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Use of the Fluorescence-Activated Cell Sorter to Quantitate and Enrich for Subpopulations of Human Skin Cells

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A variety of immunologic staining techniques were compared in a quantitative study of antigen expression by human epidermal cells. Virtually all nucleated epidermal cells express β_2-microglobulin, which is associated with HLA-A, -B, and -C antigens, whereas only about 4% expressed T6, an antigen expressed by Langerhans cells but not other cells in the skin. With the fluorescence-activated cell sorter (FACS), epidermal cell suspensions were selectively enriched 10- to 15-fold for T6-positive Langerhans cells.

An average of 6.5% of cells were specifically stained by anti-HLA-DR antibody. When dispersed cells stained with anti-DR plus peroxidase were examined with the technique of immunoelectron microscopy, only mononuclear leukocytes (probably Langerhans cells) were stained. After separating HLA-DR positive skin cells with the FACS, the DR-positive population but not the DR-negative population stimulated proliferation of allogeneic responder lymphocytes, indicating that sorted cells are metabolically active. We conclude that HLA-DR antigen is not expressed by keratinocytes in normal human skin cell suspensions and that the FACS can be used to selectively enrich or deplete skin cell suspensions of antigenically distinct subpopulations such as Langerhans cells.

The cells of the epidermis consist predominantly of keratinocytes in various stages of differentiation and 3 additional cell types: Langerhans cells (LC), melanocytes, and Merkel cells [1]. The 4 epidermal cell types can be distinguished from one another on the basis of their ultrastructural characteristics [2,3] and histochemical staining patterns [4]. Moreover, T6, a recently described cell surface antigen found only on LC in epidermis, also can be used as a marker [5,6].

The human major histocompatibility gene complex (HLA), located on the sixth chromosome [7], controls the expression of a group of polymorphic cell surface glycoproteins, the HLA-A, -B, -C, and -Dr antigens [8]. HLA-A, -B, and -C antigens comprise the classic histocompatibility antigens and are associated with β_2-microglobulin on the cell surface. HLA-DR antigens represent the human analogues of the murine immune response-associated antigens (Ia) [9]. Our knowledge of the functions of HLA antigens is incomplete but recent evidence suggests that they mediate a variety of immune functions [10] and that these antigens or products of closely linked genes may play a role in susceptibility to certain diseases [11].

Like most nucleated cells, epidermal cells express HLA-A, -B, and -C antigens [12]. On the other hand, evidence in mice indicating that the majority of dispersed epidermal cells express Ia antigens has been disputed by investigators of both rodent and human epidermis who report that only LC demonstrate this antigen [13–17]. If HLA-DR antigens play a critical role in immunoregulation, it is important to determine which cells of the epidermis demonstrate these antigens in order to delineate the possible role of the epidermis in the afferent limb of the immune system.

Preliminary experiments in this laboratory demonstrated that the fluorescence-activated cell sorter (FACS) could be used to analyze and sort dispersed skin cell suspensions into fractions expressing or not expressing a particular antigenic marker. In the current study, monoclonal antibodies were used to enrich for subpopulations of cells expressing particular cell surface proteins. As shown, this approach yields enriched subpopulations of epidermal cells that retain their immunologic functions.

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Abbreviations:
- BRBC: bovine red blood cells
- DR^-: cells that do not express detectable DR antigen
- DR^+: cells that express detectable DR antigen
- EA-rosettes: Fc receptor-bearing rosetted cells
- FACS: fluorescence-activated cell sorter
- G/R-FITC: goat antirabbit fluorescein isothiocyanate-conjugated immunoglobulin
- HLA: human leukocyte antigen
- Ia antigen: immune response-associated antigen
- IF: immunofluorescence
- LC: Langerhans cell
- PBML: peripheral blood mononuclear leukocytes
- PBS: phosphate-buffered saline
- R/M-FITC: rabbit antimouse fluorescein isothiocyanate-conjugated immunoglobulin
- SLR: skin cell lymphocyte reaction
- T6: an antigen on LC recognized by antibody OKT6

MATERIALS AND METHODS

Antibodies

Murine monoclonal anti-HLA-DR antibodies were produced by somatic cell hybridization as previously reported [18]. The antibodies used in the current study are designated 2.06 and L203, and are $gamma_2$ and $gamma_1$ immunoglobulins, respectively [19,20]. Antibody L203 was a generous gift of Ronald Levy, Stanford University. The preparation of antibody 2.06 used in the study has a titer of 10^7, based on results of radioimmune cell-binding assays. Murine monoclonal anti-T-cell antibody (designated L17F12 or anti-Leu-1) was also produced by somatic cell hybridization [21]. This antibody is also a $gamma_2$ immunoglobulin. Antibody OKT6 was obtained from Ortho Pharmaceuticals, Raritan, New Jersey. This antibody is specific for LC in the epidermis [5]. Rabbit antihuman β_2-microglobulin was purchased from Accurate Chemical and Scientific Corp., Hicksville, New York. Fluorescein isothiocyanate conjugated rabbit antimouse immunoglobulin (Ig)(R/M-FITC) was obtained from Miles Lab., Elkhart, Indiana.

Preparation of Frozen and Dispersed Skin Cells

To prepare frozen sections of fresh skin, a 3-mm biopsy was obtained from the neck of a healthy adult following local infiltration with 1% xylocaine. Specimens were immediately frozen in liquid nitrogen and cut into sections with a microtome (Lipshaw, Detroit, Michigan).

Single-cell suspensions of skin cells were prepared from cadaver skin or skin obtained at surgery as described previously [22]. Briefly,

trimmed skin was cut into 1 × 5 cm strips and split-cut with a Castroviejo keratotome set at 0.1 mm. The resulting slices were treated for 30 min at 37°C with 0.3% trypsin (ICN Pharmaceuticals, Cleveland, Ohio) in 0.8% NaCl, 0.04% KCl, 0.1% glucose, pH 7.3. In later experiments using the OKT6 antibody, 0.1% EDTA was added to the trypsin solution to decrease cell clumping. Dispersed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum, 100 units/ml penicillin, 100 μg/ml gentamicin, 25 mM Hepes buffer, and 2 mM L-glutamine (complete RPMI medium). Viability, as determined by trypan blue exclusion immediately after trypsinization, was 80% or better. Viability after 18 hr maintenance exceeded 70% with cell recovery 60–80%.

Separation of LC from Keratinocytes

LC were separated from heterogeneous skin cell suspensions by Fc receptor binding to IgG antibody-coated bovine red blood cells (BRBC) [23]. Dispersed skin cells were suspended at 4×10^6 cells/ml in TC199 (Microbiological Assoc.) supplemented with 20% fetal calf serum (Gibco, Grand Island, New York) and 50 μg/ml gentamicin. An aliquot of this cell suspension was layered onto Ficoll-Hypaque (9% Ficoll:33% Hypaque—2.4:1) and centrifuged for 30 min at 400 × *g* at 4°C. The resultant pellet was depleted of LC and was designated fraction I. The lymphoid-like LC-enriched cells at the interface (fraction II) were washed and resuspended in supplemented TC199. Aliquots of the original cell suspension and fractions I and II were processed for immunoelectron microscopy.

In a similar experiment, the lymphoid-like LC-enriched cells at the interface (fraction II) were washed, resuspended at 10^7 cells/ml in supplemented TC199, and mixed with an equal volume of 1% suspension of IgG antibody-coated BRBC. This mixture was centrifuged at 300 × *g* for 5 min, incubated on ice for 2–3 hr, gently resuspended, and relayered on a second Ficoll-Hypaque gradient as described above. The nonrosetted cells at the interface depleted of LC were designated fraction III. The pellet containing Fc receptor-bearing rosetted cells (EA-rosettes) (fraction IV) was recovered after lysis of the BRBC with ice-cold 0.83% ammonium chloride in 0.17 M Tris buffer, pH 7.2. The percentage of EA-rosettes was enumerated in unfractionated preparations and all fractions by rosetting (or re-rosetting) with antibody-coated BRBC as described above. Unfractionated epidermal cells as well as fractions I, III, IV were resuspended in complete RPMI for use in the skin cell lymphocyte reaction (SLR). Viability in all fractions was determined by trypan blue exclusion and was always 70% or above.

Adenosine Triphosphatase (ATPase) Stain

To identify LC among dispersed skin cells, skin sections were trypsinized as described above, maintained for 18 hr in complete RPMI medium, centrifuged, washed with phosphate-buffered saline (PBS), fixed in 5% cold buffered formalin (2% formaldehyde/15 mM NaH_2PO_4/23 mM Na_2HPO_4, pH 7) for 30 min and stained for ATPase as previously described [24]. These cells were compared microscopically to cells treated in an identical manner, except that the $MgSO_4$ was omitted from the incubation medium [4]. To identify LC in intact epidermis, frozen skin sections were air dried at 35°C for 1 hr, fixed in 5% cold buffered formalin for 30 min, and then treated as above.

Immunofluorescence (IF) Staining

The binding of antibodies to epidermal cells *in situ* was studied by IF with either R/M-FITC or fluorescein isothiocyanate-conjugated goat antirabbit Ig (G/R-FITC), a gift of Dr. Roland Scollay. Both reagents had been absorbed with Sepharose-conjugated human Ig and the R/M-FITC was additionally absorbed with dispersed human skin cells.

Skin sections were stained with anti-DR antibody or anti-β_2-microglobulin antibody in humidified glass Petri dishes for 30 min at room temperature [25]. All antibodies were titered and used at concentrations determined to yield optimal staining. After 2 washes with PBS, the sections were labeled with the appropriate fluoresceinated second-step reagent for 30 min at room temperature and washed twice in PBS. Preparations were covered with fluorescent antibody mounting fluid (Difco, Detroit, Michigan) and examined with a Zeiss fluorescence microscope using excitation filters LP 455 plus SP 490 and a barrier filter of LP 520. Dispersed skin cells also were examined by fluorescence microscopy following staining with mouse monoclonal antibodies plus R/M-FITC, or β_2-microglobulin plus G/R-FITC, as described below.

Analysis and Cell Separation Using the Fluorescence-Activated Cell Sorter

Trypsinized skin cells were stained by suspending 3×10^7 cells in 0.5 ml antibody diluted in 0.02% sodium azide in PBS (PBS/azide) for 30 min at 25°C. The cells were washed twice with PBS and incubated for 30 min at 25°C with 0.5 ml R/M-FITC diluted 1:150 in PBS/azide. After washing, the labeled cells were resuspended in 5% fetal calf serum in PBS and processed in a FACS III (Becton Dickinson Electronic Laboratories, Mountain View, California) at 1000 cells per second. Background fluorescence was determined by analyzing skin cells, labeled with either normal mouse serum diluted 1:50 in PBS, or normal rabbit serum diluted 1:50 in PBS. To obtain skin populations enriched for cells expressing T6 antigen ($T6^+$) or HLA-DR antigen (DR^+) and cells lacking detectable T6 ($T6^-$) or HLA-DR antigen (DR^-), cells were labeled with either anti-T6 or anti-DR antibody as described and then separated by the FACS. After sorting, cells were either studied immediately or incubated for 15 min with 0.05% trypsin (Gibco), in Hanks' balanced salt solution (Gibco) in order to remove cell-bound antibody.

Skin Cell Lymphocyte Reaction (SLR)

The SLR was performed as described previously [26]. Peripheral blood mononuclear leukocytes (PBML) were obtained from healthy volunteers by Ficoll (Sigma Chemical Co., St. Louis, Missouri)-Hypaque (Winthrop Laboratories, New York) gradient centrifugation of fresh defibrinated blood [27]. Fifty thousand PBML were coincubated in microtiter plates with varying numbers of allogeneic skin cells in complete RPMI medium in a final volume of 0.2 ml. The cultures were maintained in air/10% CO_2 for 6 days at 37°C. [^{3}H]-thymidine was then added (1 μCi/well) and the plates harvested 18 hr later. All SLR were performed in triplicate using lymphocytes from healthy donors unrelated to the skin donor.

Light and Electron Microscopy

Sorted $T6^+$ skin cells were aliquoted and centrifuged. An aliquot was fixed for 30 min in Karnovsky's fixative and routinely processed for electron microscopy [28]. Other aliquots were resuspended in PBS, smeared on glass slides, air dried, and stained with hematoxylin and eosin.

Immunocytologic staining was performed with a three-stage murine monoclonal antibody/biotinylated goat antimouse IgG/avidin-horseradish peroxidase technique [29]. This immunohistologic technique was modified for cell suspension staining. Cells were washed twice before and after each staining stage by centrifugation through PBS for 5 min at 650 × *g*. Five to fifty μg/ml titers of reagents were used in 100–200 μl aliquots per $1–12 \times 10^6$ cells. The entire procedure was performed at 4–10°C. Cell pellets were fixed for 30 min in Karnovsky's fixative and routinely processed for electron microscopy. Toluidine blue-stained 1-μm thick sections were examined with a 100× oil objective. Thin sections were examined ultrastructurally. Controls included parallel staining with monoclonal antibodies devoid of specificity for the target antigen and staining with one or more stages deleted. Biotinylated goat antimouse IgG (heavy and light chains) was obtained from Tago, Inc., Burlingame, California. Avidin-horseradish peroxidase was obtained from Vector Laboratories, Inc., Burlingame, California.

RESULTS

Antigen Expression by Intact and Dispersed Skin Cells

Sections of human epidermis labeled with either antibodies 2.06, L203, or OKT6 revealed specific staining of approximately 5% of cells. Positively stained cells consisted mainly of dendritic epidermal cells that by distribution and morphology corresponded to LC (not shown). The majority of this type of cell demonstrated ATPase activity, thereby confirming their identity as LC. By contrast, tissue sections stained with anti-β_2-microglobulin revealed specific fluorescence of virtually all nucleated epidermal cells as has been described [12].

To obtain suspensions of skin cells, skin sections were treated with trypsin, and incubated for 2–18 hr in medium free of trypsin. Thereafter, aliquots of cells were labeled with anti-DR antibody. With fluorescence microscopy, individual cells showed specific fluorescence with antibody 2.06 (3–6%) (Fig 1*A*). A similar percentage of cells labeled with antibody OKT6 also showed specific fluorescence (not shown). Approximately 3% of dispersed cells were stained by ATPase. Cell suspensions labeled with anti-β_2-microglobulin antibody revealed fluorescence of virtually all cells (Fig 1*B*). Unlabeled skin cells and cells labeled with normal mouse serum or normal rabbit serum plus the appropriate fluoresceinated Ig showed no fluorescence.

One to two hours after dispersion with trypsin and EDTA, suspensions of skin cells were incubated with OKT6 and stained

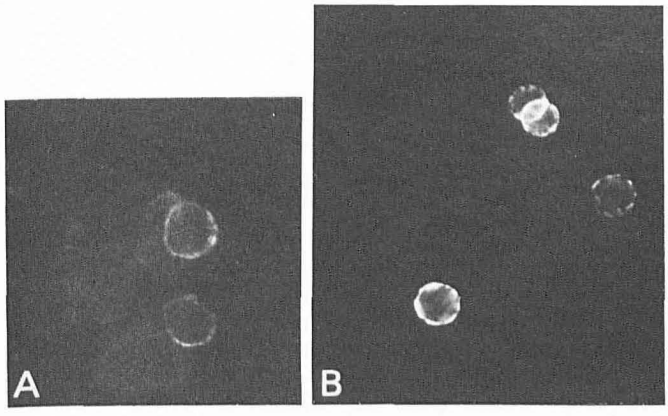


FIG 1. Detection of HLA-DR antigen and β_2-microglobulin on dispersed skin cells. Human skin was trypsinized, resuspended in complete RPMI medium and maintained at room temperature for 18 hr before staining with anti-DR plus R/M-FITC (*A*), or antibody to β_2-microglobulin plus G/R-FITC (*B*). ×400

with R/M-FITC. Analysis of these cells in the FACS revealed 3–5% brightly positive cells (Fig 2*A*). Some weakly stained cells were also seen.

Suspensions of skin cells were incubated with monoclonal anti-HLA-DR antibody (2.06) plus R/M-FITC. Analysis of these cells in the FACS revealed a distinct shoulder of brightly stained cells (DR^+), as well as a large number of unstained or weakly stained cells (DR^-) (Fig 3*A*). This pattern has been obtained in 20 separate experiments and the percentage of specifically stained cells has varied 1–14% (mean 6.5%).

To confirm the validity of our staining technique, dispersed cells were labeled with anti-β_2-microglobulin antibody and analyzed in the FACS. Eighty to 100% of human skin cells in suspension were brightly stained with this antibody (Fig 3*B*). Moreover, cell suspensions showed no net fluorescence with monoclonal anti-Leu-1 which recognizes peripheral T lymphocytes and is of the same subclass as anti-DR. When epidermal cell suspensions were stained with L203 and examined ultrastructurally, mononuclear leukocytes but not keratinocytes were stained (Fig. 4).

Separation of Subpopulations of Epidermal Cells with a Rosetting Method

Skin cell suspensions were prepared and an aliquot removed for immunocytologic staining with antibody L203. The remaining cells were rosetted with antibody-coated BRBC and centrifuged through a Ficoll-Hypaque gradient. The pellet (fraction I) and interface (fraction II) were collected and also stained immunocytologically with antibody L203. The original cell suspensions contained lymphoid cells which bound antibody against HLA-DR (Fig 4). As expected, fraction II contained more lymphoid cells (10%) than were found in the whole, undepleted preparation. However, occasional $HLA\text{-}DR^+$ mononuclear lymphocytes also were seen in fraction I (< 1%). Very low concentrations of such LC-depleted fractions (5×10^3 cells/well) retained the capacity to stimulate allogeneic lymphocytes (Table I). None of the keratinocytes observed in the undepleted cells and fractions I and II bound antibody L203 on their surface. Endothelial cells were not seen in any of the samples examined ultrastructurally.

Separation of Subpopulations of Epidermal Cells with the FACS

Skin cell suspensions were incubated with T6 antibody and R/M-FITC, and separated into $T6^+$ and $T6^-$ subpopulations with the cell sorter. With a scatter setting of 40–200 (to eliminate large cells and cell clumps) and a fluorescence window of >150 (to eliminate most weakly stained cells), we were able to enrich 10- to 15-fold for $T6^+$ cells. Of these, approximately 40% were cells other than mononuclear leukocytes as judged by light microscopic examination of aliquots smeared and stained with hematoxylin and eosin. By contrast, repeat FACS analysis of sorted $T6^+$ cells revealed greater than 90% positively stained cells (Fig 2*B*). Ultrastructural study of the sorted, positive cells indicated that the mononuclear leukocytes had nuclear and cytoplasmic features identical to those seen in LC. Several of these cells displayed characteristic LC granules on random sections (not shown). The remaining cells in the $T6^+$ pellet were keratinocytes.

Fluorescence light microscopic examination of cells from the sorted $OKT6^+$ population corroborated the ultrastructural findings, that is, a significant minority (40%) of sorted $T6^+$ cells were keratinocytes. However, only the mononuclear leukocytes

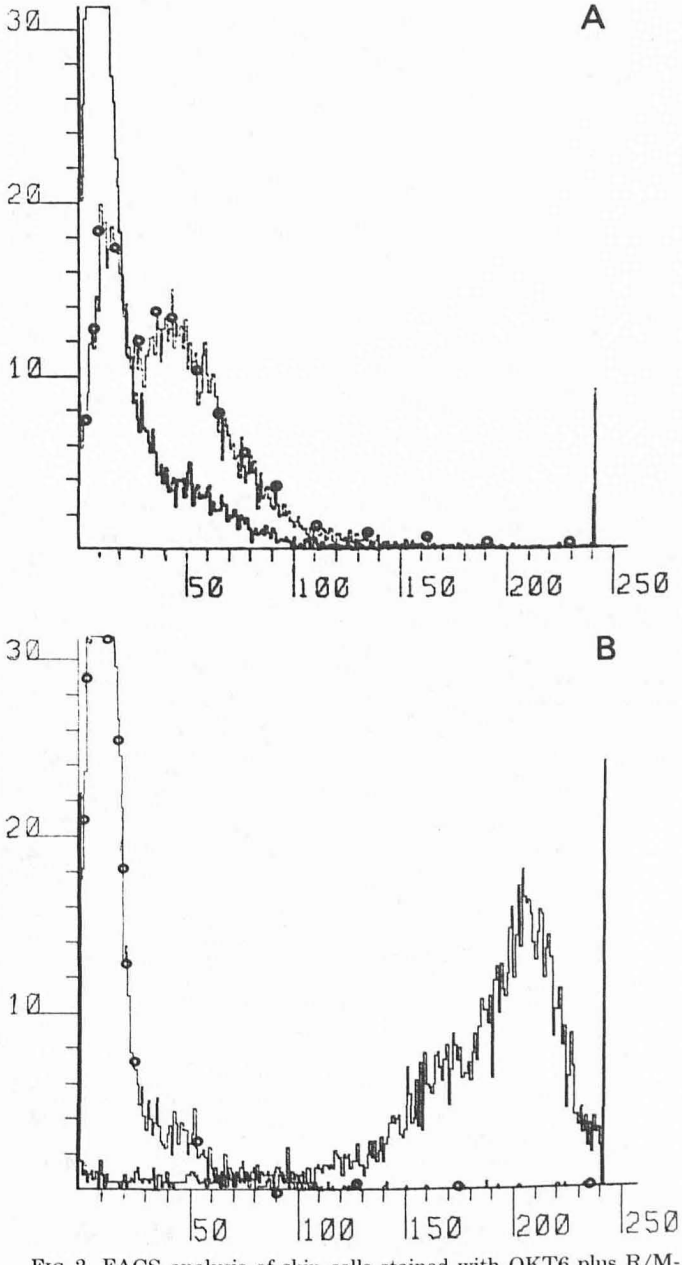
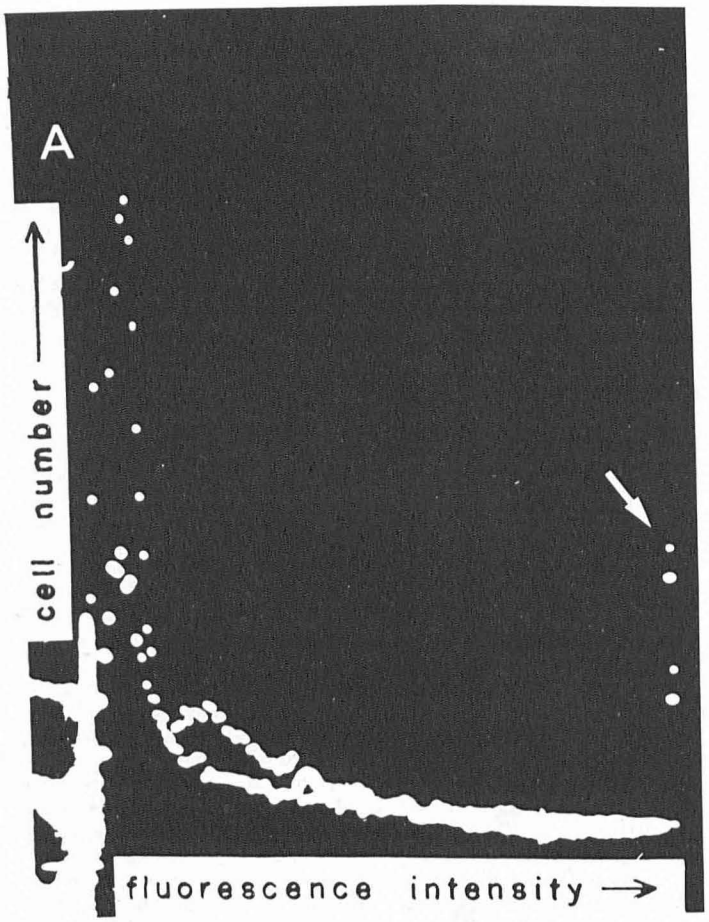


FIG 2. FACS analysis of skin cells stained with OKT6 plus R/M-FITC. *A*, Histogram of dispersed cells stained with OKT6 plus R/M-FITC O–O–O and control cells stained with R/M-FITC alone ————. *B*, At the end of the sort, aliquots of cells sorted in the $OKT6^-$ channel (O–O–O) and the $OKT6^+$ channel (————) were reanalyzed. One percent $OKT6^+$ cells contaminate the "negative" sample, and 8% $OKT6^-$ cells contaminate the "positive" sample. The fluorescence intensity is plotted on the abscissa and the fraction of total cells $\times 10^{-3}$ per channel is plotted on the ordinate.



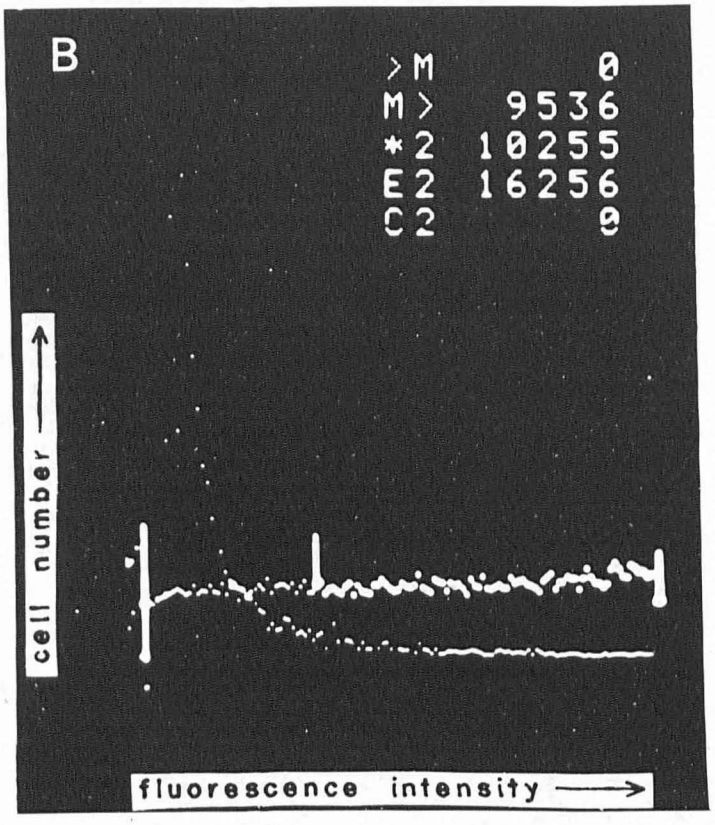


FIG 3. FACS analysis of skin cells stained with anti-DR or anti-β_2-microgobulin antibodies. Human skin was trypsinized, resuspended in complete RPMI medium, and maintained at room temperature for 18 hr before staining with anti-DR antibody plus R/M-FITC (*A*), or anti-β_2-microglobulin antibody plus G/R-FITC (*B*) and analysis in the FACS. In each histogram, the lower curve indicates cells stained with normal mouse or rabbit serum plus the appropriate FITC-Ig. The *arrow* indicates brightly stained cells.

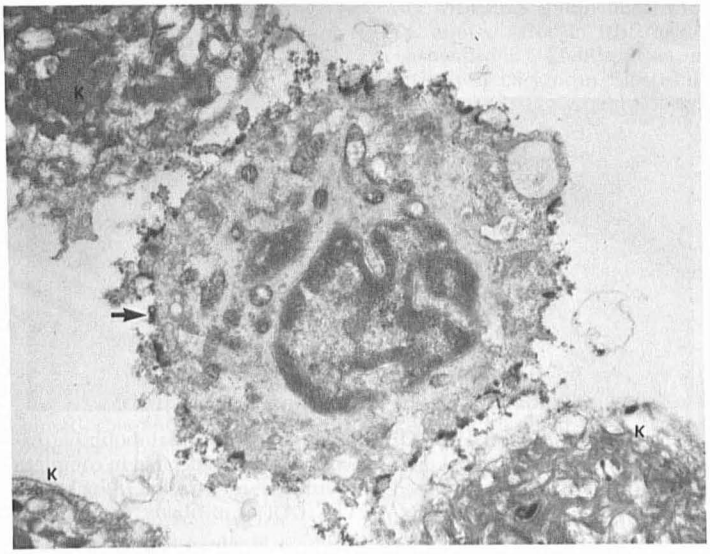


FIG 4. Dispersed skin cells were processed for immunoelectron microscopy. In the center is a mononuclear leukocyte stained with antibody against HLA-DR. The cell surface binds peroxidase (→). At the periphery are 3 keratinocytes (*K*) which do not bind the antibody.

TABLE I. *Capacity of various concentrations of LC-depleted skin cells to stimulate the proliferation of allogeneic lymphocytes*

Number of skin cells/well ($\times 10^3$)	Treatment of skin cell suspension	Response of allogeneic lymphocytes (cpm ± S.E.M.)	
		Donor A	Donor B
1.0	None[a]	1,207 ± 138	1,381 ± 143
	LC-depleted[b]	1,368 ± 141	2,393 ± 245
5.0	None	38,048 ± 3,935	14,291 ± 168
	LC-depleted	13,288 ± 1,220	9,732 ± 931
10.0	None	46,725 ± 5,280	14,265 ± 133
	LC-depleted	17,615 ± 1,592	15,921 ± 147
50.0	None	60,862 ± 6,924	28,456 ± 276
	LC-depleted	49,308 ± 5,148	25,418 ± 259

[a] Undepleted skin cells.
[b] That is, fraction I after first Ficoll-Hypaque gradient centrifugation.

were positively stained with OKT6 antibody under the fluorescence light microscope. Thus, although cell sorting with OKT6 antibody resulted in enrichment of LC, significant contamination with keratinocytes remained.

In similar experiments, skin cells were enriched for or depleted of DR^+ cells by staining with antibody 2.06 plus R/M-FITC and sorting into DR^+ and DR^- populations. Aliquots of each population were removed and examined ultrastructurally. The majority of cells in both DR^+ and DR^- subpopulations consisted of keratinocytes, probably representing a contamination analogous to that described for the OKT6 cells. Each population was then tested for the capacity to stimulate proliferation of allogeneic lymphocytes in the SLR. To rule out the possibility that the presence of antibody plus R/M-FITC might influence the response, mild trypsinization was used to remove antibody from the sorted cells before testing in the SLR. By immunofluorescence light microscopy, trypsinization was shown to eliminate all fluorescence from positively stained cells without affecting stimulation in the SLR (not shown). As shown in Fig 5, DR^+ cells but not DR^- cells stimulated allogeneic lymphocytes to proliferate. Moreover, even 2×10^4 to 5×10^4 DR^- cells, concentrations of skin cells that usually result in optimal stimulation of allogeneic lymphocytes, were incapable of stimulation above background levels [26].

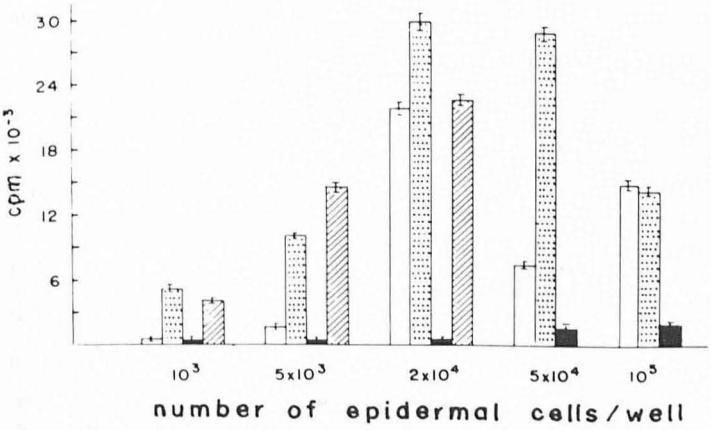


FIG 5. Capacity of DR^+ and DR^- skin cells to stimulate in the SLR. Skin sections were trypsinized and the cells were maintained for 18 hr in complete RPMI medium to allow reconstitution of cell surface proteins. These dispersed cells were added to the SLR □ or treated briefly with trypsin □ prior to culture in the SLR. The remaining cells were stained with anti-HLA-DR antibody plus R/M-FITC, sorted with the FACS into DR^+ ▨ and DR^- ■ populations, and trypsinized briefly to remove the antibodies before use in the SLR. Varying concentrations of viable skin cells were coincubated with 50,000 allogeneic responder lymphocytes in the SLR. Unstimulated responder cells incorporated 384 cpm. *Brackets* indicate standard error of the mean.

These results were compared to those obtained with another method of LC separation—the rosetting of LC with antibody-coated BRBC, detailed above [23]. Unfractionated cell suspensions contained about 2% EA-rosettes; fraction I contained virtually no rosetted cells—of 500 cells counted—whereas fraction II contained about 10% rosettes which is in agreement with our immunoelectron microscopic results. Fraction III contained no rosetted cells and fraction IV contained approximately 30% EA-rosettes. As shown in Table II, LC-depleted skin cells retain the capacity to stimulate allogeneic lymphocytes, suggesting that this method is not as rigorous a method for separating LC from keratinocytes as the FACS method.

TABLE II. *Stimulation of allogeneic lymphocytes by LC-enriched and LC-depleted skin cells*

Skin cell population	Response of allogeneic responder lymphocytes (cpm[a])	
	Donor A	Donor B
USC[b]	51,323	7,641
Fraction I[c]	45,462	8,189
Fraction III[d]	43,377	13,811
Fraction IV[e]	48,941	37,275

[a] The results represent the mean of triplicate values. Standard errors were always less than 15%. Proliferation of responder lymphocytes cultured alone was 5,200 cpm for Donor A and 746 cpm for Donor B.
[b] USC = Undepleted skin cells.
[c] Fraction I = LC-depleted pellet after first Ficoll-Hypaque gradient centrifugation.
[d] Fraction III = LC-depleted cells at interface of second Ficoll-Hypaque gradient centrifugation using EA-rosetted cells.
[e] Fraction IV = LC-enriched cells containing EA-rosetted cells in pellet after second Ficoll-Hypaque gradient centrifugation.

DISCUSSION

These data demonstrate that the FACS provides a semiquantitative method for estimating the number of skin cells expressing a given cell surface antigen. The percentage of labeled cells in the intact tissue corresponded well with the percentage obtained by FACS analysis of dispersed cells. Using the FACS, specific subpopulations of cells (e.g., LC) can be depleted or enriched 10- to 15-fold and remain functionally intact after sorting. Finally, we have shown that immunoelectron microscopy can be carried out successfully on dispersed skin cell preparations. With this method, we have confirmed other reports that skin-associated lymphoid cells express HLA-DR antigen [15–17] and that dispersed keratinocytes obtained from normal skin do not demonstrate this antigen.

When we depleted skin cell suspensions of LC by rosetting with antibody-coated BRBC and Ficoll-Hypaque gradient centrifugation [23], very low concentrations (5×10^3 cells/well) of the "LC-depleted" fraction (fraction I) retained the capacity to stimulate allogeneic lymphocytes. Subsequent electron microscopic examination of the "LC-depleted" fraction demonstrated the presence of mononuclear leukocytes, although they were considerably reduced (< 1%) compared to the nondepleted cell suspension (about 3%). On the other hand, we cannot rule out the possibility that the stimulation of allogeneic lymphocytes by cells in fraction I is due to endothelial cells even though none was visualized on electron microscopic examination. By contrast, when skin cell suspensions were depleted of LC by sorting cells into DR^+ and DR^- subpopulations, the DR^- fraction was incapable of stimulating allogeneic lymphocytes in the SLR. Thus, LC depletion with the FACS was superior to the rosetting/centrifugation method. Similarly, it was possible to achieve a greater LC enrichment with the FACS (10- to 15-fold) than with the rosetting/centrifugation method (6-fold).

We have recently described another method of separating keratinocytes from skin-associated mononuclear cells using velocity sedimentation [30]. This method has the advantage of separating large quantities of cells quickly and inexpensively and results in cell fractions devoid of cells expressing HLA-DR activity as well as fractions expressing HLA-DR activity. By contrast, the FACS method reported herein is more expensive but results in subpopulations of antigen-positive cells that are enriched 10- to 15-fold, which is the best enrichment we have achieved to date.

The spurious sorting of keratinocytes into the DR^+ and $T6^+$ populations by the FACS cannot be explained by nonspecific binding of anti-DR antibody to cell surface Fc receptors [31] because keratinocytes do not express this cell surface protein [32] and a control murine monoclonal antibody did not bind to epidermal cells. Further, the contaminating cells are not due to cell clumping since this was ruled out by light microscopic examination of sorted cells. Since a small number of cells was present even in the higher-fluorescence intensity channels in the control staining (Fig 2*A*), it is unlikely that the FACS can be used to obtain pure LC subpopulations. Potential sources of false-positive staining include keratin autofluorescence [33] and presence of a fluorophore emitting in the portion of the spectrum beyond the visible range. The reason for the presence of a larger number of weakly stained cells in the cells stained with mouse Ig (Fig 2*A*) is also unclear but may be due to the effects of prior trypsinization on mouse antibody binding.

The data obtained in this study indicate that the FACS can be used to obtain viable skin cell preparations that are functionally LC-depleted or LC-enriched with over 50% purity. This method will allow seeding of skin-derived mononuclear cells in culture and possibly allow the growth of LC-like cells *in vitro*. Experiments are in progress in this laboratory to further delineate optimal conditions for long-term culture of these cells.

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