot}09$). Lymphocyte blastogenic responses to Con A, as determined by [^{3}H]thymidine uptake, increased in a similar dose-dependent fashion (data not shown).

The effect of variable preactivating time on suppression was determined by incubation of 4×10^6 PBML/ml with 50 μg/ml Con A or no mitogen (control) for 24, 48, 72 or 96 hr, followed by coculture with autologous or allogeneic responders for 72 hr. Significant differences ($P < 0{\cdot}03$) were observed among

Table 1. Suppression of autologous and allogeneic proliferative responses

Suppressor animal no.	Con A-induced cells	Responder animal no.	Mitogenic assay* (c.p.m. ± SD)†			Mitogenic assay (c.p.m. ± SD)		Mitogenic assay (c.p.m. ± SD)	
			0‡	Con-A (50 μg/ml)	%S§	PHA-P (5 μg/ml)	%S	PWM (25 μg/ml)	%S
752	–	752	3110 ± 1080	188,609 ± 710	61	128,741 ± 5346	68	114,776 ± 12,019	53
	+		2858 ± 75	75,241 ± 2390		65,482 ± 8362		55,571 ± 1281	
752	–	867	5292 ± 904	208,227 ± 8939	60	153,568 ± 11,312	61	129,539 ± 11,858	53
	+		12,895 ± 2956	93,969 ± 894		70,546 ± 4130		71,864 ± 1201	
867	–	867	2112 ± 213	112,266 ± 13,783	51	78,144 ± 8013	48	50,400 ± 860	33
	+		6683 ± 712	61,044 ± 9400		46,388 ± 4773		38,873 ± 4019	
867	–	756	7597 ± 613	160,124 ± 38,096	51	120,082 ± 15,092	44	124,634 ± 10,305	64
	+		8876 ± 812	83,775 ± 5124		71,556 ± 18,105		55,233 ± 11,966	
860	–	860	3252 ± 1546	168,079 ± 4854	69	139,901 ± 2572	69	112,843 ± 10,606	67
	+		2349 ± 412	53,252 ± 3866		44,814 ± 5111		38,448 ± 3185	
860	–	867	8959 ± 1771	200,396 ± 9168	64	179,720 ± 21,069	70	140,496 ± 8786	71
	+		6120 ± 390	76,064 ± 6757		58,234 ± 11,197		43,362 ± 4974	

* Suppressors, 4×10^5, and responders, 2×10^5, cocultured for 72 hr.
† Counts per minute ± SD calculated on the basis of means of triplicate cocultures.
‡ Background used for all mitogens.
§ S = suppression.

Table 2. Enhancement of responses to LPS

Suppressor animal no.	Con A-induced cells	Responder animal no.	Mitogenic assay* (c.p.m. ± SD) 0	LPS (20 μg/ml)	%S†
121	−	121	3294 ± 274	28,109 ± 6962	−152
	+		4422 ± 207	66,877 ± 4269	
121	−	123	2958 ± 111	49,125 ± 9590	−334
	+		4220 ± 52	162,635 ± 13,802	
123	−	123	1277 ± 790	29,708 ± 3788	−321
	+		1642 ± 136	121,399 ± 10,175	
123	−	121	3886 ± 1178	39,527 ± 6285	−114
	+		10,162 ± 2451	86,313 ± 8207	
751	−	757	2539 ± 1018	11,544 ± 3601	−334
	+		3426 ± 955	42,460 ± 726	
751	+	751	4749 ± 2227	20,081 ± 1627	−130
	−		7314 ± 1691	42,579 ± 5052	

* Suppressors, 4×10^5, and responders, 2×10^5, cocultured for 72 hr.
† S = suppression.

Table 3. Reproducibility of suppression

Suppressor no.	Responder no.	Δ c.p.m. [(cocultured cells + mitogen) − (cocultured cells + no mitogen)]* Media-preactivated cells	Con A (50 μg/ml). preactivated cells	%S†
123	121	194,248	40,689	79
123	123	78,300‡	23,317	70
123	124	160,574	19,257	88
867	123	128,983	17,551	87
867	867	152,527	74,899	51
867	867	148,633	42,548	71
38	38	130,872	14,350	89
38	5	61,647‡	23,898	61
38	867	153,832	26,540	83
121	123	129,804	101,345	22
121	121	59,612‡	36,317	39
121	121	98,521	41,335	58

* Calculated on basis of means of triplicate cocultures of 2×10^5 suppressors or controls, and 2×10^5 responders and 50 μg/ml Con A.
† S = suppression.
‡ Coculture of 2×10^5 suppressors or controls with 1×10^5 responders.

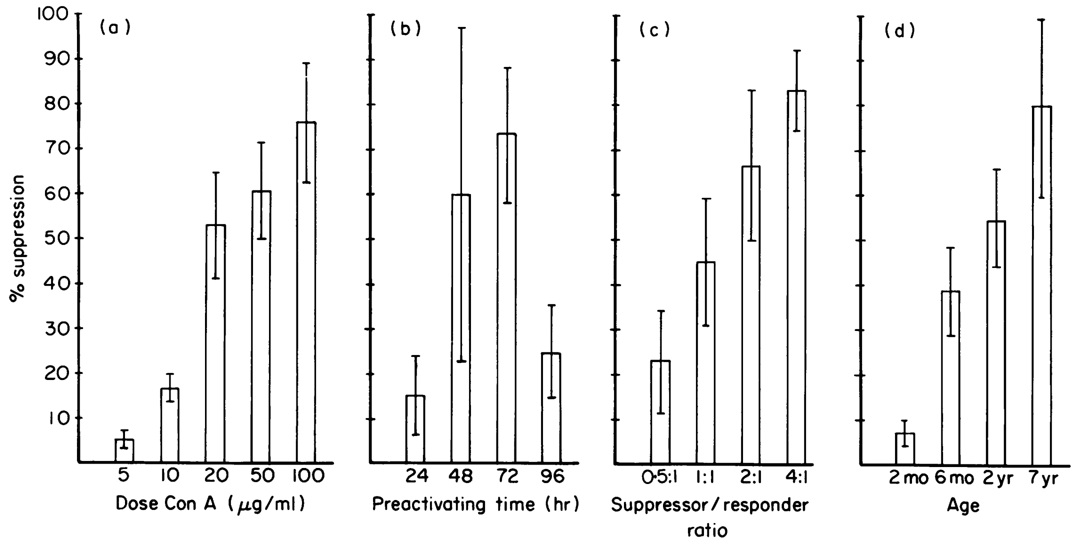

Figure 1. Factors affecting levels of Con A-induced suppressor cell activity. (a) Suppressors (2×10^5 cells) preactivated with variable doses of Con A for 48 hr were cocultured with autologous responders (2×10^2 cells) and 50 μg/ml Con A for 72 hr ($n=5$); (b) suppressors (2×10^2 cells) preactivated with 50 μg/ml Con A for variable times were cocultured with autologous responders and 50 μg/ml Con A for 72 hr ($n=3$); (c) variable concentrations of suppressors (5×10^4–4×10^5 cells) preactivated with 50 g/ml Con A for 48 hr were cocultured with autologous responders (1×10^5 cells) and 50 g/ml Con A for 72 hr ($n=5$); (d) suppressors (2×10^5 cells) preactivated with 50 μg/ml Con A for 48 hr from animals of various ages were cocultured with autologous responders (2×10^5 cells) and 50 μg/ml Con A for 72 hr (2 mo. $n=6$, 6 mo. $n=5$, 2 yr $n=10$, 7 yr $n=3$).

different preactivation times (Fig. 1), with increases in suppression noted following 48 hr ($P<0{\cdot}07$) and 72 hr ($P<0{\cdot}002$) incubation periods when compared to a 24 hr preactivation period. No significant differences were found between 24 hr and 96 hr ($P<0{\cdot}22$), and 48 hr and 72 hr ($P<0{\cdot}23$) incubation periods.

In order to determine the optimal ratio of Con A-preactivated cells required to mediate suppression, variable numbers of Con A- or media-preactivated cells were cocultured with 1×10^5 autologous PBML responders (Fig. 1). Significant increases in suppression ($P<0{\cdot}001$) were noted with increasing suppressor/responder ratios from 0·5/1 to 1/1 ($P<0{\cdot}001$), 1/1 to 2/1 ($P<0{\cdot}005$) and from 2/1 to 4/1 ($P<0{\cdot}05$) (Fig. 1). This effect was not simply due to an increase in cell numbers per well, as equivalent proliferation of responders occurred in control cocultures irrespective of the ratio of control cells.

Role of macrophages in mediating suppression

PBML which had been preactivated with Con A for 48 hr were harvested and treated with mitomycin C as above. A portion of the treated, Con A-preactivated cells were then passaged through nylon wool columns, and the percentages of surface immunoglobulin-bearing cells (B cells) and neutral red-containing cells (monocyte/macrophages) were determined in aliquots of this population, counting 200 cells. Similar or greater levels of suppression were observed in cocultures of responders with non-plastic adherent nylon wool separated cells, compared to preactivated unseparated suppressor cells, suggesting that suppression is mediated by a nylon wool non-adherent cell, presumably a T cell (Table 4).

Con A-induced suppressor cells as a function of age

As indicated in Fig. 1, significant differences were found among the different age groups of animals tested in this study ($P<0{\cdot}001$). There was a progressive increase in the level of Con A-generated suppressor cell activity in peripheral blood with increasing animal age. In each case, there was a significant difference noted between groups: 2 months to 6 months ($P<0{\cdot}002$), 6 months to 2 years ($P<0{\cdot}025$), 2 years to 7 years ($P<0{\cdot}010$).

Table 4. Absence of a macrophage requirement for the mediation of suppression

Exp.	Source of cells	% sIg+	% Neutral red +	Mitogen assay*		
				No mitogen	Con A (50 μg/well)	%S†
1	Fresh PBL	21	5			
	Unseparated control			1602±343	81,581±1797	
	Unseparated suppressor	6	0	1814±3	59,248±7065	28
	Separated suppressor	1	0	1140±93	18,906±3	78
2	Fresh PBL	18	6			
	Unseparated control			22,873±42	260,519±10,831	
	Unseparated suppressor	5	0	13,388±2521	197,252±1324	24
	Separated suppressor	1	0	14,417±2239	193,748±1832	26
3	Fresh PBL	20	5			
	Unseparated control			1803±385	58,425±6264	
	Unseparated suppressor	6	0	1838±507	27,083±551	55
	Separated suppressor	2	0	1106±446	11,798±2196	81

* Suppressors, 4×10^5, and responders, 2×10^5, cocultured for 72 hr.
† S = suppression.

Mechanism of suppression: evidence for the role of Interleukin-2 (IL-2)

In order to examine the possibility that one mechanism by which Con A-preactivated cells mediate suppression in this assay is via decreasing the availability of IL-2, lymphocyte-conditioned medium (LyCM) containing IL-2 activity was added to various ratios of suppressor/responder cocultures. Significant abrogation of suppressor activity ($P < 0{\cdot}05$), as determined by increased [^{3}H]thymidine incorporated by responders, was observed when a source of IL-2 was added to different coculture combinations at the beginning of the 72-hr coculture period (Table 5). Addition of IL-2 at 48 hr of the coculture period was less effective in abrogating suppression. A one-half media change at 48 hr did not result in the abrogation of suppression. The addition of complete media with 50 μg/ml Con A (equivalent to the dose of Con A in LyCM) did not abrogate suppression (data not shown).

In order to test whether IL-2 abrogation of T-cell suppression could result from binding of IL-2 to receptors on suppressor cells, Con A-preactivated, mitomycin C-treated cells were preincubated with LyCM for 4 hr at 37° and, after washing the cells, were tested for their ability to suppress in the coculture assay. Preincubation of Con A-preactivated suppressor cells with IL-2 containing LyCM resulted in a reduction of their capacity to suppress Con A-stimulated mitogenic responses (Table 5). This reduction in suppressor cell activity was not demonstrated in Con A-preactivated suppressor cells which were incubated under identical conditions in supplemented RPMI instead of LyCM. Conversely, LyCM in which mitomycin C-treated suppressor cells had been preincubated showed a decreased capacity to abrogate the suppression observed in suppressor/responder cocultures. Suppression in these cocultures was not due to the carry-over of mitomycin C, as samples of supplemented media which had been similarly incubated with mitomycin C-treated suppressors did not inhibit blastogenesis when used in culture with responders alone.

DISCUSSION

This study demonstrates that preactivating ovine peripheral blood and lymph node mononuclear cells with Con A induces a population of lymphocytes to suppress the mitogenic response of autologous and allogeneic cells to Con A, PHA and PWM, but not to LPS. Similar to other species, PHA and Con A are T-cell mitogens in sheep (Burrells & Wells, 1977; Jun, Johnson & Mills, 1979). PWM has been shown to stimulate T and B cells in cattle (Smith *et al.*, 1981), while LPS is a T-cell independent B-cell mitogen (Waldmann & Broder, 1982). These results suggest that Con A-induced suppressor cells may be directed primarily against the T-cell system. Suppression of B-cell responses in this assay (Miyawaki *et al.*, 1981)

Table 5. Effect of lymphocyte conditioned medium with interleukin-2 activity on suppressor cell function

Experiment no.	1		2		3	
SUP* source (sheep no.)	121 (2×10^5 cells)		53 (1×10^5 cells)		38 (2×10^5 cells)	
RES† source (sheep no.)	121 (2×10^5 cells)		53 (1×10^5 cells)		860 (2×10^5 cells)	
LyCM‡ source (sheep no.)	124		860		125	
	c.p.m. $\times 10^{-3}$	%S§	c.p.m. $\times 10^{-3}$	%S	c.p.m. $\times 10^{-3}$	%S
CON¶¶ + RES no mitogen	3·9		2·2		7·1	
CON + RES + mitogen	245·0		67·7		118·2	
SUP + RES no mitogen	2·3		2·4		2·9	
SUP + RES + mitogen	86·4	65	44·0	36	68·5	41
SUP + mitogen + 50% LyCM 0 hr	263·9	−9**	66·4	2**	126·6	−7**
SUP + RES + mitogen + 25% LyCM 0 hr	251·5	−3	53·5	2		
SUP + RES + mitogen + 50% LyCM 48 hr	99·3	60	52·7	23		
SUP + RES + mitogen + 50% media 48 hr	81·4	67	41·7	40		
SUP(preincub. w/ LyCM) + RES + mitogen					130·4	−3
SUP(preincub. w/ media + RES + mitogen					92·9	24
SUP + RES + mitogen + 50% LyCM (preincub. w/SUP					67·7	50
RES + mitogen + 50% media (preincub. w/SUP)					112·8	

* SUP = suppressor cells
† RES = responder cells.
‡ LyCM, lymphocyte conditioned medium (with interleukin-2 activity).
§ %S = suppression.
¶¶ CON = control cells.
** *P* values = 0·05 (comparing c.p.m. of triplicate cultures of suppressors and responders with and without the addition of LyCM).

may be an indirect manifestation of suppression of regulatory (helper) T cells. This suppression of mitogenic responses of autologous, as well as allogeneic, combinations suggests that the mechanism of suppression in this assay is not genetically restricted. The degree of suppression was directly related to preactivating mitogen concentration and suppressor/responder ratio in coculture. A greater degree of suppression was mediated by cells preactivated for 48 or 72 hr compared to a 24 or 96 hr preactivation period.

Reproducible levels of suppression were measured at different points in time from different animals. Similar studies in humans (Ratliff, McCool & Catalona, 1982) suggest that temporal variability can be reduced by reporting the data in terms of a peak suppression value for multiple sets of conditions.

Levels of Con A-induced suppressor cell activity increased with increasing age of the donor, as reported in other species (Kay & Makinodan, 1981). Others (Jun *et al.*, 1979) have reported a similar age-dependent increase in T-cell mitogen-induced blastogenic responses in sheep. Con A- and PHA-induced lymphocyte proliferation is well developed in the newborn human (Schecter *et al.*, 1977). Miyawaki *et al.* (1981)

implicated deficient lymphokine production by neonatal lymphocytes (Eife *et al.*,1974) to explain decreased ability to generate Con A-induced suppressor cells.

Previous studies suggest that macrophages are not required for either mitogen-induced proliferation (Usinger, Smith & Splitter, 1981) or the generation of Con A-induced suppressor cells in cattle (Smith *et al.*, 1981). While the requirement for accessory cells in the generation of ovine Con A-induced suppressor cells was not directly examined in this study, the apparent necessity for small numbers of macrophages in ovine lectin-induced blastogenesis (DeMartini, Fiscus & Pearson, 1983) suggests that complete removal of macrophages from preactivation cultures would result in the failure to generate Con A-induced suppressor cells. Macrophages have been shown to mediate suppressor functions following 3–5 days in culture (Rollwagen & Stutman, 1981). The failure to identify neutral red-positive cells (macrophages) and surface immunoglobulin cells (B cells) following successive plastic and nylon wool adherence suggests that the effector cell in the Con A-preactivated cell population is a nylon wool non-adherent T cell, as in human (Sakane & Green, 1977) and bovine (Smith *et al.*, 1981) systems.

Mitomycin C or irradiation are commonly employed in the Con A-induced suppressor cell assay to prevent DNA replication in control and suppressor cells, distinguishing the proliferation in responder cells. In this system and others (Smith *et al.*, 1981; Herscowitz *et al.*, 1980), Con A-induced suppressor cells were functional after both irradiation and mitomycin C treatment, demonstrating that cell division is not necessary for expression of the regulatory effect. In contrast, the demonstration of radiation sensitivity (Tsokos, Christian & Balow, 1982) and dependency on DNA or protein synthesis for both induction and activity of Con A-induced suppressor cells (Fineman *et al.*, 1979; Rich & Pierce, 1973), suggest heterogeneity in suppressor cell populations or mechanisms of suppression.

Fernandez & MacSween (1980) reported that coculture of responders with media-preactivated cells yielded heightened [^{3}H]thymidine uptake in the responding cells, compared to equivalent lower levels of proliferation in responders cultured alone or in coculture with Con A-preactivated cells, suggesting that, rather than suppression, media preactivation resulted in the generation of enhancing capability. This enhancing effect could be the result of a loss of a labile population of suppressor cells (Bresnihan & Jasin, 1977), or altered self-regulatory activity in responding cells as a result of interactions with preactivated cells. In the studies reported here, cells cocultured with Con A-preactivated cells generally incorporated less [^{3}H]thymidine than responding cells alone, thus suggesting that some suppression occurs, despite a degree of enhancement in control cocultures.

Farrant & Newton (1981) suggested that the observed suppressive effects in cocultures of responders with preactivated cells is a function of cellular concentration and the ability to provide 'helper' functions. The abrogation of suppression by the addition of lymphocyte-conditioned medium with demonstrable IL-2 activity lends credence to this argument. In the absence of increased cytotoxicity in Con A-preactivated cocultures (Fineman *et al.*, 1979), suppression in this assay is likely to be a manifestation of reduced helper function, resulting from depletion of IL-2, by Con A-stimulated lymphoblasts expressing IL-2 receptors (Robb, Munck & Smith, 1981) as previously suggested (Palacion & Möller, 1981). The failute to functionally demonstrate suppressor factors in the supernatants from Con A-stimulated ovine and feline lymphocytes (Langweiler & Cockerell, 1982) further support this mechanism. These results do not preclude the existence of such factors (Jegasothy & Battles, 1979; Greene, Fleisher & Waldmann, 1981), but question their role in this commonly used assay.

These and similar results (Palacion & Möller, 1981) provide alternative interpretations of this assay and proposed mechanisms of *in vitro* and *in vivo* suppression. Conservatively, this assay provides a means of evaluating the responsiveness of T-cell subsets, probably with particular reference to the capacity to express IL-2 receptors. Recent evidence, involving a more endocrinological view of the immune system, suggests that suppression of immune responses may be involved with the changing sensitivities of the populations of cells affecting responses, in concordance with receptor-mediated modulation of growth factor availability (Robb *et al.*, 1981).

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REFERENCES

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. Invest.* **59,** 106.

BURRELLS C. & WELLS P.W. (1977) *In vitro* stimulation of ovine lymphocytes by various mitogens. *Res. Vet. Sci.* **23,** 84.

CAHILL R., POSKITT D.C., FROST H., JULIUS M.H. & TRNKA Z. (1978) Behavior of sheep-immunoglobulin bearing and non-immunoglobulin bearing lymphocytes isolated by nylon wool columns. *Int. Archs. Allergy appl. Immun.* **57,** 90.

DEMARTINI J.C., FISCUS S.A. & PEARSON L.D. (1983) Macrophages in efferent lymph of sheep and their role in lectin induced lymphocyte blastogenesis. *Int. Arch. Allergy appl. Immunol.* **72,** 110.

EIFE R.F., EIFE G., AUGUST C.S., KUHRE W.L. & STAEHR JOHANSEN K. (1974) Lymphotoxin production and blast cell transformation by cord blood lymphocytes: dissociated lymphocyte function in newborn infants. *Cell. Immunol.* **14,** 435.

ELLIS J.A. & DEMARTINI J.C. (1984) Ovine interleukin 2: partial purification and assay in normal sheep and sheep with ovine progressive pneumonia. *Vet. Immunol. Immunopathol.* (in press).

FARRANT J. & NEWTON C. (1981) Relative ability to provide help: an explanation for Con A-induced suppression. *Clin exp. Immunol.* **45,** 504.

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

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

FISCUS S.A., DEMARTINI J.C. & PEARSON L.D. (1982) Mitogen-induced blastogenesis of peripheral blood and efferent lymphocytes from sheep. *Am J. vet. Res.* **43,** 629.

GERSHON R.E. & KONDO. K. (1970) Cell interactions in the induction of tolerance. The role of thymic lymphocytes. *Immunology,* **18,** 723.

GREENE W., FLEISHER T. & WALDMANN T. (1981) Soluble supernatants elaborated by concanavalin A-activated human mononuclear cells. I. Characterization of a soluble suppressor of T cell proliferation. *J. Immunol.* **126,** 1185.

HERSCOWITZ H., SAKANE T., STEINBERG A. & GREEN I. (1980) Heterogeneity of human suppressor cells induced by Concanavalin A as determined in simultaneous assays of immune function. *J. Immunol.* **124,** 1403.

JEGASOTHY B & BATTLES D. (1979) Immunosuppressive factors. I. Purification of inhibitor of DNA synthesis to homogeneity. *J. exp. Med.* **150,** 662.

JUN M.H., JOHNSON R.H. & MILLS J.M. (1979) *In vitro* response of lymphocytes of normal and ovine squamous cell carcinoma-bearing sheep to phytomitogens and tumor extracts. *Res. Vet. Sci.* **27,** 144.

KAY M.B. & MAKINODAN T. (1981) Relationship between aging and the immune system. *Prog. Allergy,* **29,** 134.

LANGWEILER M. & COCKERELL G.L. (1982) Generation of Concanavalin A-induced suppressor cells in the cat. *Int. Arch. Allergy appl. Immun.* **69,** 148.

LEDERMAN M., ELLNER J. & RODMAN H. (1981) Generation of a soluble immune response suppressor factor (IRSF) by unstimulated leukocytes of healthy subjects. *Clin. exp. Immunol.* **45,** 191.

MACA R.D., BONNARD G.D. & HERBERMAN R.B. (1979) The suppression of mitogen and alloantigen-stimulated peripheral blood lymphocytes by cultured human T lymphocytes. *J. Immunol.* **123,** 246.

MAWAS C., CHARMONT D., CONROY A. & SASPORTES M. (1977) Suppression of generation of human cytotoxic effectors by lectins or lectin activated peripheral blood lymphocytes. *Eur. J. Immunol.* **7,** 11.

MISHELL B.B. & SHIIGI S.M. (eds) (1980) *Selected Methods in Cellular Immunology,* p. 20. W. H. Freeman and Co., San Francisco.

MIYAWAKI T., MORIYA N., NAGAOKI T & TANIGUCHI N. (1981) Maturation of B-cell differentiation ability and T-cell regulatory function in infancy and childhood. *Immunol. Rev.* **57,** 61.

PALACION R. & MÖLLER G. (1981) T cell growth factor abrogates Concanavalin-A induced suppressor cell function. *J. exp. Med.* **153,** 1360.

RATLIFF T.L., MCCOOL R.E. & CATALONA W.J. (1982) Optimum conditions for the reproducible measurement of concanavalin A-activated suppressor cell activity. *Transplantation,* **33,** 505.

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

RICH R.R. & PIERCE C.W. (1974) Biological expressions of lymphocyte activation. III. Suppression of plaque-forming cell responses *in vitro* by supernatant fluids from concanavalin A-activated spleen cell cultures. *J. Immunol.* **42,** 1360.

ROBB R.J., MUNCK A. & SMITH K. (1981) T cell growth factor receptors: quantitation, specificity, and biological relevance. *J. exp. Med.* **154,** 1455.

ROLLWAGEN F.M. & STUTMAN O. (1981) Culture generated suppressor cells: evidence for an adherent cell component. *Cell. Immunol.* **64,** 371.

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

SCHECTER B., HANDZEL Z.T., ALTMAN Y., NIR E. & LEVIN. (1977) Cellular immunity in newborn infants and children: stimulation of lymphocyte protein synthesis as a measure of immune competence. *Clin. exp. Immunol.* **27,** 478.

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

SMITH W.G., USINGER W.R. & SPLITTER G.A. (1981) Bovine Con A induced suppressor cells: generation, macrophage requirements and possible mechanisms of regulatory action. *Immunology,* **43,** 91.

TSOKOS G.C., CHRISTIAN C.B. & BALOW J.E. (1982) Concanavalin-induced suppressor cells: characterization on the basis of corticosteroid and radiation sensitivity. *Immunology,* **47,** 85.

USINGER W.R., SMITH W.G. & SPLITTER G.A. (1981) Bovine T cells do not require auxiliary cells for response to selected mitogens. *Vet. Immunol. Immunopathol.* **2,** 381.

WALDMAN T.A. & BRODER S. (1982) Polyclonal B-cell activators in the study of the regulation of immunoglobulin synthesis in the human system. *Adv. Immunol.* **32,** 1.