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The effect of retinoids on dendritic cell function

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SUMMARY

The effect of retinoid administration on the antigen presenting function of mouse dendritic cells (DC) was assessed. Culturing spleen cells with retinoic acid (10^{-10}–10^{-4}M) had no effect on the numbers of DC separated from these cultures. However, DC isolated from retinoic-acid-treated cultures were less stimulatory than DC from untreated cultures when added to allogeneic lymphocytes in a mixed leucocyte culture. Allogeneic stimulation by DC was also inhibited by pulsing separated DC with retinoic acid (10^{-6}–10^{-4}M) for 2 h. Pulsing DC with lower doses of retinoic acid (10^{-14}–10^{-20}M) enhanced this response. DC isolated from animals maintained on VAA-enriched diets had a reduced capacity to stimulate allogeneic lymphocytes. The response of unseparated lymph node cells pulsed with retinoic acid to untreated allogeneic DC was inhibited by 10^{-10}–10^{-4}M retinoic acid but enhanced by lower doses (10^{-14}M). However, the inhibitory effect of retinoids on the function of responding lymphoid populations was abolished on removal of DC from responding cells. The results indicate that immunomodulation by retinoids could occur via an effect on the efficiency of antigen presentation by DC.

Keywords dendritic cells retinoids antigen presenting cells mixed leucocyte culture dendritic cell functions immunoregulation by retinoids

INTRODUCTION

Vitamin A and its analogues, the retinoids, influence many immune functions. Treatment of animals with retinoids inhibits the growth of chemically and virally induced tumours, either by suppressing tumour growth (Lotan, 1980), or by potentiating tumour-specific immunity (Dennert, 1984). Retinoid treatment of mice enhances delayed type hypersensitivity (DTH) responses, rendering animals sensitive to lower doses of contact sensitizers (Miller, Maisey & Malkovsky, 1984; Colizzi & Malkovsky, 1985). Similar enhancement of DTH reactions by vitamin A has also been observed in man (Micksche *et al.*, 1977). Enhancement of host versus graft (HvG) reactivity by retinoids has been demonstrated in mice fed vitamin-A-enriched diets, by the induction of HvG responses with suboptimal numbers of semiallogeneic cells (Malkovsky *et al.*, 1983). Stimulation of cell-mediated immunity by retinoids has also been demonstrated by their ability to induce tumour-specific cytotoxic T cells (Dennert & Lotan, 1978), and the reversal of neonatally induced transplantation tolerance by administration of vitamin A acetate (VAA) (Malkovsky *et al.*, 1985).

In contrast, however, there is evidence that retinoids may inhibit some immune responses. Retinoid treatment reduces primary IgM antibody responses, whilst enhancing secondary IgG responses (Barnett, 1983). Mitogen-induced proliferation of lymphocytes is also inhibited (Bauer & Orfanos, 1981). Retinoids suppress the expression of Fc receptors on macrophages, and subsequent phagocytosis of opsonized cells (Rhodes & Oliver, 1980). These inhibitory properties may play a role in the anti-inflammatory effects exhibited by oral administration of retinoids in the treatment of inflammatory dermal disorders (Orfanos & Bauer, 1983).

The initiation of immune responses requires the presentation of antigen. One possible mechanism of action of some immunomodulatory drugs may be via a modulation of antigen presentation. One mode of action of the immunosuppressive drug cyclosporine A may be via a direct effect on the antigen presenting capacity of DC. Previous studies have indicated that acquisition and presentation of antigen by DC is blocked by treatment with cyclosporine A (Knight *et al.*, 1988). Similarly treatment of patients with prednisolone and azathioprine results in a reduction in the number of Langerhans cells, the antigen presenting cells in the skin, and in an alteration of their morphology (Sontheimer *et al.*, 1984). These cells also have a reduced capacity to stimulate allogeneic responses *in vitro* (Sontheimer *et al.*, 1984). Retinoids may also influence antigen presentation. Retinoid treatment has a direct effect on Langerhans cells by prolonging the expression of HLA-DR and CD1 antigens on epithelial cells in culture (Walsh, Seymour & Powell, 1985). The abnormal distribution of Langerhans cells in the epidermis of patients with psoriasis is also restored after treatment with retinoids (Haftek *et al.*, 1983). Furthermore,

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long term feeding of vitamin-A-enriched diets increases the numbers of DC in the spleen and lymph nodes of mice (Drzymala *et al.*, 1984). The effect of retinoid treatment, *in vitro* and *in vivo*, on DC function was therefore investigated.

MATERIALS AND METHODS

Mice

CBA or C57BL/10 mice were between 6 and 12 weeks of age and were bred in the specific pathogen-free unit at the Clinical Research Centre. They were maintained on a conventional diet (Spratts Laboratory Diet 1, Spillers) ad libitum, either with or without supplementary vitamin A acetate (VAA) in the form of stable gelatinized beadlets (0·5 g/Kg conventional diet, Roche). The VAA diet was maintained for at least 3 weeks prior to isolation of lymphoid cells from these animals.

Retinoic acid

All trans retinoic acid (Sigma) was dissolved in absolute alcohol to give stock solutions of between 10^{-18} and 10^{-2} M retinoic acid, and it was stored in the dark at 4°C.

Cell suspensions

Suspensions of cells from spleens or lymph nodes were obtained by pressing tissues through a metal gauze, and washing in medium (RPMI 1640 Dutch modification, Flow Labs., with 100 iu/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-mercapto-ethanol, and 10% fetal calf serum). Spleen cell suspensions were cultured at between 5 and 10×10^{6} cells/ml of medium in tissue culture flasks (Nunc, 25 ml) for between 24 and 72 h at 37°C. Between 5 and 8 ml of the non-adherent cells were layered onto 2 ml of metrizamide (Nyegaard Oslo, 14·5 g added to 100 ml of medium) and centrifuged at 600 ***g*** for 10 min. The mononuclear cells at the interface were collected, washed once and resuspended in medium. These cells were > 75% dendritic, indicated by their sensitivity to the monoclonal antibody 33D1, with fewer than 5% macrophages and small numbers of lymphocytes (Macatonia *et al.*, 1987). Retinoic acid was added to some cell suspensions to give final concentrations of between 10^{-20} M and 10^{-4} M retinoic acid and 1% alcohol. One per cent alcohol was added to some suspensions as a diluent control. Treated cell suspensions were washed twice before being added to cultures.

Some lymph node cell preparations were depleted of DC by treatment with cytotoxic anti DC antibody, 33D1, and rabbit complement (Buxted) at 37°C for 30 min.

Culture in vitro

Cells were in 20 μl hanging drops in inverted Terasaki plates which contained between 1 000 and 100 000 lymph node cells/well. Some cultures received 500 DC which were irradiated with 2 000 rads (^{60}Co source). After 3 days the cultures received 1 μl of [^{3}H]-thymidine (2 Ci/mM, Amersham) to give a final concentration of 1 μg/ml of thymidine. After 2 h the cultures were harvested by blotting onto filter discs and the acid-insoluble material was counted using a liquid scintillation counter. Analysis of variance on log transformed data was used to assess differences in counts significantly greater than replication variability (Knight, 1987).

RESULTS

Effect of retinoic acid treatment on dendritic cell separation

Treatment of spleen cell cultures with 10^{-5} M retinoic acid, for up to 96 h, had no significant effect on the total number of cells recovered from the cultures. Similarly there was no difference in the number, or purity, of DC isolated from treated or untreated cultures. Greater than 70% of the cells in these preparations were of dendritic morphology (see Macatonia *et al.*, 1987).

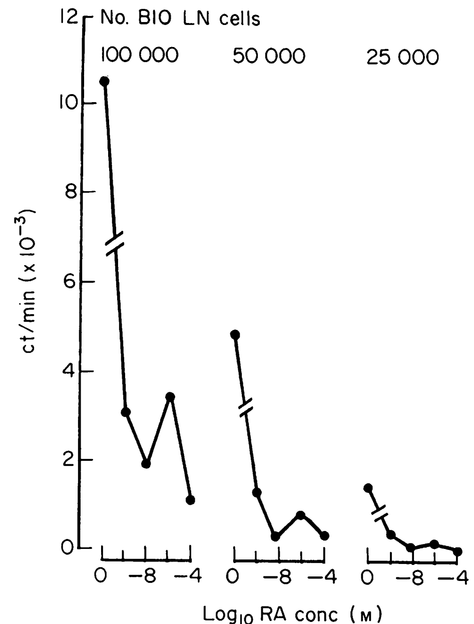

Fig. 1. Overnight treatment of spleen cell cultures with RA. Uptake of ^{3}H-thymidine by 25 000 to 100 000 lymph node cells in response to 500 allogeneic DC isolated from spleen cell cultures treated with between 0 and 10^{-4} M retinoic acid. Points represent means of triplicate cultures.

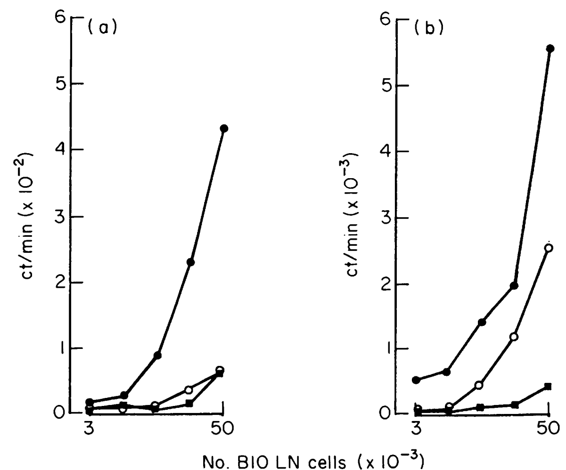

Fig. 2. Inhibition of MLC by 10^{-4} M RA. Uptake of ^{3}H-thymidine by 3000 to 50000 lymph node cells alone (■) or with 500 untreated allogeneic DC (●). (a) Complete inhibition of this response was induced by pulsing DC with 10^{-4} M retinoic acid (○) $P=0{\cdot}0121$. (b) Partial inhibition of the response was induced by pulsing DC with 10^{-4} M retinoic acid (○) $P=0{\cdot}0781$. Note difference in scale on ordinate. Points represent means of triplicate counts. P values represent significance of differences in stimulation by treated and untreated DC over the range of responder cell concentrations.

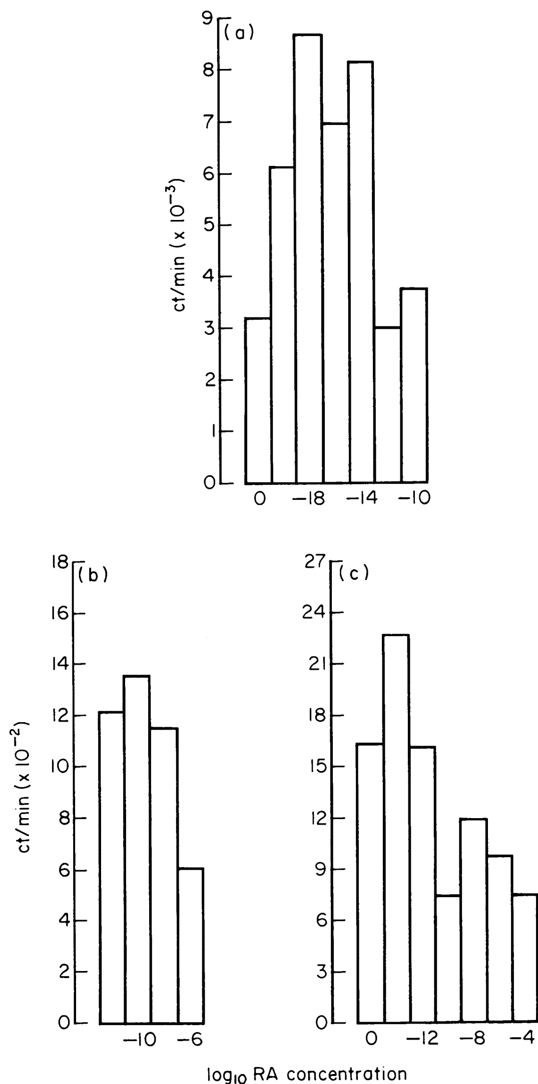

Fig. 3. Dosimetry of RA treatment. Uptake of ^{3}H-thymidine by 100 000 (a–b) untreated lymph node cells in response to 500 allogeneic DC either untreated or pulsed with between 10^{-6} M and 10^{-20} M retinoic acid, or 100 000 (c) lymph node cells pulsed with between 0 and 10^{-14} M retinoic acid in response to 500 untreated allogeneic DC. Points represent means of triplicate counts. Similar results were obtained with a range of responder cell concentrations down to 3 000 cells/well. *P* values for the effect of retinoid treatment over the complete cell concentration curve are as follows: (a) 10^{-20}–10^{-14} M $P<0{\cdot}0001$, 10^{-12}–10^{-10} M $P=$NS. (b) 10^{10}–10^{-8} M $P=$NS, 10^{-6} M $P=0{\cdot}034$. (c) 10^{-14} M $P=0{\cdot}0176$, 10^{-12} M $P=$NS, 10^{-10}–10^{-4} M $P=0{\cdot}0018$.

Maximal numbers of DC were obtained after incubation of spleen cell suspensions for 24 h. In subsequent experiments DC isolated after 24 h of culture were used.

Effect of retinoic acid treatment on stimulator capacity of dendritic cells

Addition of 500 splenic DC to allogeneic lymph node cells induced significant proliferation. However, DC isolated from spleen cells cultured for 24 h with varying doses of retinoic acid were less effective in stimulating allogeneic responses (Fig. 1), significant inhibition being caused by as little as 10^{-10} M retinoic acid ($P=0{\cdot}0015$). Treatment of separated DC with retinoic acid

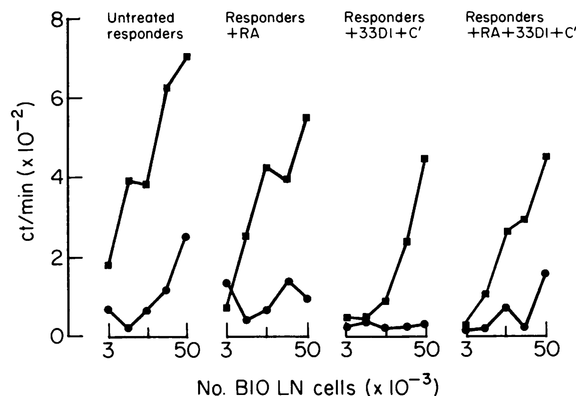

Fig. 4. Comparative effect of RA treatment and DC depletion of responder cells. Uptake of ^{3}H-thymidine by 3 000 to 50 000 lymph node cells, or lymph node cells depleted of DC (●). These populations were either untreated or pulsed with 10^{-4} M retinoic acid, and stimulated with 500 allogeneic DC (■). Points represent means of triplicate counts.

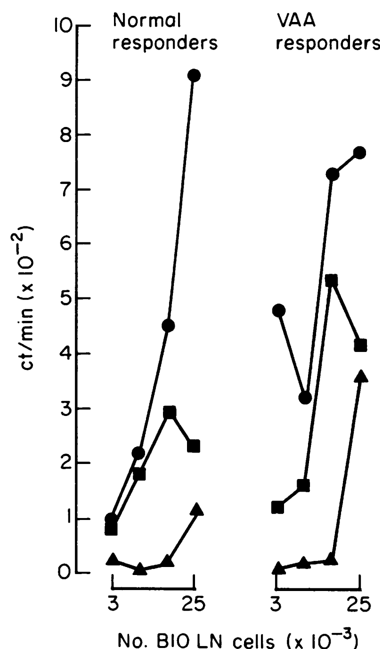

Fig. 5. Effect of retinoid treatment *in vivo*. Uptake of ^{3}H thymidine by 3000 to 25 000 lymph node cells from animals on conventional or VAA enriched diets (▲), in response to 250 allogeneic DC isolated from animals on conventional (●) or VAA enriched diets (■). Points represent means of triplicate counts.

also inhibited their capacity to stimulate allogeneic responses, whilst having no effect on cell viability, as assessed by trypan blue exclusion. The effect of 10^{-4} M retinoic acid on separated DC was dependent upon the duration of retinoic acid treatment. Significant inhibition was seen after treating DC with retinoic acid for 1 h ($P=0{\cdot}0317$), and pulsing for 2 h gave maximal inhibition ($P=0{\cdot}0224$) (data not shown). On six out of 11 occasions pulsing for 2 h with 10^{-4} M retinoic acid reduced the response to the level of background turnover (eg. Fig. 2a).

Table 1. Effect of VAA-enriched diets on the number of lymph node and dendritic cells recovered

Duration of diet (weeks)		Number of lymph node cells recovered				Number of dendritic cells recovered		
	n	Control	VAA	*P*	*n*	Control	VAA	*P*
3–10	9	34·9 (7·4)	36·4 (8·2)	NS	9	1·10 (0·4)	1·10 (0·6)	NS
10–18	4	26·7 (8·5)	38·2 (6·4)	NS	6	1·8 (1·1)	3·7 (0·8)	0·008

Results are expressed as number of lymph node cells or DC $\times 10^{-6}$ ($\pm$s.e.) isolated from mice fed VAA-enriched diets, or from control mice on normal diets.
NS = not significant.

However on five other occasions, when the initial allogeneic response was greater, this treatment resulted in only partial inhibition of the response (eg. Fig. 2b), and this inhibition usually remained statistically significant. The effect of treating DC for 2 h on the stimulatory capacity of these cells was dependent on the dose of retinoic acid used. Significant inhibition of DC function was seen with 10^{-4}–10^{-6} M retinoic acid (Fig. 3b), whereas lower doses (10^{-8}–10^{-12} M) had no significant effect. However, reducing the dose even further (10^{-14}–10^{-20} M) resulted in a significant enhancement of the response (Fig. 3a).

Effect of retinoic acid treatment on responder lymph node cells
Treatment of the responding lymph node cell population with 10^{-4} M retinoic acid for 2 h reduced the level of response to allogeneic DC ($P=0·0003$), but did not completely inhibit the response (Fig. 4). This effect was not generally as marked as treating DC used for stimulation. Significant inhibition of the response was seen when lymph node cells were treated with between 10^{-4} M and 10^{-10} M retinoic acid for 2 h, and 10^{-12} M retinoic acid had no significant effect on the proliferation. However, pulsing lymph node responder cells with 10^{-14} M retinoic acid for 2 h resulted in an enhancement of the proliferative response (Fig. 3c).

Lymph node cell preparations were depleted of DC by treatment with an anti-DC antibody, 33D1, and complement. These DC-depleted populations exhibited reduced background turnover and a reduced ability to respond to an allogeneic stimulus ($P=0·0038$). Subsequent treatment of the DC-depleted preparations with 10^{-4} M retinoic acid had no effect on either the unstimulated proliferation or the allogeneic response of these cells (Fig. 4).

Effect of in-vivo retinoid treatment
The effect of VAA-enriched diets on both the number of lymph node cells and the number of splenic DC isolated was studied. Diets were maintained for up to 18 weeks. On the basis of previous reports showing increases in both DC and lymph node cells after feeding VAA-enriched diets for 12 weeks (Drzymala *et al.*, 1984), animals were divided into two groups, those fed VAA-enriched diets for less than 10 weeks or animals maintained on VAA-enriched diets for longer than 10 weeks, prior to statistical analysis of the numbers of cells recovered. The total number of cells isolated from lymph nodes of mice on VAA-enriched diets was not significantly different from the number of cells obtained from mice on normal diets in either group. Similarly there was no difference in the numbers of DC isolated from spleens of mice fed VAA-enriched diets for less than 10 weeks, compared with mice on normal diets. However, spleens from mice maintained on VAA-enriched diets for periods longer than 10 weeks yielded significantly greater numbers of DC than spleens from control animals (Table 1). Since previous work has indicated that functional changes occur in animals on VAA diets after only 3 weeks (Malkovsky *et al.*, 1984), subsequent experiments were carried out on cells isolated from animals between 3 and 10 weeks after the diets commenced. DC isolated from mice fed the VAA-enriched diet had a reduced capacity to stimulate allogeneic responses (Fig. 5) ($P=0·032$). The VAA diet had no significant effect on the ability of lymph node cells from these animals to respond to an allogeneic stimulus (Fig. 5).

DISCUSSION

In-vitro treatment of spleen cell cultures, prior to the separation of DC, had a profound effect on the functional capacity of these cells, although it did not significantly alter the yield of DC from these cultures. DC isolated from spleen cells cultured with retinoic acid in concentrations as low as 10^{-10} M exhibited a reduced capacity to stimulate proliferation of allogeneic lymphocytes. Pulsing separated DC with retinoic acid also blocked their stimulatory capacity. This effect was dependent upon both the duration and dose of retinoid treatment. The inhibitory effect of retinoic acid treatment on the stimulatory capacity of separated DC suggests that retinoic acid has a direct effect on DC. Since there was also a reduction in the stimulatory capacity of DC from mice fed on a retinoid-enriched diet, similar effects on antigen presenting function may occur *in vivo*.

Previous studies on the effect of retinoids on immune function have suggested that these compounds act at the induction, rather than the effector, phase of the immune response (Dennert & Lotan, 1978). DC are known to be potent antigen presenting cells for the induction of primary allogeneic

responses (Steinman & Witmer, 1978). It is therefore possible that retinoids may influence immune responses via a direct effect on DC function. A similar mode of action has previously been proposed for the immunosuppresssive drug cyclosporine A (Knight *et al.*, 1986; Knight *et al.*, 1988). Retinoids do not, however, alter the level of expression of major histocompatibility antigens on DC (Bedford & Knight, unpublished observations).

Treatment of unseparated lymph node cells with retinoic acid decreased their response to untreated allogeneic DC. This suggested that retinoids may have a direct effect on the responding lymphocyte population. However, this could reflect an alteration in the function of DC in the responding cell population. The presence of DC in the responder cells increases the response to an allogeneic stimulus (Goodacre Bedford & Knight, unpublished observations). This may be via a mechanism similar to that seen *in vivo*, in mice receiving skin allografts, where acquisition and presentation of alloantigen by host antigen presenting cells occurs (Sherwood, Brent & Rayfield, 1986). Further evidence for the importance of responder type DC in mixed leucocyte reactions was indicated by the reduced responsiveness of lymph node cells depleted of DC (Fig. 4). The lack of effect of retinoic acid in lymph node cells depleted of DC supports the idea that the retinoids are affecting the DC in the responder population.

Retinoids have previously been shown to enhance graft rejection across minor histocompatibility barriers (Floersheim & Bollag, 1972; Jurrin & Tannock, 1972). However, in this study, treatment with 10^{-4}–10^{-6} M retinoic acid inhibited the capacity of DC to induce proliferation of allogeneic lymphocytes. Inhibition of mixed leucocyte responses by a similar concentration of retinoic acid (10^{-5} M) has previously been reported (Dennert & Lotan, 1978). These observations appear at variance with the effect *in vivo* of retinoids on allografted tissue. This may reflect a retinoid-induced change in the kinetics of the allogeneic response. Enhancement of cytotoxic, HvG, and DTH responses by retinoids is seen in suboptimal conditions (Dennert & Lotan, 1978; Malkovsky *et al.*, 1983; Miller *et al.*, 1984). Under 'optimal' conditions in these systems retinoids either have no effect or cause inhibition (Dennert & Lotan, 1978; Hunt *et al.*, 1982). These results might be explained if retinoid treatment caused a shift in the kinetics of the immune response, rendering animals and/or cells sensitive to smaller immunological stimuli. The level of stimulus causing suppression would also be lowered, resulting in inhibition under previously 'optimal' conditions. On the basis of this hypothesis enhancement by retinoids would be expected on reducing the dose of retinoid administered. Enhancement of the mixed leucocyte response was observed when DC were pulsed for 2 h with very low doses of retinoic acid (10^{-14}–10^{-20} M), and similar enhancement was seen when responding lymph node cells were pulsed with 10^{-14} M retinoic acid. Similarly, Dillehay *et al.*, (1987) report an enhancement of mitogen responses by low doses of retinoic acid (10^{-15} M), particularly at superoptimal doses of mitogen. Also, pulsing DC with 10^{-12} M retinoic acid increased their capacity to present suboptimal doses of ConA to syngeneic lymph node cells (Bedford & Knight, unpublished observations). Thus retinoid treatment appears to shift the kinetics of the immune response, resulting in an increased sensitivity of the system (see Knight, 1982).

Long-term feeding of retinoid-enriched diets leads to hypertrophy of lymphoid organs and increased numbers of both T cells and DC (Hunt *et al.*, 1982; Turton *et al.*, 1985; Drzymala *et al.*, 1984). The increase in the numbers of DC isolated from the spleens of animals after long-term feeding (> 10 weeks) of VAA-enriched diets, is confirmed in this study. The total number of lymph node cells recovered from these animals also tended to be higher. This latter increase did not, however, reach statistical significance, possibly due to the relatively small number of observations in this group. Since previous studies have shown that immunological functions are altered after only 3 weeks on VAA-enriched diets (Malkovsky *et al.*, 1984) functional assays were carried out on cells isolated from animals between 3 and 10 weeks after the commencement of VAA-enriched diets. Thus, since the functional change in these cells occurred before the increase in number, this is unlikely to be due to any form of compensatory mechanism brought about by changes in the numbers of cells.

The immunodulatory effects of retinoids are well documented. The results presented in this study show that retinoid treatment modulates mixed leucocyte responses via a direct effect on the stimulatory capacity of DC, and suggests that the function of DC within the responding population is also affected by retinoids. Further detailed studies on the alteration of the kinetics of alloproliferative responses by retinoids may help to clarify the conflicting reports of retinoid-induced effects on these responses.

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