The effect of oral retinoid therapy on the normal human immune system

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SUMMARY

Twenty four patients were studied prior to and after 6 and 12 weeks therapy with isotretinoin (17 patients) for acne and related disorders, or with etretinate (7 patients) for psoriasis and related disorders. Patients treated with isotretinoin had a significant reduction in natural killer cell activity at an effector: target cell ratio of 100:1 at 12 weeks and also a reduction in natural killer cell numbers at this time. Patients treated with etretinate had elevated natural killer cell activity and a significant elevation of natural killer cell numbers at 12 weeks. Other tests which were performed and showed no significant change at 6 or 12 weeks compared with starting levels included lymphocyte transformation in response to phytohaemagglutinin, pokeweed mitogen and concanavalin A, total numbers of circulating T lymphocytes, B lymphocytes and T helper and T suppressor subsets, numbers of epidermal Langerhans cells and serum levels of IgA, IgM and IgE. In view of the involvement of natural killer cells in the initial phase of organ rejection, these results suggest that isotretinoin is the safer of the two retinoids if administration to renal transplant recipients is considered, particularly in the immediate post-transplant period.

In the past decade the introduction of oral retinoid therapy has been a major step forward in dermatological therapeutics. The two retinoids currently prescribable in both Europe and North America are isotretinoin (Roaccutane® U.K., Accutane® U.S.A.) and etretinate (Tigason® U.K., Tegison® U.S.A.). Isotretinoin is of great value in control of severe nodulocystic acne, and etretinate is an advance in our management of the dyskeratoses, in particular psoriasis. A third major potential area for clinical use of oral retinoids is in the treatment and prevention of premalignant and malignant skin disease. Following encouraging early reports in the mouse papilloma model, a number of clinical studies have reported encouraging results in actinic keratoses, basal cell carcinoma, and squamous cell carcinoma. From these reports it would appear that relatively high doses of retinoid are required to have an effect, and that continuous treatment is required to prevent relapse.

It is now well established by the work of several groups that a long term complication of renal
transplantation and the necessary associated therapeutic immunosuppression is the development of large numbers of both premalignant and also malignant skin tumours, mainly on light exposed skin.\textsuperscript{11-13} The numbers of lesions appear to increase with time after transplantation. Although one study has suggested that a metabolite of azathioprine might be responsible,\textsuperscript{14} there appears at present to be no reduction in the rate of development of cutaneous lesions associated with the current switch in many transplant centres from azathioprine to cyclosporin. Animal work suggests that cutaneous malignancy is likely to remain a problem in patients given cyclosporin, with no prior azathioprine,\textsuperscript{15} and clearly, as technical problems of organ transplantation are overcome, and a cohort of long-term survivors is established, the cutaneous problems are likely to assume greater importance. Oral retinoid therapy might be of great value in this cohort of patients, provided that it was firmly established that retinoid treatment did not increase the risk of rejection of the transplanted organ.

The current literature on the immunological effects of retinoid therapy is confused, with varying results reported for animal models and man. In general the studies in man are confined to small studies of patients looking at only one test of immune function.\textsuperscript{16-18} Against this background, we have carried out a battery of tests of immune function, studying patients prior to and during therapy with both etretinate and isotretinoin. We felt it essential that this data was available before proceeding to consider oral retinoid therapy in organ transplant recipients, so that the choice between the two currently available retinoids could be made on logical grounds.

**METHODS**

**Patients**

Twenty four patients were studied in all. Seventeen patients (12 males, five females, mean age 26 years, range 18–49 years) received isotretinoin 1 mg/kg/day for 12 weeks. Fourteen of this group were treated for severe acne vulgaris non-responsive to oral antibiotics, two for rosacea, and one for hidradenitis suppurativa.

Seven patients (three males, four females, mean age 57 years, range 44–76 years) were treated with etretinate 0.5–0.75 mg/kg daily. Six of this group were treated for severe psoriasis vulgaris resistant to topical therapy, and one for ichthyosis vulgaris. Ethical Committee approval was obtained for the study.

**Immunological methods**

The immunological tests carried out included the following: Serum levels of circulating IgG, IgA and IgM were measured by standard radial immunodiffusion, and of IgE by a radioallergosorbent technique. Lymphocyte responses were measured to two concentrations of the mitogens phytohaemagglutinin (PHA) (0.25 \( \mu \)g and 0.1 \( \mu \)g PHA/10\(^6\) lymphocytes) pokeweed mitogen (1:10 and 1:100 dilutions of a 1 mg/ml solution) and concanavalin A, (0.25 \( \mu \)g and 0.025 \( \mu \)g/10\(^6\) lymphocytes). For the mitogen assays a concentration of 10\(^6\) purified lymphocytes/ml was assayed in triplicate; 256 \( \mu \)l autologous plasma was added to each well, and control wells contained only 25 \( \mu \)l of RPMI containing L-glutamine 2 mmol/ml, penicillin/streptomycin mixture 2 U/ml, and 5% heat inactivated foetal calf serum (all from Gibco, Paisley, U.K.). After 72 h culture at 37\(^\circ\)C in 5% CO\(_2\), plates were pulsed with 25 \( \mu \)l \(^3\)H thymidine per well (1 \( \mu \)Ci per well) and further incubated overnight. Thymidine was omitted from one control well to provide the background level. After 18 h exposure to tritiated thymidine, the plates were harvested on a Skatron cell harvester, and samples counted in a
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Tricarb series 3 scintillation counter (Packard). Results were calculated with the mean taken of each of the triplicate assays, and the mean of controls without tritiated thymidine subtracted. Results are expressed as counts per 10^6 cells.

Langerhans cells were quantitated by the standard suction blister technique. A 2 cm diameter area of normal epidermis was obtained by raising a blister on the upper inner arm by applying a 2 cm diameter suction cup and negative pressure for 45 min. The epidermis was then removed, snap frozen and stored at -20°C. All epidermal sheets were examined within 20 days of removal. Once thawed, the epidermal sheet was stained using a standard indirect immunoperoxidase technique.\textsuperscript{19} Anti-HTA\textsubscript{1} (Sera Laboratories) was used as a marker for Langerhans cells at a 1:100 dilution in Tris buffer. After visualization of Langerhans cells with 0.03% diamino benzidine, sections were permanently mounted and Langerhans cells counted per square millimetre. Langerhans cell bodies were only counted if a minimum of three dendrites were clearly identified emerging from the body of the cell.

For quantitation of T cell subsets, B cells and NK cells, all patients were venesected between 0830 and 0930 h to minimize effects due to diurnal variation. Heparinized blood samples were diluted in Hanks balanced salt solution without calcium and magnesium. Mononuclear cells were separated on a Ficoll-Hypaque (lymphoprep) gradient and the cells washed twice for 10 min in phosphate buffered saline. Twenty microlitres of antibody was added to 200 µl of phosphate buffered saline (PBS) containing 1 x 10^6 mononuclear cells, and incubated on ice for 30 min. The antibodies used were CD3 pan T marker UCHT-1 (Seward, Bedford, U.K.), CD4 Dako T4 helper marker (Dako, U.K.), CD8 T suppressor/cytotoxic marker (Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland) B cell marker Bl (Coulter) and natural killer cell marker Leu 7 (Becton Dickinson). After two further washes in PBS, cells were incubated for a further 30 min on ice with 100 µl of a 1:10 dilution of rabbit anti-mouse immunoglobulin F (ab)\textsuperscript{2} fragment (Dako) in PBS, conjugated to fluorescein isothiocyanate. After two further washes, cells were counted using a FACS analyser (Becton Dickinson Immuno Cytometry Systems, Mountain View, CA, U.S.A.). Correlation of wide angle light scatter and cell volume enabled lymphocytes to be resolved from monocytes, and also from any contaminating granulocytes. Gates were placed around the lymphocyte population to exclude all other cells, and fluorescence signals from 1 x 10^6 lymphocytes were analysed for each antibody.

For natural killer cell function, the mononuclear cell fraction of heparinized blood was isolated as detailed above. The lymphocytes were then washed three times in RPMI containing L-glutamine 2 mmol/ml, 2 U/ml of penicillin/streptomycin mixture and 5% heat inactivated foetal calf serum (Gibco). Cells were then resuspended in a small volume of this medium and counted. Viability was assessed by trypan blue exclusion and was never less than 95%. A final cell concentration of 2 x 10^6/ml was achieved and two further dilutions made for concentrations of 1 x 10^6/ml and 5 x 10^5/ml.

Target cells used in this assay were the erythroleukemic cell line K562, maintained in continuous culture in RPMI with additions as detailed above. Cells were sub-cultured routinely every 2 days and always 24 h before performing a cytotoxicity assay. An aliquot of culture medium containing 3 x 10^6 cells was incubated with 0.2 ml of sodium chromate\textsuperscript{41}, containing 20 MBq/ml activity, at 37°C for 1 h. The cells were then washed six times in RPMI plus additions and the final concentration adjusted to 2 x 10^4/ml. For the cytotoxicity assay, V bottomed micro titre plates (Flow) were used; 0.1 ml of each of the effector cell concentrations (2 x 10^6, 1 x 10^6 and 5 x 10^5) were incubated with 0.1 ml of the target cell preparation to achieve effector to target cell ratios of 100:1, 50:1 and 25:1. Effector cells and labelled target cells were incubated together for 4 h at 37°C in 5% CO\textsubscript{2}. Two controls were used, the first to estimate the degree of
spontaneous release of isotope from 0.1 ml of labelled target cells that had been incubated with 0.1 ml of medium alone, and the second to estimate the total activity by completely lysing target cells by the addition of 0.1 ml of 5 M sodium hydroxide to wells containing 0.1 ml of labelled target cells. All tests and controls were carried out in quadruplicate, and, following incubation, 0.1 ml of the cell free supernatant was removed from each well and counted for 3 min in a gamma counter (LKB 1282 Compugamma) to assess radioisotope release. Results were expressed as percentage lysis according to the following formula:

\[
\text{Percentage lysis} = \frac{\text{mean CPM sample} - \text{mean CPM spontaneous release}}{\text{mean CPM total activity} - \text{mean CPM spontaneous release}} \times 100
\]

where CPM is counts per minute.

All changes at 6 and 12 weeks compared with baseline levels were investigated for levels of significant variation by means of the paired Student’s t-test. Statistical significance was calculated at the 5% level.

RESULTS

Isotretinoin treated patients
In this group of patients, natural killer cell activity declined at all effector:target ratios at 6 and
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TABLE I. Natural killer (NK) cell numbers during etretinate and isotretinoin therapy

<table>
<thead>
<tr>
<th>NK cell numbers/10^6 lymphocytes</th>
<th>Prior to therapy</th>
<th>Week 6</th>
<th>Week 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotretinoin</td>
<td>551</td>
<td>516</td>
<td>470</td>
</tr>
<tr>
<td>Etretinate</td>
<td>636</td>
<td>628</td>
<td>809*</td>
</tr>
</tbody>
</table>

* P < 0.05 compared with baseline.

12 weeks and a significant reduction was seen at 12 weeks with an effector: target ratio of 100:1 (Fig. 1). Numbers of circulating natural killer cells fell during this period, but this change did not achieve statistical significance (Table 1).

A statistically significant rise in levels of circulating serum IgG was seen at 12 weeks by comparison with pre-treatment levels, but levels at 0, 6 and 12 weeks are all within the normal range for our laboratory. There were no significant changes in any of the other tests performed (Table 2).

If the two patients with rosacea and one with hidradenitis are excluded from analysis, the 14 patients with acne vulgaris also showed a significant decline in natural killer cell activity and an elevation of serum IgG.

TABLE 2. Lymphocyte numbers, mitogen responses, total immunoglobulins and Langerhans cell numbers in seven patients prior to and after 6 and 12 weeks of isotretinoin therapy

<table>
<thead>
<tr>
<th>Circulating lymphocyte numbers/10^6 mononuclear cells</th>
<th>Total T lymphocytes</th>
<th>Helper T cells</th>
<th>Cytotoxic suppressor T cells</th>
<th>B lymphocytes</th>
<th>Circulating immunoglobulin levels</th>
<th>IgG mg/100 ml</th>
<th>IgA mg/100 ml</th>
<th>IgM mg/100 ml</th>
<th>IgE IU/ml</th>
<th>Mitogen stimulation indices</th>
<th>PWM (1:10)</th>
<th>PWM (1:100)</th>
<th>Con A (1:10)</th>
<th>Con A (1:100)</th>
<th>PHA (1:100)</th>
<th>PHA (1:250)</th>
<th>Langerhans cells/mm^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week no.</td>
<td>Normal range</td>
<td>0</td>
<td>6</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>650-1900</td>
<td>1825 (631)</td>
<td>1109 (409)</td>
<td>1264 (221)*</td>
<td>1086 (228)</td>
<td>1109 (235)</td>
<td>1264 (221)</td>
<td>1086 (228)</td>
<td>57.2</td>
<td>69.5</td>
<td>77.7</td>
<td>14.4</td>
<td>26.14</td>
<td>200.4</td>
<td>882 (112)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>470-1300</td>
<td>1095 (409)</td>
<td>1135 (438)</td>
<td></td>
<td>1122 (542)</td>
<td>1095 (409)</td>
<td>1135 (438)</td>
<td>1122 (542)</td>
<td>79.3</td>
<td>82.6</td>
<td>86.2</td>
<td>15.7</td>
<td>38.7</td>
<td>267.3</td>
<td>939 (212)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>180-750</td>
<td>712 (274)</td>
<td>770 (333)</td>
<td></td>
<td>820 (388)</td>
<td>712 (274)</td>
<td>770 (333)</td>
<td>820 (388)</td>
<td>102.1</td>
<td>107</td>
<td>109</td>
<td>107</td>
<td>107</td>
<td>107</td>
<td>ND</td>
<td>998 (188)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>251 (128)</td>
<td>267 (150)</td>
<td>340 (228)</td>
<td></td>
<td>251 (128)</td>
<td>267 (150)</td>
<td>340 (228)</td>
<td>251 (128)</td>
<td>102.1</td>
<td>107</td>
<td>109</td>
<td>107</td>
<td>107</td>
<td>107</td>
<td>ND</td>
<td>998 (188)</td>
</tr>
</tbody>
</table>

* P = 0.001 compared with week 0.
ND = not determined.
Values in parenthesis are standard deviations.
TABLE 3. Lymphocyte numbers, total immunoglobulins, mitogen responses and Langerhans cell numbers in 17 patients prior to and after 6 and 12 weeks of etretinate therapy

<table>
<thead>
<tr>
<th></th>
<th>Normal range</th>
<th>Week no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Circulating lymphocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>numbers/10⁶ mononuclear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total T lymphocytes</td>
<td>650-1900</td>
<td>1599 (231)</td>
</tr>
<tr>
<td>Helper T cells</td>
<td>470-1300</td>
<td>1012 (412)</td>
</tr>
<tr>
<td>Cytotoxic suppressor T</td>
<td>180-750</td>
<td>665 (353)</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td>303 (176)</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Circulating immunoglobulin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG mg/100 ml</td>
<td>700-1900</td>
<td>1009 (221)</td>
</tr>
<tr>
<td>IgA mg/100 ml</td>
<td>90-450</td>
<td>1104 (113)</td>
</tr>
<tr>
<td>IgM mg/100 ml</td>
<td>45-180</td>
<td>108 (29)</td>
</tr>
<tr>
<td>IgE IU/ml</td>
<td>0-100</td>
<td>111 (138)</td>
</tr>
<tr>
<td>Mitogen stimulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>indices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PWM (1:10)</td>
<td>56±4</td>
<td>36±5</td>
</tr>
<tr>
<td>PWM (1:100)</td>
<td>44±2</td>
<td>33±7</td>
</tr>
<tr>
<td>Con A (1:10)</td>
<td>41±4</td>
<td>32±1</td>
</tr>
<tr>
<td>Con A (1:100)</td>
<td>10±4</td>
<td>6±9</td>
</tr>
<tr>
<td>PHA (1:100)</td>
<td>89±3</td>
<td>161±6</td>
</tr>
<tr>
<td>PHA (1:250)</td>
<td>91±9</td>
<td>108±6</td>
</tr>
<tr>
<td>Langerhans cells/mm²</td>
<td>882 (112)</td>
<td>812 (212)</td>
</tr>
</tbody>
</table>

ND = not determined.
Values in parentheses are standard deviations.

Etretinate treated patients
In this group of patients, natural killer cell numbers were significantly raised at 12 weeks and natural killer cell activity was also raised at 12 weeks, although this elevation did not reach statistical significance by comparison with pre-treatment levels. No other significant changes in tests of immune function were observed in the etretinate treated group (Table 3). Analysis of the six patients with psoriasis, with the exclusion of the one patient with ichthyosis, also revealed a significant elevation of natural killer cell activity at 12 weeks and a significant elevation in natural killer cell numbers at both 6 and 12 weeks.

DISCUSSION

The finding of particular interest in this study is the fact that natural killer cell numbers and function alter over a 12-week treatment period with both isotretinoin and etretinate, but in opposite directions. With isotretinoin the NK cell numbers and function are reduced, while with etretinate they are increased. The observed elevation of serum IgG levels in the isotretinoin treated group confirms an earlier study, but is unlikely to be of biological significance in view of the fact that the highest levels reached were still within the normal range of our laboratory.

A previous study carried out by Jansen et al. studied six patients with psoriasis, one patient with verruca vulgaris, one patient with condylomata accuminata and one patient with acne vulgaris. Seven patients received etretinate therapy in doses of 25-75 mg daily and two received isotretinoin in daily doses of 10-20 mg. The authors report an 'increasing trend' for NK activity during the first two months of treatment. Pigatto et al. assessed natural killer cell function in
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eight patients with psoriasis who received etretinate and nine patients with acne who received isotretinoin only, 2 weeks after starting therapy. They report increased NK function in the psoriatic etretinate treated group and no change in the acne isotretinoin treated group at this point in time. Kang and Ellis\textsuperscript{22} report no change in natural killer cell activity in patients treated with etretinate, 28 days after starting therapy.

All of these studies and our own, clearly make the basic assumption that the diseases for which retinoids are given are not associated with any general underlying immune defect which might alter either the initial findings or the observed changes during retinoid therapy. The fact that pre-treatment levels in all tests used in our study were within the normal range supports this assumption. There were no significant differences in pre-treatment results between our etretinate and isotretinoin treated groups, which do of course differ in their age range because of the use of isotretinoin in young acne patients. The relatively small number of patients in the etretinate treated group reflects the number of patients in this centre commencing etretinate over the 9-month period of this study, and continuing therapy for at least 12 weeks.

Our results show that it is important to report the effects of isotretinoin and etretinate on the immune system separately. It is not acceptable to group together results obtained in patients treated with these two retinoids. Their effects on the pilosebaceous follicle in acne are also very different and this difference clearly extends to their effect on natural killer cells. The possibility that natural killer cells are involved in graft rejection has been discussed in the past. Mowatt and Felstein\textsuperscript{23} investigated natural killer cell activity in murine graft-versus-host disease and suggest that natural killer cells may well be a non-specific component of immunological tissue damage. Similarly, Nemlander, Saksela and Hayry\textsuperscript{24} have identified natural killer cells in rat renal allografts during rejection and have also found natural killer cell activity to be high in the early rejection phase. Roy \textit{et al.}\textsuperscript{25} have also reported increased natural killer cell activity in the early phase of the graft-versus-host reaction, and Dokhelar \textit{et al.}\textsuperscript{26} demonstrate increased natural killer cell activity 9 days after receipt of bone marrow transplantation and also report a statistically significant correlation between high numbers of natural killer cells and acute graft-versus-host disease in the 1st month after receipt of a transplant. All these studies suggest that natural killer cells may well be involved in the early phases of graft rejection and graft-versus-host disease.

These data, together with the results of our study, suggest that if oral retinoids are to be used in renal transplant patients, particularly in the immediate post-transplant period, isotretinoin is less likely than etretinate to be associated with any increased risk of organ rejection. The work carried out to date on the relative effects of isotretinoin and etretinate in reversing or preventing premalignant cutaneous lesions suggests that both are effective in this situation.\textsuperscript{27} It should, however, be emphasised that, to date, these results are associated with long term continuous use of retinoid. The fact that at 12 weeks the trend for both NK cell numbers and activity shows a steep upward curve for etretinate is a further point to suggest that isotretinoin is likely to be the safer drug over a longer period. A further note of caution should be added with regard to etretinate in view of the recent case reports of patients developing malignancies during or shortly after etretinate therapy.\textsuperscript{28,29} No such reports are in print for isotretinoin.

Further work on NK cell numbers and function in patients treated for longer than 3 months with etretinate is in progress.

ACKNOWLEDGMENT

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