Synthetic Retinoids Inhibit the Antigen Presenting Properties of Epidermal Cells in Vitro

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The clinical efficacy of retinoids in benign and malignant skin diseases involving immune mechanisms suggests that they affect the immunologic functions of the epidermis. However, these effects have yet to be demonstrated. The action of vitamin A (retinol) and the synthetic retinoids, isoretinoin, etretinate, acitretin, and arotinoid-free acid have been studied on the lymphocyte proliferation induced by phytohemagglutinin (PHA), by the mixed lymphocyte reaction (MLR), and the mixed epidermal cell-lymphocyte reaction (MECLR). The results for PHA-induced proliferations were highly variable for all the retinoids. However, in MECLR, the synthetic retinoids consistently reduced the proliferation by 20%–30%. This occurred at therapeutic drug concentrations of about 10^{-6}M. In MLR, a minor decrease of 10%–15% was only found for higher concentrations (10^{-6}M). Retinol induced no effect in either reaction.

Further analysis of acitretin on MECLR showed that it reduced lymphocyte proliferation in a dose-dependent fashion. This reduction was combined with a decrease in cytotoxic T-lymphocyte induction (CTL). Addition of 10^{-6}M acitretin at various times also revealed that its presence at cell culture initiation was necessary to inhibit proliferation significantly. Furthermore, cell treatments prior to MECLR showed that exposure of epidermal cells to acitretin was essential to produce this inhibition, suggesting that it acts directly on epidermal cells. Consequently, it is suggested that the specific inhibitory effect of synthetic retinoids on lymphocyte activation in MECLR may partly account for their therapeutic action on the skin. J Invest Dermatol 93:455–459, 1989

Vitamin A derivatives (i.e., retinoids) have beneficial effects not only on skin diseases with abnormal epidermal proliferation and differentiation such as psoriasis or ichthyosis, but also on diseases involving abnormal interactions between lymphocytes and epidermis (e.g., lichen planus or cutaneous T-cell lymphoma)[1,2]. This implies that they might alter immune skin functions. In fact, retinoids have been shown to act on most components of the immune system such as T and B lymphocytes and macrophages. Retinoids can either enhance or decrease phytohemagglutinin (PHA)-induced T-cell proliferations, depending on the retinoids used, and their concentrations [3,4]. Most studies suggest that, in vivo, retinoids rather enhance T-cell functions such as delayed-type hyper-sensitivity [5], skin allograft rejection [6], and antitumoral immunity in mice [7]. However, delayed-type hypersensitivity reaction also can be inhibited if retinoids are given during the sensitization phase [8]. Contradictory data have been reported concerning macrophages [9].

The immunologic properties of the skin are now well documented. Epidermal Langerhans cells are dendritic bone marrow-derived cells that synthesize and express class II histocompatibility antigens (e.g., HLA-DR) [10]. They are effective antigen-presenting cells in allogenic and antigen specific proliferation and in cytotoxic T-cell responses. Like macrophages, they produce interleukin-1 (IL-1). Keratinocytes also secrete soluble factors that may enhance or inhibit T-cell proliferation and differentiation, including IL-1 [11], thymopoetin [12], and prostaglandins [13]. On the other hand, lymphocytes may also affect the epidermis. For instance, the lymphokine interferon-γ induces HLA-DR expression by keratinocytes [14]. In short, although epidermal cells and lymphocytes are known to influence each other’s proliferation and differentiation via cellular and soluble factors, nothing is known about the modulation of these factors by the retinoids. The mixed epidermal cell-lymphocyte reaction (MECLR) is a well-defined reaction in which epidermal cells strongly stimulate proliferation of allogeneic lymphocytes. This reaction was recently shown to be a reliable predictive model in graft-versus-host disease, which involves epidermal cell-lymphocyte interactions [15]. Therefore, MECLR appears to be a useful tool for studying pharmacologic modulation in cutaneous immune reactions.

In the present work, we investigated the action of retinol and synthetic analogues upon the MECLR in humans. We demonstrated that vitamin A derivatives specifically inhibit the lymphocyte activation by allogeneic epidermal cells. Furthermore, the observation that the inhibition of the MECLR by
retinoids requires either early addition of the drugs to the cultures or pretreatment of the epidermal cells, suggests that retinoids act directly on the antigen presenting properties of these cells.

MATERIALS AND METHODS

Materials Sixteen healthy adult volunteers (ten men and six women) were recruited with the agreement of the hospital ethics committee. Their ages ranged from 18 to 39 years, with a mean age of 26.

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by centrifugation over Ficoll Hypaque density gradients (Eurobio, Paris, France). Epidermal sheets were obtained from suction blister roofs on thigh skin. Each blister was removed with scissors and washed in McKeehan’s solution (Flow Laboratories, Irvine, United Kingdom). The sheets were cut into small pieces, incubated in a 0.3% solution of trypsin for 15 min, and again washed in McKeehan’s solution. The resulting dispersed cells were centrifuged and resuspended in culture medium. Using these procedures, we obtained more than 80% of viable epidermal cells.

Medium consisted of RPMI 1640 (Flow Laboratories, Irvine, United Kingdom) supplemented with 1-glutamine (1%), nonessential amino acids (1%), penicillin-streptomycin (1%), HEPES buffer (3%), and decomplemented human AB serum (10%).

Molecules studied were the natural retinoid retinoic acid (Ro 0-5488) and four synthetic retinoids: isotretinoin (Ro 0-3780), etretinate (Ro 10-9359), acitretin (Ro 10-1670), and aridotinoid-free acid (Ro 13-7410). These retinoids were initially stored in 10% dimethyl sulfoxide (DMSO) at −20°C. Because retinoids are light-sensitive, they were diluted in the medium just before addition to the wells, and cultures were incubated in the dark. Cultures containing similar amounts of DMSO only were used as controls.

Cell Viability The direct cytotoxic effects on lymphocytes and epidermal cells of various concentrations of retinoids and their solvents (ethanol and DMSO) were checked using trypan blue exclusion after periods of incubation up to 24 h. Further investigation on cell viability with 10−6 M etretin was conducted in the same conditions using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, as described [16].

PHA-Induced Lymphocyte Proliferation Assays Lymphoid cells (106/ml) were cultured with 1% PHA in 200 μl round-bottom microtiter plates (Corning Glass Works, Corning, NY). The retinoids and DMSO were put at the initiation of the cultures. The cultures were incubated at 37°C for 4 days in a dark chamber containing a humidified 5% CO2 atmosphere. [3H]thymidine (0.8 μCi/well) (CEA, Gif-sur-Yvette, France) was added to cultures 18 h before harvesting with a Mash II apparatus. Counts per minute (cpm) were determined by an LKB liquid scintillation counter.

Mixed Lymphocyte Reactions and Mixed Epidermal Cell-Lymphocyte Reactions Allogeneic mixed lymphocyte reactions (MLR) and mixed epidermal cell-lymphocyte reactions (MECLR) proceeded in round-bottomed microtiter plates by mixing 106 viable responder lymphocytes and 106 irradiated (2400 R) stimulator allogeneic cells (either peripheral blood mononuclear cells (PBMC) or epidermal cells) in a final volume of 200 μl. In both reactions, the presence of 106 stimulator cells in culture was able to up-stimulate lymphocyte proliferation, as demonstrated by previous titration experiments [17]. The retinoids were added at culture initiation. Cultures were set up in quadruplicate. [3H]thymidine incorporation was measured for 18 h, after 5 days of culture. In some MECLR cultures, 10−6 M acitretin was added once on day 0, 1, 2, 3, or 4, and in others it was added cumulatively either on days 0, 1, 2, 3, and 4, or on days 1 and 3. In other MECLR cultures, responder lymphocytes and stimulator epidermal cells were incubated separately with either 10−6 M etretin or 0.01% DMSO for 2 h overnight, and washed five times in fresh medium. The two types of cells were then combined as either lymphocytes treated with DMSO + epidermal cells treated with DMSO; lymphocytes treated with acitretin + epidermal cells treated with DMSO; lymphocytes treated with DMSO + epidermal cells treated with acitretin; or lymphocytes treated with acitretin + epidermal cells treated with acitretin.

Generation of Cytotoxic T Lymphocytes in MECLR 5 × 104 responder lymphocytes and 5 × 105 allogeneic epidermal cells were cultured in 25 cm2 tissue culture flasks containing 10 ml of medium in the presence of acitretin (10⁻⁶ or 10⁻⁸ M). After 6 days of culture, responder cells were tested by chromium release on PHA-induced target PBMC, autologous to the stimulating epidermal cells. The cytotoxicity assay was performed by incubating 100 μl of various concentrations of responder cells for 4 h at 37°C with 100 μl of allogeneic or control autologous target cells, in sharp bottom-microtiterplates. Results were expressed as specific chromium release:

\[
\frac{\text{percent specific release} \times 100}{\text{spontaneous release}}
\]

Statistical Analysis Results were analyzed for statistical significance using the two-way-analysis of variance, and the Student's t-test.

RESULTS

Direct Cytotoxicity of the Retinoids and Their Solvents A direct cytotoxic effect appeared after a 4-h incubation. After 18 h, 1% DMSO or ethanol significantly reduced the viability of both lymphocytes and epidermal cells (p < 0.02). A more toxic effect was observed with 10⁻⁶ M retinol, acitretin or aridotinoid-free acid in the presence of 1% DMSO (p < 0.01), whereas isotretinoin and etretinate at the same concentrations did not produce any effect in addition to that of DMSO. For up to overnight (16 h), cell viability in the presence of both 10⁻⁵ M retinoid and 0.1% DMSO was about 60% of the initial concentration, i.e., similar to that observed in control medium, except for 10⁻⁶ M retinol, which was still slightly toxic on lymphocytes (p = 0.08). With 10⁻⁶ retinol, no decrease of lymphocyte viability was observed. Additional experiments using the MTT assays showed again that 10⁻⁶ M acitretin induced no reduction on lymphocyte and epidermal cell viability, compared with the controls (medium, and 0.1% DMSO) (data not shown).

Synthetic Retinoids Significantly Decreased the Lymphocyte Proliferation in MECLR Four independent experiments on the lymphocyte proliferation induced by PHA, MLR, and MECLR were conducted for each retinoid. Figure 1 illustrates the comparative results with 10⁻⁶ M acitretin.

At concentrations between 10⁻⁵ M and 10⁻⁸ M, etretinate and acitretin tended to inhibit PHA-induced proliferation. The other retinoids often exhibited no effect. However, none of these results was consistently observed.

In MLR, isotretinoin, acitretin and aridotinoid-free acid only inhibited lymphocyte proliferation slightly by 10%–15%. Moreover, this inhibition only occurred at the high concentration of 10⁻⁸ M. Neither retinol nor etretinate had any inhibitory or stimulatory effect.

In MECLR, the inhibition of lymphocyte proliferation by all the retinoids except retinol was consistently higher than that observed in MLR. At drug concentrations between 10⁻⁴ M and 10⁻⁷ M, isotretinoin, acitretin, and aridotinoid-free acid reduced [3H]thymidine incorporation into lymphocytes, by 20%–30%. In some acitretin-treated cultures, addition of 5 × 10⁻⁶ M indomethacin (CIRD, Sophia-Antipolis, France), which is known as an inhibitor of prosta­ glandin synthesis, did not lead to a reduction of this inhibition. Errotinate induced less inhibition than the other synthetic retinoids (10%–20%). At retinoid concentrations of 10⁻⁵ M, there was no difference between proliferation in the retinoid groups and the DMSO control groups.

A two way-analysis of variance was realized on the results obtained with 10⁻⁶ M retinol and acitretin, in MLR and MECLR.
Dose-Response Curves With Acitretin in MLR and MECLR

To establish dose-response curves, lymphocyte proliferation in MLR and MECLR was studied in the presence of various concentrations of acitretin. The final concentration of DMSO in each well was 0.01%. The results of two separate experiments confirmed those of the described studies, because inhibition of cell proliferation was significantly higher in MECLR than in MLR (Fig. 2). In MECLR, the decrease in \(^{3}H\)thymidine uptake by the lymphocytes was clearly dose dependent, and reached a plateau of inhibition at about 30% for an approximate acitretin concentration of 10\(^{-6}\)M.

Acitretin Inhibits the CTL Induction in MECLR

In two separate experiments, the induction of a cytotoxic lymphocyte (CTL) activity in the presence of 10\(^{-6}\) and 10\(^{-8}\)M acitretin was tested in MECLR. In this reaction, 10\(^{-8}\)M acitretin significantly reduced not only the proliferation but also CTL activity (Fig. 3), whereas no effect was observed when PBMC stimulator cells were used (data not shown). At 10\(^{-6}\)M, CTL activity during MECLR was within normal limits.

Acitretin Acts on the Stimulator Epidermal Cells in MECLR

The action of acitretin on lymphocyte activation in MECLR was further examined using two sets of experiments. In the first set, the respective effects of one and several additions of 10\(^{-6}\)M acitretin on the proliferative response were tested. Addition of acitretin at the initiation of cultures (day 0) was crucial for inducing significant inhibition of proliferation (\(p<0.02\), Fig 4). Further additions, either on days 0, 1, 2, 3, and 4, or on days 0, 2, and 4, did not increase inhibition. Addition of acitretin to an ongoing MECLR

Table I. Two-Way Analysis of Variance Obtained With 10\(^{-6}\)M Retinol (A) and Acitretin (B) in the Four Subject Group (a, b, c, and d), During MLR and MECLR*

<table>
<thead>
<tr>
<th>% control</th>
<th>Retinol</th>
<th>MECLR</th>
<th>% control</th>
<th>Acitretin</th>
<th>MECLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLR</td>
<td>MECLR</td>
<td>MLR</td>
<td>MECLR</td>
<td>MLR</td>
<td>MECLR</td>
</tr>
<tr>
<td>a</td>
<td>105</td>
<td>95.5</td>
<td>a</td>
<td>91</td>
<td>78.75</td>
</tr>
<tr>
<td>b</td>
<td>96</td>
<td>103</td>
<td>b</td>
<td>94</td>
<td>82</td>
</tr>
<tr>
<td>c</td>
<td>97</td>
<td>95</td>
<td>c</td>
<td>88</td>
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</tr>
<tr>
<td>d</td>
<td>102</td>
<td>98</td>
<td>d</td>
<td>86.5</td>
<td>80</td>
</tr>
</tbody>
</table>

*The first factor F(subj) was the individuals, and the second F(reac) the reactions (MLR and MECLR). An interaction term F(int) between these two factors was systematically tested. MLR, mixed lymphocyte reaction; MECLR, mixed epidermal cell-lymphocyte reaction.
Table II. Effects of Cell Treatments with Acitretin on Subsequent MECLR

<table>
<thead>
<tr>
<th></th>
<th>LY (DMSO) +EC</th>
<th>LY (Acitretin) +EC</th>
<th>LY (DMSO) +EC</th>
<th>LY (Acitretin) +EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>MECLR Conditions</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Exp. 1</td>
<td>73452 ± 5210</td>
<td>59657 ± 4118</td>
<td>69358 ± 2014</td>
<td>60859 ± 3743</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>32840 ± 3824</td>
<td>22689 ± 5039</td>
<td>28673 ± 3683</td>
<td>25814 ± 2850</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>39749 ± 3060</td>
<td>30764 ± 3258</td>
<td>42100 ± 6416</td>
<td>31429 ± 4557</td>
</tr>
</tbody>
</table>

* Cell treatments with 10^{-6} M acitretin vs exposure to 0.01% DMSO (control) before MECLR, in three independent experiments. Responder lymphocytes [LY (DMSO) or LY (Acitretin)] and stimulator epidermal cells [EC (DMSO) or EC (Acitretin)] were treated separately overnight. Afterwards, cells were washed five times, and cultured in the MECLR system. Values represent mean [3H]thymidine incorporation ± SD. p values are given for DMSO-treated LY proliferations with acitretin-treated EC compared to those with DMSO-treated EC.
their HLA-DR antigen production. It might also act through the accessory functions of epidermal cells, by modulating the production of the cytokines quoted above. The retinoid effects on prostaglandin-E2 seem unlikely because here the prostaglandin inhibitor, indomethacin, had no action on the actretin inhibition. Alternatively, retinoids might also act directly on epidermal cell membranes.

It is noteworthy that, in our data, retinoids with a free carboxyl group in the C_5 position significantly inhibited the MECLR, whereas retinol and etretinate, which respectively have an alcohol and a dimethyl ether group in this position, induced a weaker or nonsignificant action. Because the availability of this free carboxyl function is a necessary condition for binding to the cellular retinoic acid binding protein (CRABP) [33], this latter might mediate the effects of active retinoids on epidermal cells during MECLR.

Thus, the predominance of CRABP in epidermal cells may explain in part the specific action of the synthetic retinoids in MECLR [34].

We observed that the synthetic analogues of vitamin A had a specific action in MECLR, whereas retinol did not. New active derivatives such as actretin, the main metabolite of etretinate, and arvinoid-free acid induced a greater inhibition than already commercialized retinoids such as isotretinoin and etretinate. MECLR might therefore be a useful tool for assessing the in vitro immunologic action of new molecules. However, it remains to be established that the down-modulation by retinoids of lymphocyte activation, and the magnitude of this regulation in vitro reflect the situation in vivo. The changes caused by retinoids in the interaction between epidermal cells and lymphocytes may partly explain their therapeutic effects on the skin.

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REFERENCES


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