Regulation of accessory cell function by retinoids in murine immune responses

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Summary. This study examines the effects of in-vivo immune regulation by vitamin A acetate (VAA) and 13-*cis*-retinoic acid (13-CRA) on in-vitro accessory cell function. Mice were fed a control diet, or diet containing VAA or 13-CRA, and monitored by body weight gains and diet consumptions at weekly intervals. At 4, 7 and 12 weeks mice were killed, differential blood counts performed and accessory cells isolated from lymphomedullary tissues. Histology confirmed that the chief feature of the lymphomedullary organs of the VAA-fed animals was an expansion of the splenic marginal zone and the paracortical region of the lymph nodes. There was an increase in the number of accessory cells present, and this included both dendritic cells and macrophages. The accessory cell function of these cells was also increased, as evidenced by both alloproliferative and allocytotoxic responses *in vitro*. In 13-CRA-fed animals the effects were similar to those seen with VAA, but were less pronounced. We suggest that the primary effects of these compounds on in-vivo immunoregulation could be due to their promotion of accessory cell function.

Keywords: vitamin A, retinoids, immunoregulation, dendritic cells, macrophage, mouse

Retinoids, i.e. natural and synthetic analogues of vitamin A, have profound effects on both normal and neoplastic cells (Wolbach & Howe 1925; Lotan 1980). The interaction of retinoids with specific receptor sites in cell membranes and their hormone-like activity in controlling cell differentiation have been the subjects of intensive study (Lotan *et al.* 1983). An attractive hypothesis is that retinoid effects on cell growth and differentiation, and on immunomodulatory activities (Dresser 1968; Dennert 1985), are mediated by a common mechanism. In one in-vitro system a linked relationship between cellular and immune-mediated events has been clearly demonstrated (Patek *et al.* 1979), but the precise nature of the immunological processes involved still require clarification.

During retinoid feeding experiments in these laboratories a consistent enlargement of the lymphomedullary organs was observed in retinoid-fed mice (Medawar &

Hunt 1981; Turton *et al.* 1985; Drzymala *et al.* 1985). The present studies were designed to investigate the pattern of cellular changes involved in this enlargement, and to define the functional attributes of the lympho-medullary cells from retinoid-fed animals by comparison with controls.

Materials and methods

Animals, diet and retinoid administration

Weanling female mice of the strains C57BL/ 10 × DBA/2 F_1 (B10D2F_1) (Olac 1976 Ltd, Bicester, Oxon) and CBA (Department of Immunology, Middlesex Hospital Medical School and the Clinical Research Centre) were fed *ad libitum* a control ground maintenance diet (Rat and Mouse No. 1, SDS Ltd, Witham, Essex). Groups of mice were fed diet supplemented with retinoids. Stabilized beadlets (Dry Vitamin A Acetate Type 500, Roche Products Ltd, Chemical Division, Welwyn Garden City, Herts) containing vitamin A acetate (VAA) were blended into the control diet to give a concentration of 1.0 mmol/kg diet (1.91 g beadlets containing 329 mg VAA/kg diet) and beadlets containing 13-*cis*-retinoic acid (13-CRA) (a gift from Roche Products Ltd) were added to the control diet to give a level of 1.8 mmol/kg (4.50 g beadlets containing 541 mg 13-CRA /kg diet). Diets were stored at 4°C and fed in pots designed to reduce spillage and contamination. Animals were maintained up to 12 weeks at 19–22°C in a relative humidity of 50–60% on a 12:12 h, light:darkness cycle. They were weighed and 24 h diet consumptions carried out weekly to the 10th week.

Tissue and cell samples

Histology. Tissues were taken at predetermined times at autopsy, fixed in 2.5% glutaraldehyde in phosphate buffered saline, paraffin embedded and sections cut and stained with haematoxylin and eosin.

Peripheral blood. Mice were fed a control or VAA-supplemented diet for 10 to 12 weeks, anaesthetized with CO_2 and blood collected by cardiac puncture into K_2EDTA after making a thoracotomy incision. Total and differential white blood cell counts were recorded.

Single cell suspensions. Spleens were teased apart and the resulting cell suspension used to prepare a low density accessory cell (AC) population on a discontinuous bovine serum albumin gradient. Where appropriate, the low density cells were further subfractionated into dendritic cell and macrophage enriched fractions (Sunshine *et al.* 1980). In parallel with the AC preparation, either non-adherent cells or high density cells from the same gradients were used as T-cell enriched populations.

Assay Systems

Proliferation assay. A standard mixed leucocyte culture system was used. Known numbers of AC from control and retinoid-fed animals were irradiated (1200 rad from a ^{60}Co source) before being used as stimulators at a ratio of 1 stimulator to 10 responders. Non-adherent high density cells from both syngeneic and allogeneic mice were used as responders.

Cells were cultured in 200 μl RPMI-1640 medium (GIBCO) supplemented with 5% heat inactivated fetal calf serum, 10 mM HEPES buffer, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol and antibiotics, in flat bottom, 96-well plates. Plates were incubated for 96 h at 37°C in humidified 5% CO_2 in air. For the final 6 h, 0.5 μCi ^{125}iododeoxyuridine (^{125}IUDR) in 10 μl medium was added. The cells were harvested using an automated cell harvester and radioactive macromolecular DNA was assessed by gamma counting. Results, expressed as counts per minute (ct/min), are the mean of triplicate assays in which the standard deviation was less than 15%.

Cytotoxicity assay. Allogeneic responder cells, stimulated as outlined above, were

Table 1. Mean body weight increases, diet and retinoid consumptions*

Dietary supplement	Body weight (g)			Diet consumption/day		Retinoid consumption/day	
	Week 0†	Week 10	Increase (%)	g/mouse	body g/kg weight	mg/mouse	body mg/kg weight
Control diet	16.1	23.2	44	5.7	280	—	—
13-CRA	14.7	19.8	35	5.6	316	3.0	171
VAA	15.2	22.0	45	5.8	303	1.9	99

* Body weights and diet consumptions were recorded weekly for 10 weeks of the 12-week trial.
† Beginning of the experiment; 40 mice per group.

used as effector cells, target cells were 24 h-cultured concanavalin A blast cells labelled with ^{51}Cr at a concentration of 2×10^3/ml. Triplicate effector cell samples at a range of effector: target ratios from 30:1 to 1:1 were used in a 4 h assay at 37°C and specific ^{51}Cr release was calculated according to the formula:

$$^{51}\text{Cr release (\%)} = \frac{\text{(Experimental} - \text{Medium control) ct/min}}{\text{(Maximum} - \text{Medium control)}} \times 100.$$

The medium control was always less than one third of maximum release; maximum release was determined by detergent lysis.

Results

Body weight increases, diet and retinoid consumptions

All animals appeared healthy and in good condition throughout the trial. Body weights, diet and retinoid consumptions from the beginning of the experiment to week 10 are presented in Table 1. In all groups of mice body weights increased satisfactorily, although the percentage increase was slightly reduced in the 13-CRA-fed animals. Diet consumptions were similar in all groups.

Histology

Spleens and lymph nodes from mice fed the control diet did not enlarge or alter in general architecture during the course of the 12-week study. The VAA-fed animals showed splenomegaly and lymphadenopathy with progressive expansion of the marginal zone region of the spleen (Fig. 1) and the paracortical region of the lymph nodes. This selective zonal expansion was also seen in the 13-CRA fed mice; however, with this retinoid the effects were maximal at 7 weeks and by 12 weeks prominent reactive germinal centres could be identified.

Peripheral blood

Leucocyte counts of mice fed control or VAA diets for 10 to 12 weeks are shown in Table 2. There was no significant difference in the total leucocyte count of the 2 groups, but in mice fed the VAA diet there was a decrease in the ratio of neutrophils to lymphocytes which was not statistically significant. The monocyte and eosinophil counts were not affected in VAA-fed mice.

Tissue-derived cell counts

In contrast to the peripheral blood values, but in concordance with the histological observations, there was a considerable and progressive increment in the number of low density AC isolated from the retinoid-fed animals (Table 3). The increase was greater with VAA than with 13-CRA.

Allogeneic responses

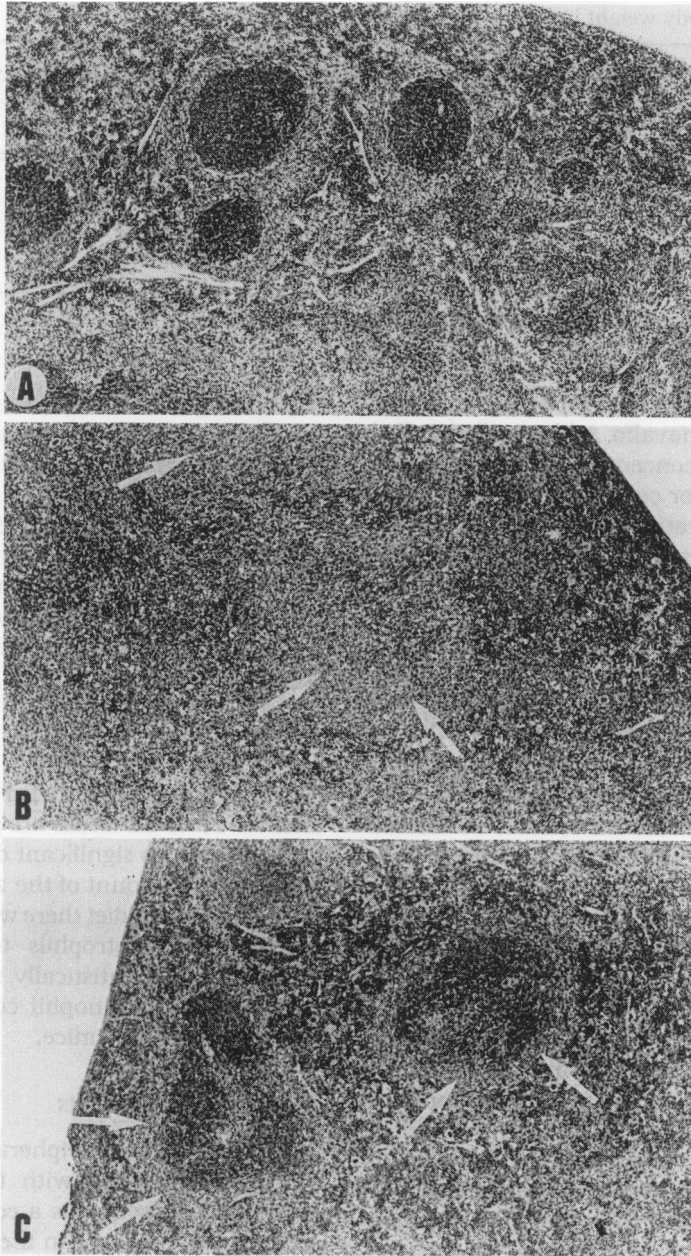Functional experiments were performed comparing low density cells from mice fed

Fig. 1. *A*, Normal spleen from mouse fed a control diet showing a normal distribution of red and white pulp. H & E, × 70. *B*, Spleen from mouse fed a vitamin A acetate-containing diet for 7 weeks illustrating a loss of normal white pulp distribution due to a massive expansion of the marginal zone region (arrows). There is little of the compact peri-arteriolar small lymphoid aggregates seen in the normal spleen. H & E, × 70. *C*, Spleen from mouse fed on 13-cis-retinoic acid-containing diet for 7 weeks showing an intermediate appearance between (*A*) and (*B*). There is expansion of the marginal zone (arrows) and the small lymphoid aggregates are less well defined than in the normal spleen. H & E, × 70.

control or retinoid diets. Table 4 shows the results for B10D2F$_1$ (H-2$^{b/d}$) macrophages and dendritic cells in a representative series of experiments using the same CBA (H-2^k) responder cells. There was an increase in relative inducer capacity of AC cells in the VAA- and 13-CRA-fed mice compared to the controls, but as with previous findings, in the

Table 2. Total and differential white blood cell counts for mice fed a control or VAA-supplemented diet for 10 to 12 weeks*

Dietary supplement	Total count ($\times 10^9$/l)	White blood cell count			
		Differential count (1%)			
		Neutrophils	Lymphocytes	Monocytes	Eosinophils
Control diet	15,119 (4,717)	29.2 (8.6)	63.4 (9.5)	6.5 (2.1)	0.9 (0.8)
VAA	13,024 (1,691)	21.8 (6.4)	70.2 (7.2)	6.8 (2.2)	1.0 (0.9)

* Mean values; s.d. in parentheses. Twenty mice per group.

Table 3. Mean numbers of low density accessory cells* from mice fed control or retinoid-supplemented diets†

Dietary supplement	4 weeks	7 weeks	12 weeks
Control diet	2.0	3.0	8.4
13-CRA	2.5	8.0	15.5
VAA	5.0	12.0	64.0

* Total dendritic cells plus macrophages ($\times 10^5$); results are from eight animals per group in a representative series of experiments.

† Diets were fed for 4, 7 or 12 weeks.

Table 4. Alloproliferative responses at 4 and 7 weeks in mice fed control or retinoid-supplemented diets*

Time of diet administration	Dietary supplement	Macrophages	Dendritic cells
4 weeks	Control diet	8.3	13.9
4 weeks	13-CRA	10.0	15.9
4 weeks	VAA	12.4	18.8
7 weeks	Control diet	13.2	16.6
7 weeks	13-CRA	15.9	24.8
7 weeks	VAA	18.8	29.7

* Results expressed as ^{125}IUDR incorporation 96 h (ct/min $\times 10^{-4}$) and are the mean of triplicate assays where s.d. $< 15\%$.

Table 5. Allocytotoxic responses at 7 weeks in mice fed control or retinoid-supplemented diets*

	Effector: Target cell ratios		
Dietary supplement	30:1	15:1	1:1
Control diet	12.2	7.0	2.3
13-CRA	15.5	10.5	4.3
VAA	22.9	14.1	7.2

*Results expressed as per cent specific lysis of allogeneic target cells.

13-CRA-fed animals the increase was not as great as in those fed the VAA diet.

Allocytotoxic responses

The capacity of the retinoids to induce effector cytotoxic cells was compared at week 7 (Table 5). CBA ($H-2^k$) responder cells were activated by B10D2F$_1$ ($H-2^{b/d}$) accessory cells and tested against target cells from the stimulator strain. A similar pattern of changes to the proliferative response was observed, with increased cytotoxic inducer capacity of non-adherent high density cells in the retinoid-fed animals. This was more marked with VAA than with 13-CRA.

Discussion

The considerable literature dealing with the role of retinoids in the immune response reflects an awareness that these compounds may be potential in-vivo immunomodulatory agents (Dennert 1985). However, the sequential systemic changes which occur in the immune system *in vivo* following retinoid ingestion have not been established. Thus while it is known that the effector cell for retinoid immunomodulation is a $Ly1^+2^-$ cell (Malkovský *et al.* 1983) and that the effector function is linked to interleukin 2 (IL-2) production (Malkovský *et al.* 1984; 1985; Colizzi & Malkovský 1985), the mechanisms which trigger the generation of these $Ly1^+$ cells is not known.

The initial observations which formed the basis for this study were morphological (Medawar & Hunt 1981; Turton *et al.* 1985). In this study we have confirmed that neither the lymphadenopathy nor the splenomegaly were random events; both processes selectively and predominantly involved the region around the germinal centres, rather than the B-cell area of the centre itself. In both the spleen and lymph nodes this could be accounted for by expansion of either T-cell or AC pools within the cell populations normally present at these sites.

In-vitro quantitative analysis of the AC derived from control and retinoid-fed animals confirmed that there was a progressive increase in the number of cells of low density AC type present in the lymphomedullary tissues of the retinoid-fed mice. This increase was more pronounced with the natural analogue, VAA, than with the synthetic retinoid, 13-CRA. The increase was not reflected in any detectable significant shift in the peripheral blood populations. However, it was reflected as an increased potential for the induction of both proliferative and functional T-cell responses by these AC.

It is generally accepted that most tissue AC are bone marrow derived and travel to the tissues via the blood stream as the blood monocyte pool (Fossum & Ford 1985). In the retinoid-fed animals, there is no quantitative increase (reflected in peripheral blood values) in the migratory fraction of these cells as they pass to the tissues, and histological examination of femoral bone marrow of control and retinoid-fed mice shows normal

cellularity. Thus the most likely site for the retinoid effects lies in the tissues themselves.

The augmented AC activity may account for some of the previously observed retinoid effects. For example, where the earlier studies on immunomodulatory function refer to an adjuvant effect (Spitznagel & Allison 1970; Taub *et al.* 1970) this could be due to a non-specific increase in reactivity. Another systemic effect of retinoids in a murine model was reported by Dennert *et al.* (1979) who demonstrated that retinoic acid induced an increase in T cytotoxic activity. We believe that a common link between retinoid activity and both AC and cytotoxic effects might be mediated via gamma-interferon, which is known to modulate both cell types. The possibility that retinoids act systematically by promoting local interferon release is being investigated further.

Acknowledgements

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