Induction of an Immature T-Cell Phenotype in Malignant Helper T Cells by Cocultivation With Epidermal Cell Cultures

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The possible inductive effect of epidermal cells on T-cell maturation has been examined employing an in vitro cocultivation technique. Mononuclear cells from 6 patients with cutaneous T-cell lymphoma (CTCL) and from 12 healthy volunteers were studied. In the 6 CTCL patients, all showed an expansion of the helper T-cell subpopulation and in one patient with leukemic CTCL, there was almost complete replacement of peripheral blood mononuclear cells by malignant cells with a helper T-cell phenotype. Epidermal cells derived from normal human skin were cultured to confluent monolayers, and were cocultivated with the mononuclear cells from CTCL patients or normal controls for 48 h at a density of 10^6/ml. Following cocultivation, the surface phenotype of the cells from the 12 healthy volunteers and 5 of the patients with CTCL showed no significant phentotypic change. In the patient with leukemic CTCL, however, the surface phenotype of the malignant T cells had changed, with the acquisition of the T6 antigen by the majority of the cells. Cells cocultivated in medium alone and with human fibroblast monolayers showed no change in surface phenotype. The malignant T cells from the leukemic CTCL patient failed to react in a mixed lymphocyte culture to lymphocytes from 2 different healthy donors, and showed no phenotypic change following culture with these lymphocytes, indicating that the phenotypic change seen was not due to allogeneic stimulation. *J Invest Dermatol 89:358–361, 1987*

The skin is now recognized as an immunologically active organ of the body, and over the last 5 years the epidermis has been shown to produce soluble substances that can influence T-cell function [1] and T-cell maturation [2,3]. Luger and associates [1] have demonstrated that epidermal cells produce a substance called epidermal thymocyte-activating factor, which is chemically and functionally identical to monocyte-derived interleukin 1. This substance is produced by Langerhans cells (LC) and keratinocytes. Chu and coworkers [3] have shown the presence of a substance immunologically identical to thymopoietin in basal keratinocytes. This substance appears to be produced by the keratinocytes but the functional activity of this substance is yet to be characterized.

Studies by Rubenfeld and colleagues [2] have demonstrated, in both murine and human systems, that in vitro cocultivation of bone marrow cells or circulating T cells will induce the production of the enzyme terminal deoxyribonucleotide transferase in a percentage of the mononuclear cells. Similar studies by MacKie and Hughes [4] have shown that phenotypic maturation of thymocytes can be induced by supernatants from epidermal cell cultures.

To further investigate the possible influence of human epidermis on T-cell maturation, we have used a cocultivation technique to look at changes in surface phenotypes in normal and malignant T cells.

MATERIALS AND METHODS

Mononuclear Cells Mononuclear cells were separated from 20 ml of heparinized venous blood taken from 12 healthy adult volunteers and 6 patients with leukemic cutaneous T-cell lymphoma (CTCL). Five patients with CTCL had stage 3 disease with plaques and tumors of the skin. These patients had white cell counts within normal limits. One patient was erythrodermic and had a circulating white cell count of 38×10^9/dl. At the time of the study none of the patients was having systemic chemotherapy. Three patients were being treated with psoralen + UVA (PUVA), one with local radiotherapy, one was on no therapy, and one was being treated by regular leukophoresis. Mononuclear cells were separated from the blood within 2 h of collection by velocity sedimentation on Ficoll Hypaque. Mononuclear cells were washed 3× in Hanks' buffered saline solution (HBSS) without calcium and magnesium, and were resuspended at a density of 10^7 cells/ml in RPMI with 10% fetal calf serum.

Epidermal Cell Cultures Epidermal cells were separated from skin obtained from surgical specimens into a single cell suspension by overnight trypsinization of the cells at 4°C as previously de-

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Abbreviations:

CTCL: cutaneous T-cell lymphoma
FACS: fluorescence-activated cell sorter
HBSS: Hanks' buffered saline solution
LC: Langerhans cell(s)
PBS: phosphate-buffered saline

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scribed [5]. The viability of the cells was tested by trypan blue exclusion and was constantly over 90%. Epidermal cell cultures were then established in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 0.4% μg/ml hydrocortisone in plastic Petri dishes as described by Eisinger and colleagues [5]. After 7 days, the cultures had achieved a confluent monolayer and at this stage were used in the cocultivation studies.

Cocultivation of Mononuclear Cells and Epidermal Cells Mononuclear cells from volunteers and the patients with CTCL were washed in HBSS and resuspended in epidermal growth medium at a density of 10^6 cells/ml. Epidermal cell monolayers were drained of their growth medium and 5 ml of the mononuclear cell suspension was introduced per 25 cm^2 Petri dish. Mononuclear cells were also plated onto human fibroblast monolayers derived from normal human skin, and also incubated in clean Petri dishes in epidermal cell growth medium.

Cells were cultured for 48 h at 37°C and 5% CO_2 and were then harvested and washed 3× in HBSS. These studies were repeated on 3 different occasions. On one occasion with patient AJ, the cells were incubated with epidermal cell supernatants for 48 h at 37°C and 5% CO_2 before harvesting.

Monoclonal Antibodies The monoclonal antibodies used in this study were kindly provided by Dr. Gideon Goldstein, Ortho Pharmaceuticals, Raritan, New Jersey. These were OKT3, which recognizes a pan T-cell antigen [6]; OKT4, which recognizes the helper/inducer subpopulation of T cells [7]; OKT8, which recognizes the suppressor/cytotoxic subpopulation of T cells [7]; OKT10, which recognizes a pan thymocyte antigen and also some myeloid and monocytic bone marrow precursors [7]; OKT6, which recognizes the cortical thymocytes and LC in the skin [7]; and OKI, which recognizes the HLA-DR antigen [7]. As a second antibody, at light microscopy a fluorescein-conjugated rabbit anti–mouse immunoglobulin (Miles Laboratories, Cambridge, U.K.) was used.

T-Cell Subpopulations T-cell subpopulations were estimated using an indirect immunofluorescent technique as previously described [8]. In brief, 100 μl of a 10^7 cell/ml suspension of the mononuclear cells were incubated with an appropriate dilution of the monoclonal antibody for 30 min at 4°C. After 3 washes in phosphate-buffered saline (PBS), the cells were incubated for 30 min at 4°C with a fluorescein-conjugated rabbit anti–mouse antibody. After a further 3 washes in PBS, the cells were resuspended in 50% glycerol in PBS and examined under a Leitz fluorescent microscope. Two hundred cells were counted under light microscopy, and the percentage of fluorescent cells assessed.

Langerhans Cell Status of the Epidermal Cell Cultures To ensure that LC did not contaminate the epidermal cell cultures and provide a possible exogenous source of T6 antigen, cultures were examined by indirect immunofluorescent and electron microscopy at day 1, day 7 of culture, and following cocultivation with mononuclear cells.

At these times, cells in the supernatant were harvested and examined with 3 washes of the adherent cell layer and the adherent cells were removed by trypsinization and examined after washing in HBSS.

Cells from the nonadherent and adherent populations were divided into 2 parts: one part was used in a standard indirect immunofluorescent technique [8] using OKT6 and OKI, and part was fixed in 2% glutaraldehyde and prepared for standard transmission electron microscopy as previously described [9].

Mixed Lymphocyte Reaction Mononuclear cells from the patient with leukemic CTCL and 2 healthy controls were separated from blood as described and used in the mixed lymphocyte reaction. The normal controls were used as inducer cells and also to establish a normal response. Inducer cells were irradiated prior to use with 3440 cGy and were used at a density of 3×10^6 cells/ml. One hundred microliters of the inducer cell suspension was mixed with an equivalent number of responder cells in 96-well flat-bottomed plates in triplicate. After 4 days incubation at 37°C and 5% CO_2 wells were pulsed with [^{3}H]thymidine and incubated for a further 18 h before harvesting onto filter papers. Stimulation was measured using a β counter.

Phenotypic change of the cells following allogeneic stimulation was assessed using a similar system employing 1 ml volumes of cells in flat-bottomed tubes. The cells were harvested at 5 days without Tritium labeling and the T-cell subpopulations examined using monoclonal antibodies in an indirect immunofluorescence technique.

RESULTS

Langerhans Cells in the Epidermal Cell Cultures At day 1 of the epidermal cell cultures, LC were observed in both adherent and nonadherent cell populations by indirect immunofluorescence and electron microscopy, representing 3 ± 1% of the nonadherent and 2 ± 1% of the adherent cells. By day 7, no OKT6 or OK1 cells were identifiable in the epidermal cell cultures by immunofluorescence and no cells with a micromorphology similar to LC could be found, even on extensive examination of 30 different grids on electron microscopy. Following cocultivation with the mononuclear cells, the adherent cell population was examined (this was performed in 3 CTCL stage 3 patients and 6 normal volunteers) and once again, LC could not be found on indirect immunofluorescence or electron microscopy.

T-cell Subpopulations (Table I) Prior to cocultivation, the T-cell subpopulations in the normal controls were within normal limits, 74 ± 7% T cells, 45 ± 7%, helper-T cells and 28 ± 7% suppressor-T cells. No cells in the normal controls expressed the T6 antigen. The 5 patients with stage 3 CTCL showed a mild elevation of the total T cells (80 ± 11%) but a significant expansion of the helper-T cells compared with controls (60 ± 13%) with normal suppressor-T cell numbers (19 ± 6%). No OKT6 + T cells were identified. The patient with leukemic CTCL showed a major expansion of T cells (98%) which was composed of T4 + helper-T cells (87%). No OKT8 or OKT6 + cells were identified. In all preparations background fluorescence was very low, and in the OKT6 preparations, the cells were completely unlabeled.

Table I. T-Cell Subpopulations Before and After Cocultivation With Epidermal Cell Cultures

	OKT3	OKT4	OKT6	OKT8	OKT10
Prior to cocultivation					
Control (n = 12)	74 ± 7	45 ± 7	0	28 ± 7	5 ± 4
CTCL-AJ (n = 1)	98	87	0	0	0
CTCL stage 3 (n = 5)	80 ± 11	60 ± 13	0	19 ± 6	4 ± 3
Post cocultivation					
Control (n = 12)	69 ± 5	55 ± 6	0	10 ± 3	3 ± 1
CTCL-AJ (n = 1)	88	89	80	0	0
CTCL stage 3 (n = 5)	75 ± 6	61 ± 9	0	11 ± 7	5 ± 3

CTCL, Cutaneous T-cell lymphoma; AJ, patient.

Following cocultivation with epidermal cells, viability of the cells, as shown by trypan blue exclusion tests, were shown to be >90%. The lymphocytes from controls and 5 stage 3 CTCL patients showed a marginal loss of suppressor (OKT8 + ve) T cells, but no cells expressing the T6 antigen. In the leukemic CTCL cells from patient AJ, the majority of cells had acquired the T6 antigen (98%) and showed very bright fluourescence. This was a consistent finding in all 3 experiments performed and in the single experiment in which epidermal cell supernatants were used.

In cells cocultivated with normal human fibroblasts or cultured alone, no phenotypic change was noted in the lymphocytes present.

Mixed Lymphocyte Cultures (Table II) Leukemic CTCL cells from patient AJ failed to respond to allogeneic stimulation in these tests. Normal controls gave positive responses. Following the 5-day incubation with inducer cells, the CTCL cells also failed to show any phenotypic change and no OKT6 + ve cells were identified.

DISCUSSION

Rubenfeld and colleagues [2] first described the inductive effect of epidermal cells on T-cell maturation. In their study, they described the induction of the enzyme terminal deoxynucleotidyl transferase in bone marrow cells, null-enriched peripheral blood cells, and E-rosette + cells by cocultivation with epidermal cells. Terminal deoxynucleotidyl transferase is the earliest marker of the T-cell lineage and is usually only demonstrable in bone marrow and thymus cells, being lost by mature and circulating T cells [10]. The finding of the induction of an immature T-cell marker in mature circulating T cells was particularly interesting, and the authors suggested that this was due to an inductive effect on postthymic precursor T cells [11].

The demonstration by Chu and colleagues [3] of a thymopoietin-like substance in normal human epidermal cells suggested a possible mediator for the phenotypic changes seen in the study by Rubenfeld and associates [2]. The functional characteristics of this substance, however, remain to be elucidated.

In our study, we have demonstrated the induction of an immature T-cell antigen, T6, on leukemic T cells which express a mature helper T-cell phenotype. OKT6 recognizes the HTA-1 antigen [12] on 70% of cortical thymocytes [7], but also recognizes epidermal LC and indeterminate cells [9,13]. Our data confirm that this is a specific effect of epidermal cells on the leukemic T cells which is not related to allogeneic stimulation.

There are 3 possible explanations for this phenomenon: (1) that the leukemic CTCL cells expressed very low levels of T6 antigen and this was intensified by the inductive influence of the epidermal cells, (2) that this is due to a specific inductive influence on a particular subpopulation of T cells by the epidermis, and (3) that this is due to the presence of exogenously produced T6 antigen which is cytophilic for the leukemic T cells.

In this study, an indirect immunofluorescent technique with direct visibility was employed, as at the time of the study a fluorescence-activated cell sorter (FACS) was not available. Unfortunately, the studies on patient AJ cannot be repeated as the patient subsequently died. In the study, however, the background fluorescence was undetectable by direct visibility, and there was no observed fluorescence of AJ's cells prior to cocultivation when stained with OKT6. Although FACS are more sensitive than the naked eye, in our experience over the last 2 years with several hundred specimens, the FACS and manual counting of cells stained with indirect immunofluorescence equate very well, and we have yet to pick up positive staining on FACS which was not obtainable on manual counting.

The results of this study cannot, thus, totally discount this possible explanation but nevertheless, the results do indicate a specific inductive influence of the epidermal cells on the malignant helper T cells.

The T6 antigen is expressed on 2 diverse but interrelated cell lines. The immature thymocyte expresses the T6 antigen within the milieu of the thymus, but loses it as it matures to the medullary thymocyte and T6 antigen is not expressed by mature T cells [7]. In the skin, the immigrant population of dendritic antigen-presenting cells, the LC, appears to acquire the T6 antigen in the milieu of the skin [9]. The LC and thymocytes are of very different lineages, but LC and T cells do interact in a specific fashion in immunologic responses in the skin [14]. The thymus and skin do share a number of similarities, and authors have suggested that the skin may be capable of thymus-like activity, possibly directed to a definite subpopulation of skin-related T cells [15]. A skin-related T-cell subpopulation has not been positively identified, but it seems likely that the cells involved in CTCL, a malignant expansion of helper T cells originating in the skin, represent such a skin-related cell population [16]. The data provided in patient AJ with leukemic CTCL in this study would support this, and would indicate an inductive effect of a human epidermal cytokine on a T-cell subpopulation at a particular ontologic stage. Results of the control blood cells and the 5 other patients with stage 3 CTCL showed no phenotypic change following cocultivation with human epidermal cells but this may reflect the relative maturity or immaturity of the T cells present. Another possible explanation could be that other mononuclear cells present—i.e., suppressor T cells—could be inhibiting the response of the T cells to the epidermal cytokine. In patient AJ, no suppressor T cells were identified. To examine this further, we have purified helper (T4 + ve) T cells by E-rosetting and panning on OKT4 coated plates, and used these cells in the cocultivation studies. Two experiments failed to show any phenotypic change in the purifield helper T cells (unpublished data).

Within the skin, the only cell that produces the T6 antigen is the LC. In the epidermal culture system that we use, we have confirmed that LC are lost from the culture after 7 days and no OKT6+ cells or cells with the ultrastructural phenotype of LC are seen after this time. In this study, epidermal cell cultures were only used after 7 days culture, and thus no LC would be present. The possibility of T6 antigen being produced by the epidermal cells and bound exogenously to the leukemic T cells can thus be discounted.

Studies are now under way to examine further patients with CTCL to try to reproduce the results seen with this single leukemic CTCL patient and also to examine T-cell subpopulations at different stages in their ontogeny to identify an ontologic point at which T cells are sensitive to the inductive influences of the epidermis. We are also investigating epidermal cell supernatants to try to isolate and characterize the factor responsible for the changes we have identified.

Table II. Mixed Lymphocyte Cultures

	Cells	Counts
A-inducer	B	3168
	A + B	33,652
	C	2013
	A + C	1198
B-inducer	A	3890
	B + A	25,760
	C	1086
	B + C	3102

A, Control I; B, Control II; C, Cutaneous T-cell lymphoma.

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