Enrichment of Human Epidermal Langerhans Cells by Attachment to Erythrocyte Monolayers

Liisa Räsänen, M.D., Maili Lehto, B.S., Timo Reunala, M.D., and Pauli Leinikki, M.D.
Institutes of Biomedical Sciences and Clinical Medicine, University of Tampere, Tampere, Finland

The present investigation introduces a method for purification of human epidermal Langerhans cells (LC). The method is based on the attachment of LC to IgG-coated sheep erythrocyte monolayers via their Fc receptors. To optimize the enrichment assay, several variables were tested. The best results were obtained when epidermal cells were centrifuged against erythrocyte monolayers; the purification procedure was performed at 4°C in the presence of 5% fetal calf serum, using about 6×10^6 epidermal cells per erythrocyte plate (diameter 5 cm). The average purity of the recovered LC was 80.9% and LC-depleted fractions contained an average of 0.5% DR-positive cells. LC were able to enhance significantly leukoagglutinin- and purified protein derivative-induced T lymphocyte proliferation and leukocyte migration inhibitory factor production. *J Invest Dermatol 86:9–12, 1986*

Human epidermal Langerhans cells (LC) constitute only 3–8% of all epidermal cells [1]; nevertheless, they play an important role in lymphoepidermal interactions. They possess receptors for Fc-IgG and C3 [2] and express HLA-DR antigens, which are associated in presenting antigens for T lymphocytes [3,4]. Their function as antigen-presenting cells in cutaneous hypersensitivity reactions seems well established, and for certain antigens their capacity exceeds that of peripheral blood monocytes on a per cell basis [5,6]. Recent studies suggest that LC are related to dendritic cells, which are widely distributed in lymphoid and nonlymphoid organs and participate in immune reactions [7].

Enrichment of LC followed by coculture with other purified cells is required to obtain detailed information about their functions. In this paper we describe a purification method based on the attachment of epidermal LC to IgG-treated erythrocyte monolayers. Epidermal cells were obtained from suction blisters. To test the functional capacity of purified LC they were cocultured with antigen- and mitogen-stimulated T lymphocytes, and proliferation and lymphokine production of T cells were measured.

MATERIALS AND METHODS

Enzyme Treatment of Epidermal Sheets Epidermal sheets were obtained by a suction blister device [8] from uninvolved abdominal skin of various dermatologic patients. Epidermal sheets were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin (Gibco Ltd., Paisley, Scotland) at 37°C for 30 min with occasional shaking. DNase I (final concentration 0.01%, type III, Sigma Chemical Co., St. Louis, Missouri) was then added and the sheets were pipetted vigorously with a Pasteur pipette for a couple of minutes. RPMI-1640 (Orion Diagnostica, Helsinki, Finland) supplemented with penicillin, streptomycin, and amphotericin B (antibiotic-antimycotic solution, Gibco Ltd.), L-glutamine, and 5% fetal calf serum (FCS, Flow Laboratories Ltd., Irvine, Scotland) was added on epidermal sheets and cells, which were spun down by centrifuging at 200 *g* for 5 min. The supernatant was sucked off and discarded. Thereafter RPMI-1640 containing 0.01% DNase I and 5% FCS was added to the tubes and their contents pipetted vigorously. Single cell suspensions were removed to another tube, discarding stratum corneum sheets. We found the DNase treatments essential to avoid mucous reaggregation of the cells. The cells were washed twice with 5% FCS-RPMI. Thereafter the cell suspension was sucked into a syringe through a 25GI needle and filtered through a nylon mesh (pore size 80 μm). In preliminary experiments, crude epidermal cells were preincubated at 37°C for 30 min or not incubated before the enrichment procedure. No differences were found and thus the preincubation step was omitted in subsequent experiments.

Enrichment of LC LC were isolated by attaching them to sheep erythrocyte monolayers treated with IgG. The method described by Clayman and Schmidtke [9] for the enrichment of Fc receptor-positive cells from peripheral blood was used with modifications. Tissue culture dishes (diameter 5 cm) (Sterilin Ltd., Teddington, Middlesex, England) were first treated with 0.005% poly-L-lysine (M_r 40,000, Sigma Chemical Co.) at room temperature for 45 min. The plates were rinsed 3 times with PBS. A 2.5 ml quantity of a 1% sheep erythrocyte suspension (SRBC) was added to the dishes, which were incubated at room temperature for 45 min followed by 2 rinses with PBS. Sterile PBS (2.5 ml) was added to each dish and the plates kept at 4°C overnight. On the following day 2.5 ml of a 1:1000 dilution of rabbit antisheep red blood cell IgG (Cappel Laboratories Inc., Cochranville, Pennsylvania) was added. The plates were kept at 37°C for 45 min and then rinsed 3 times with PBS. They were used on the same day to enrich LC. Unless otherwise stated, 6×10^6 epidermal cells in 2.5 ml of 5% FCS-HEPES-RPMI were put per plate. The dishes were centrifuged at 200 *g* for 5 min to enhance the binding of skin cells to SRBC monolayers. As a rule, the dishes were then kept at 4°C for 90 min. The plates were rocked gently by hand and the supernatants removed and saved. Thereafter the dishes were rinsed twice with 2.5 ml PBS and these supernatants discarded. To detach LC from the erythrocyte monolayers, SRBC were lysed by incubating with 2.5 ml of 0.83% NH_4Cl-Tris buffer, pH 7.2, at room temperature for 5

Manuscript received March 25, 1985; accepted for publication July 24, 1985.

This work was supported by grants from the Sigrid Juselius Foundation, the Paulo Foundation, and the Orion Corporation Research Foundation.

Reprint requests to: Liisa Räsänen, M.D., Institute of Biomedical Sciences, University of Tampere, Box 607, SF-33101 Tampere 10, Finland.

Abbreviations:
- FCS: fetal calf serum
- LA: leukoagglutinin
- LC: Langerhans cells
- LIF: leukocyte migration inhibitory factor
- MI: migration index
- PBS: phosphate-buffered saline
- PPD: purified protein derivative of tuberculin
- SRBC: sheep red blood cells

0022-202X/86/$03.50 Copyright © 1986 by The Society for Investigative Dermatology, Inc.

min and then pipetted vigorously. The LC-enriched and -depleted fractions were washed twice with RPMI, checked for purity, and cocultured with T cells.

Separation of T Lymphocytes Mononuclear cells containing lymphocytes and monocytes were obtained by Ficoll-Isopaque centrifugation of heparinized venous blood [10]. Monocytes were depleted from lymphocytes in two phases. Cells attached to plastic surfaces were removed followed by depleting carbonyl iron-fed cells with a magnet. To obtain T cells, lymphocytes were rosetted with neuraminidase-treated sheep erythrocytes and centrifuged by Ficoll-Isopaque [11].

Cellular Markers The amount of LC among crude epidermal cells was assessed by Fc rosette formation or staining DR- or T6-positive cells with fluorescent antibodies. As LC-enriched and -depleted fractions rosetted erythrocytes poorly, their purity was studied with monoclonal antibodies. For rosettes, SRBC were treated with anti-SRBC IgG (final dilution 1:1000, Cappel Laboratories) at 37°C for 30 min, washed 3 times, and a 1% suspension was prepared. Epidermal cells in 5% FCS-RPMI and 1% IgG-coated SRBC were mixed, centrifuged for 5 min at 200 *g*, and incubated in an ice bath for 1–2 h. Nucleated cells were stained with 0.02% acridine orange and the rosettes counted under a fluorescence microscope; 200–500 cells were counted.

The purity of T-lymphocyte populations was studied using SRBC rosette formation to demonstrate T cells, staining DR-positive cells to demonstrate B cells or monocytes, and staining nonspecific esterase to demonstrate monocytes [12]. Staining of DR- or T6-positive cells in skin cell fractions and DR-positive cells among T cells was performed with OKIa1 and OKT6 (Ortho Diagnostic Systems Inc., Raritan, New Jersey) and fluorescein isothiocyanate-conjugated antimouse IgG antibodies (Gibco Ltd.) according to standard procedures. The viability of all cell fractions was assessed by the exclusion of trypan blue.

Cell Cultures T cells, 25 × 10³ per well of V-bottomed microplates (Sterilin Ltd.), were incubated alone or with LC-enriched or -depleted fractions. The cells were stimulated with leukoagglutinin (LA, Pharmacia Fine Chemicals, Uppsala, Sweden) or purified protein derivative (PPD, State Serum Institute, Copenhagen, Denmark) and cultured in RPMI-1640 supplemented with penicillin, streptomycin, amphotericin B, and 10% autologous plasma for 5 days.

Lymphocyte Transformation and Leukocyte Migration Inhibitory Factor (LIF) Tests Sixteen hours before harvesting, 4.625 kBq of [5-^{125}I]iodo-2′-deoxyuridine (Amersham, Buckinghamshire, England) containing 1 μM fluorodeoxyuridine was added per well of transformation cultures. The uptake of the isotope was measured with a gamma counter. The results were expressed as net cpm values: cpm value in stimulated culture − cpm value in corresponding control culture.

LIF activity was tested in the culture supernatants by the agarose migration method using purified granulocytes as indicator cells [11]. The migration index (MI) was defined as follows:

$$MI = \frac{\text{(area of migration in the presence of test supernatant)}}{\text{(area of migration in the presence of control supernatant)}}.$$

RESULTS

Optimizing the Method for LC Enrichment In order to find optimal conditions for the LC-enrichment assay, several variables were tested. First, the effect of FCS was checked. Serum, due to its IgG, might have interfered with the binding of LC to IgG-coated erythrocytes. Table I shows that the percentages of Fc rosettes in crude epidermal cells were practically the same in the presence or absence of FCS. Neither did FCS affect the purification of LC determined on the basis of DR-positive cells among LC-enriched fractions (Table I). However, serum had a clear-cut beneficial effect on the viability of epidermal cells (Table I). Thus in all subsequent experiments, 5% FCS was used throughout the separation procedure.

When the purification assay was performed at 4°C, room temperature, and 37°C, the best results were obtained at 4°C. At this temperature LC-enriched and -depleted fractions contained on the average 80.1% (range 75.9–84.1%, n = 3) and 0.4% (0.2–0.8%) DR-positive cells, respectively. However, there were no great differences between the results at 4°C and room temperature. At 37°C the purities of LC-enriched and -depleted fractions were lower, and they contained 64.5 and 2.5% DR-positive cells, respectively.

Increasing the amount of crude epidermal cells from 3 × 10⁶ to 12 × 10⁶ per plate did not affect the purity of the recovered LC (Table II). The purity of LC-depleted fractions was somewhat lower at the highest cell density tested (Table II). As a rule, about 6 × 10⁶ skin cells per plate were used in subsequent experiments.

Characterization of Skin Cell Fractions and T Lymphocytes with Different Markers Skin cell fractions and T lymphocytes were analyzed for purity and viability. T-lymphocyte populations (viability >98%) contained >90% SRBC-rosetting cells, <5% DR-positive cells, and <0.2% nonspecific esterase-positive cells, i.e., monocytes. Table III gives the results on epidermal cells. In the purification, 6 × 10⁶ cells per plate were used and the procedure performed at 4°C. The viability of crude epidermal cells and LC exceeded 80%, whereas that of LC-depleted fractions was slightly lower. The percentages of Fc rosettes and DR-positive cells among crude epidermal cells were very similar, 4.0% and 4.2%, respectively. Epidermal cells did not form SRBC rosettes. Neither could we detect lymphocytes or monocytes in epidermal cell smears stained for nonspecific esterase. Determined on the basis of DR-positive cells, the average purity of LC-enriched fractions was 80.9% (range 59.4–95.1%) and there were 0.5% (<0.2–1.1%) LC left in LC-depleted fractions. In 4 experiments LC-enriched and -depleted fractions were stained with OKIa1 and OKT6 antibodies. The results were *practically the same*. LC-enriched populations contained 84.3% DR-positive cells and 87.5% T6-positive cells, whereas LC-depleted fractions were contaminated with 0.5% DR- or T6-positive cells.

Enhancement of T-Cell Proliferation and LIF Production by LC In coculture experiments with T cells and epidermal cells, suboptimal concentrations of PPD and LA were used. It was thought that at suboptimal stimulant concentrations the possible enhancing effect of LC could be better seen. Fig 1 depicts

Table I. Effect of Serum on Cell Viability, Fc Rosette Formation, and Langerhans Cell Enrichment

Serum	Crude Epidermal Cells		Enriched Langerhans Cells	
	Viability	Fc Rosettes	Viability	DR-positive Cells
Present[a]	84.3 (81.2–89.4)[b]	3.8 (3.5–4.3)	88.8 (84.8–91.6)	77.9 (69.6–91.9)
Absent	76.1 (68.0–82.5)	3.5 (3.0–4.2)	73.5 (63.9–81.0)	81.1 (74.5–93.7)

[a]Five percent FCS.
[b]Mean percentage and range of 3 experiments.

Table II. Effect of Crude Epidermal Cell Density Per Plate on Langerhans Cell Enrichment

Cell Population	Amount of Epidermal Cells Per Plate		
	3×10^6	6×10^6	12×10^6
Langerhans cell-enriched	84.0 (75.1–91.9)[a]	84.7 (78.9–89.3)	77.5 (66.4–87.2)
Langerhans cell-depleted	0.4 (0.3–0.5)	0.5 (0.3–0.6)	0.8 (0.4–1.1)

[a]DR-positive cells, mean percentage, and range of 3 experiments.

the results of transformation experiments. T-cell proliferation was significantly augmented by LC, even 0.3×10^3 LC among 25×10^3 T cells were effective; $5–10 \times 10^3$ LC-depleted cells cocultured with T cells also enhanced their proliferation to some degree, probably due to contaminating LC. However, there was a clear-cut difference between these enriched and depleted fractions. The cpm values of T-cell cultures supplemented with 10×10^3 LC were about 4-fold (PPD) or 14-fold (LA) compared with those supplemented with the same amount of LC-depleted cells.

Fig 2 shows how LC also augmented T-cell LIF production. LIF synthesis seemed to be dependent on accessory cells, at least at suboptimal stimulant concentrations. No significant LIF production occurred in cultures containing no accessory cells or in cultures supplemented with $0.3–10 \times 10^3$ LC-depleted cells. In the case of PPD or LA, 1.3 or 0.6×10^3 LC, respectively, were sufficient to restore T-cell LIF production.

DISCUSSION

Here we describe a method to enrich functionally competent LC from the human epidermis by attaching them to IgG-coated SRBC monolayers. The average purity of the LC-enriched fractions was about 80% and these cells were able to augment mitogen- and antigen-induced T-lymphocyte proliferation and lymphokine production.

Even if LC resemble monocytes and macrophages as to origin, surface membrane markers, and function [13], they are less firmly adherent cells, at least in vitro. They cannot be isolated, unlike the monocytes, by attaching to nontreated plastic surfaces. The fact that LC carry receptors for the Fc portion of IgG [2] was utilized and the method described by Clayman and Schmidtke [9] applied with modifications in the present study. We found that centrifugation of epidermal cells against erythrocyte monolayers clearly enhanced the purity of the recovered LC. If the centrifugation step was omitted, the purity of the LC-enriched fractions did not exceed 20%. The enrichment method used here is simple, does not require expensive reagents and equipment, and yields LC of fairly high purity. Our assay has one obvious limitation due to the nonspecificity of the Fc-IgG receptor. B cells, T-cell subsets, and mononuclear phagocytes also possess this receptor and these cells may infiltrate the epidermis in inflammatory cutaneous disorders. However, neither we nor others [14–16] were able to detect other Fc receptor-positive cells in normal epidermis. If these cells are present in normal epidermis, they must be extremely few in number.

Table III. Purity and Viability of Epidermal Cell Fractions

Cell Fraction	Viability	Fc Rosettes	DR-positive Cells
Crude epidermal cells	83.3 ± 1.3[a]	4.0 ± 0.4	4.2 ± 0.3
Langerhans cell-enriched	86.7 ± 2.4	n.d.[b]	80.9 ± 4.2
Langerhans cell-depleted	78.8 ± 2.0	n.d.	0.5 ± 0.1

[a]Mean ± SEM of 12 experiments.
[b]n.d. = Not done.

Several methods have been described for the purification of LC. These techniques are based on rosette formation followed by Ficoll or Percoll flotation [17,18], attachment to pretreated surfaces on plastic plates [15,19–21], and flow cytometry sorting [15]. In the method by Bjercke et al [18], human LC coated with OKT6 were allowed to form rosettes with ox erythrocytes sensitized with antimouse IgG. Rosette-forming cells were separated by Percoll centrifugation and the average purity of the recovered LC was 79%. In the panning method of Morhenn et al [19], OKT6-treated LC adhered to plastic dishes coated with antimouse immunoglobulin. The average purity of the LC-enriched fractions was about 70%. Wood et al [20] recently published a panning method in which pan-leukocyte monoclonal antibodies were used instead of OKT6. The LC-enriched fractions contained 80–99% mononuclear cells, almost all of which had the ultrastructural features of LC. Schuler et al [21] took advantage of the binding of anti-Ia-treated LC to *Staphylococcus aureus* Cowan I monolayers. Their enriched fractions contained an average of 74% LC. Using flow cytometry sorting, Scheynius et al [15] were able to obtain LC the average purity of which was 79%. In preliminary experiments we also tested flow cytometry sorting but found it time-consuming and technically difficult. Upon standing, epidermal cells tended to form clumps. This and fluorescence of dead cells brought additional difficulties.

In functional studies, antigen- or mitogen-stimulated T cells were cocultured with LC. Using V-bottomed microplates it was possible to diminish the cell amount to a fourth part of that ordinarily used and thus more experiments could be conducted.

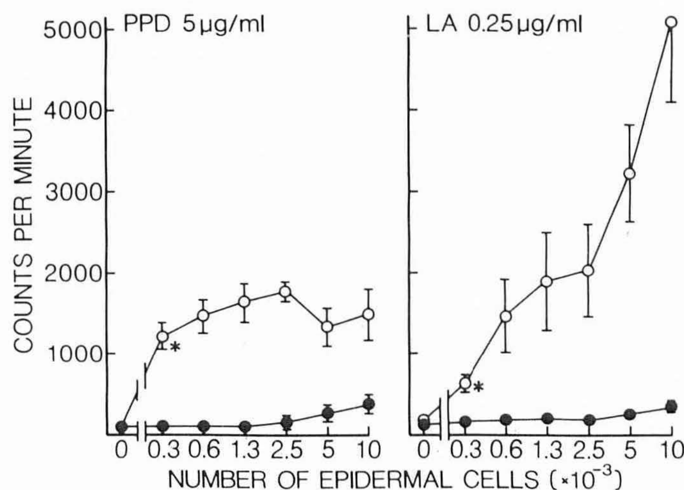

Figure 1. Augmentation of PPD- or LA-induced proliferation of T lymphocytes by Langerhans cells. 25×10^3 T cells per well were cultured in the absence or presence of indicated numbers of epidermal cells. *Closed symbols* = Langerhans cell-depleted fractions, *open symbols* = Langerhans cell-enriched fractions. Values are mean cpm ± SEM of 4–6 experiments. The *asterisks* express statistical significance of differences ($p < 0.05$ or $p < 0.01$, Mann-Whitney's U test) between cultures containing and not containing LC. Only the first significantly different values have been marked with an asterisk.

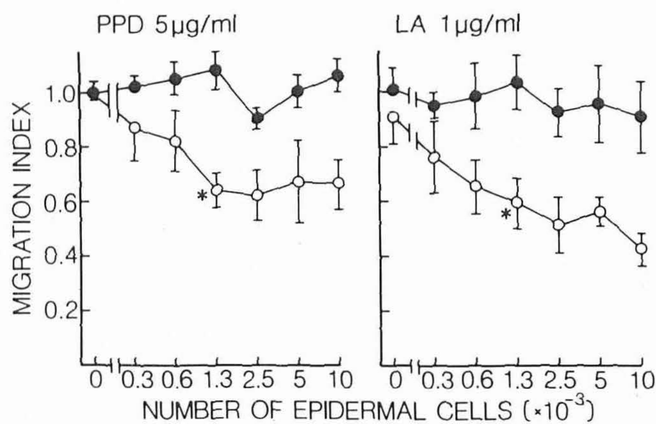

Figure 2. Production of LIF in the presence of Langerhans cell-enriched (*open symbols*) and cell-depleted (*closed symbols*) populations. Values are mean MI ± SEM of 4–6 experiments. For other explanations, see the legend to Fig 1.

LC appeared to be very potent accessory cells; about 1% of them enhanced T-cell functions. Probably even smaller amounts would have been effective if they had been tested.

In summary, the enrichment method described here is easy to perform, inexpensive, and gives relatively pure LC. Purification is a prerequisite for further studies, e.g., how LC cooperate with T lymphocytes and other cells. In the future, it will be intriguing to learn about the role of LC in various diseases and how medical treatments affect them.

REFERENCES

1. Friedmann PS: The immunobiology of Langerhans cells. Immunology Today 2:124–128, 1981
2. Stingl G, Wolff-Schreiner EC, Pichler WJ, Gschnait F, Knapp W, Wolff K: Epidermal Langerhans cells bear Fc and C3 receptors. Nature 268:245–246, 1977
3. Klareskog L, Malmnäs Tjernlund U, Forsum U, Peterson PA: Epidermal Langerhans cells express Ia antigens. Nature 268:248–250, 1977
4. Rowden G, Lewis MG, Sullivan AK: Ia antigen expression on human epidermal Langerhans cells. Nature 268:247–248, 1977
5. Braathen LR, Thorsby E: Human epidermal Langerhans cells are more potent than blood monocytes in inducing some antigen-specific T-cell responses. Br J Dermatol 108:139–146, 1983
6. Bjercke S, Elgø J, Braathen L, Thorsby E: Enriched epidermal Langerhans cells are potent antigen-presenting cells for T cells. J Invest Dermatol 83:286–289, 1984
7. Tew JG, Thorbecke GJ, Steinman RM: Dendritic cells in the immune response: Characteristics and recommended nomenclature. J Reticuloendothel Soc 31:371–380, 1982
8. Kiistala V: Suction blister device for separation of viable epidermis from dermis. J Invest Dermatol 50:129–137, 1968
9. Clayman CH, Schmidtke JR: Purification and mitogenic activation of Fc receptor bearing human peripheral blood lymphocytes. Cell Immunol 28:148–157, 1977
10. Böyum A: Separation of leucocytes from blood and bone marrow. Scand J Clin Lab Invest [Suppl 97] 21:77–89, 1968
11. Räsänen L, Karhumäki E, Krohn K: Elaboration of leukocyte migration inhibitory factor by human lymphocyte subpopulations stimulated with mitogens. Cell Immunol 37:221–228, 1978
12. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 55:283–290, 1971
13. Wolff K, Stingl G: The Langerhans cell. J Invest Dermatol [Suppl] 80:17s–21s, 1983
14. Levis WR, Miller AE: Leucocyte/skin cultures as a measure of histocompatibility in man. Lancet 2:357–360, 1972
15. Scheynius A, Klareskog L, Forsum U, Matsson P, Karlsson L, Peterson PA, Sundström C: Enrichment of epidermal Langerhans cells: Studies with a monolayer technique and flow cytometry sorting. J Invest Dermatol 82:452–455, 1982
16. Chardonnet Y, Beauve P, Viac J, Schmitt D: T-cell subsets and Langerhans cells in wart lesions. Immunol Lett 6:191–196, 1983
17. Belter S, Aberer W, Stingl-Gazze LA, Wolff K, Schuler G, Stingl G: A new method for Langerhans cell enrichment (abstr). J Invest Dermatol 76:427, 1981
18. Bjercke S, Lea T, Braathen LR, Thorsby E: Enrichment of human epidermal Langerhans dendritic cells. Scand J Immunol 19:255–263, 1984
19. Morhenn VB, Wood GS, Engleman EG, Oseroff AR: Selective enrichment of human epidermal cell subpopulations using monoclonal antibodies. J Invest Dermatol [Suppl] 81:127s–131s, 1983
20. Wood GS, Kosek JK, Butcher EC, Morhenn V: Enrichment of murine and human Langerhans cells with solid phase immunoabsorption using pan-leukocyte monoclonal antibodies. J Invest Dermatol 84:37–40, 1985
21. Schuler G, Auböck J, Linert J: Enrichment of epidermal Langerhans cells by immunoadsorption to *Staphylococcus aureus* cells. J Immunol 130:2008–2010, 1983