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Cultured Human Epidermal Cells Do Not Synthesize HLA-DR

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All nucleated cells express HLA-A, B, and C antigens. However, only a few cells, including epidermal cells, demonstrate HLA-DR antigens which are potent transplantation immunogens in man. The current study was undertaken to determine if epidermal cells continue to synthesize and/or express HLA-DR antigens after prolonged *in vitro* culture. Epidermal cells cultured for 7 days or more no longer stimulated allogeneic lymphocytes in the epidermal cell-lymphocyte reaction. Indirect immunofluorescence light microscopy of cultured cells using mouse monoclonal antibody to HLA-DR antigen confirmed that these cells do not express HLA-DR antigens whereas they retain β_2-microglobulin. Detergent extracts of 12-day cultured epidermal cells biosynthetically labeled with ^{35}S-methionine were immunoprecipitated with monoclonal anti-DR antibody and analyzed by the method of two-dimensional polyacrylamide gel electrophoresis. No radiolabeled proteins were found on these gels in the regions where HLA-DR molecules are known to migrate. These data indicate that HLA-DR antigen is absent from cultured epidermal cells. Finally, we describe a technique for growing epidermal cells on a gelatin membrane which allows subsequent removal of intact cell monolayers from the culture dish. Such monolayers may be useful for purposes of transplantation.

The human epidermis consists of a heterogeneous cell population which includes keratinocytes in various stages of differentiation, melanocytes, Langerhans cells (LC), and Merkel cells. Keratinocytes, which represent the majority of epidermal cells, are of ectodermal origin [1]. Langerhans cells which comprise only 2–6% of the total population, are thought to be bone marrow derived [2,3]. Despite their diverse origins all epidermal cells express histocompatibility antigens (HLA) A, B, and C on their surface [4]. Some epidermal cells also express immune response associated (Ia) antigens (HLA-DR in man), which, like other products of the major histocompatibility complex (MHC), are potent transplantation immunogens [5,6]. The precise epidermal population(s) which express Ia antigens remains controversial. Several investigators have reported that the majority of keratinocytes in animals demonstrate Ia antigen [7–9] whereas other investigators have presented strong evidence that only LC express this antigen [10–12].

One method for detecting HLA-DR antigens on the cell surface is the "one-way" mixed lymphocyte culture (MLC) [13]. In this assay, stimulator lymphocytes are coincubated with allogeneic responder lymphocytes and at day 7 incorporation of ^{3}H-thymidine (^{3}H-T) into newly synthesized DNA of proliferative cells is measured. In a variation of the MLC, the epidermal cell-lymphocyte reaction (ELR), epidermal cells are used as stimulator cells for allogeneic lymphocytes [14,15]. Several investigators have shown that it is the HLA-DR antigens on epidermal cells which cause stimulation of allogeneic responder lymphocytes in this reaction [16,17].

In an effort to decrease the heterogeneity of the epidermal cell population and increase the number of cells available for experimentation, we attempted to use cultured epidermal cells as the stimulator cells in the ELR. To our surprise, epidermal cells harvested from cultures grown for 7 days failed to stimulate allogeneic lymphocytes in the ELR. Since antigens of the MHC represent the major barriers for transplantation [18], we explored the effects of culture on expression of HLA antigens by epidermal cells.

MATERIALS AND METHODS

Epidermal Cell Cultures

Keratinocyte cultures were prepared from cadaver skin as described previously [19]. Briefly, 0.1 mm keratotome slices were incubated at 37°C in 0.3% trypsin (Microbiological Assoc., Bethesda, MD) in 150 mM NaCl, 0.04% KCl, 0.1% glucose, pH 7.3 for 40 min. The skin slices were washed, transferred to complete growth medium consisting of Dulbecco's Minimum Essential Medium plus 20% heat inactivated fetal calf serum, 5 ng/ml epidermal growth factor (Collaborative Research, Inc., Waltham, MA), 50 μg/ml gentamicin, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 14 mM Hepes buffer, 0.4 μg/ml hydrocortisone and the basal and malpighian cells were released into the medium by gentle agitation. Routinely, 2×10^6 viable, round refractile cells were plated on a collagen thin gel coated 3.5 cm Petri dish (Lux, Microbiological Assoc.) and incubated in 5% CO_2: 95% air at 37°C. Alternatively, 4.2×10^5 cells/cm^2 were seeded on gelatin membranes (G-membranes) floated on complete growth medium and incubated as above. Viability was determined by trypan blue exclusion.

Preparation of G-Membranes

The G-membranes were prepared according to published methods [20]. Briefly, 13-mm holes were punched in the center of 25-mm millipore filters and the filters were dipped in a 3% solution of 300 Bloom gelatin (Sigma Chemical Co., St. Louis, MO) heated to 29°C. The gelatin membrane formed across the hole in the filter was allowed to cool and the filters were removed and placed concave side up to dry. After 18 hr, the gelatin was fixed with glutaraldehyde vapor for 60 min, soaked in 12.5% glutaraldehyde solution overnight, washed extensively and incubated in growth medium for 3 to 10 days before use.

Harvesting of Cultured Epidermal Cells

To harvest epidermal cells from Petri dishes or G-membranes, cultures were incubated with 0.3% trypsin/1% ETDA (Allied Chemical,

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Abbreviations:
ATPase: adenosine triphosphatase
B cell: bone marrow derived lymphocyte
BRBC: bovine red blood cells
2D-PAGE: two dimensional polyacrylamide gel electrophoresis
EDTA: ethylenediamine tetraacetate
ELR: epidermal cell-lymphocyte reaction
FACS: fluorescence activated cell sorter
G-membrane: gelatin membrane
G/R FITC: fluorescein isothiocyanate conjugated goat anti-rabbit Ig
HLA: human leukocyte antigen
^{3}H-T: ^{3}H-thymidine
Ia: immune response associated antigen
IIF: indirect immunofluorescence
LC: Langerhans cell
MHC: major histocompatibility complex
MLC: mixed lymphocyte culture
PBS: phosphate buffered saline
R/M FITC: fluorescein isothiocyanate conjugated rabbit anti-mouse Ig

Morristown, NJ) in phosphate buffered saline (PBS) pH 7.3 for 10 min at 37°C. The detached cells were resuspended in complete growth medium, immediately centrifuged at 400 ×*g* for 5 min and then suspended in RPMI 1640 medium plus 10% heat inactivated pooled human serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 25 mM Hepes buffer and 2 mM L-glutamine (complete RPMI).

Adenosine Triphosphatase (ATPase) Stain

To identify LC in epidermal cell cultures, a stain specific for ATPase was used. Cell monolayers were washed with PBS and fixed in 5% cold buffered formalin (2% formaldehyde/15 mM NaH_2PO_4/23 mM Na_2HPO_4, pH 7) for 30 min. Following 2 washes with distilled water, the cells were treated with incubation medium (.83 mM ATP/80 mM Tris-HCl/3.6 mM $Pb(NO_3)_2$/10 mM $MgSO_4$, pH 7.2) [21] for 60 min, rinsed in distilled water, developed in 1% yellow ammonium sulfide, and rinsed in water. These cell cultures were compared microscopically to cultures treated in an identical manner, except that the $MgSO_4$ was omitted from the incubation medium [22].

Isolation of Epidermal Cells Bearing Fc Receptors

IgG antibody-coated bovine red blood cells (BRBC) were prepared as described previously [23]. Cultured epidermal cells were trypsinized and resuspended in TC199 (Microbiological Assoc., Walkersville, MD), supplemented with 20% fetal calf serum (GIBCO, Grand Island, NY), and 50 μg/ml gentamicin. An aliquot of this suspension was mixed with an equal volume of 1% IgG antibody-coated BRBC. This mixture was centrifuged for 5 min at 300 ×*g*, incubated on ice for 3 hr, and the pellet was resuspended in supplemented TC199 and examined microscopically.

Fluorescence Microscopy and Fluorescence Activated Cell Sorter (FACS) Analysis

Cultured cells were either stained with antibody in the Petri dish (*in situ*) or harvested by trypsinization. Cells *in situ* or 2×10^6 cells in suspension were washed twice with PBS, incubated for 25 min with monoclonal mouse anti-human HLA-DR antibody [24] or rabbit anti-human β_2-microglobulin antibody (Accurate Chemical Corp., Hicksville, NY) washed twice with PBS and further incubated for 25 min with fluorescein conjugated rabbit anti-mouse Ig (R/M FITC) or fluorescein conjugated goat anti-rabbit Ig (G/R FITC), a gift of Dr. Roland Scollay. All antibodies were used at concentrations which resulted in maximum binding. Before labeling, harvested cells were incubated for 18 hr in complete RPMI at room temperature to allow reconstitution of cell membrane proteins. The viability of cells, determined by trypan blue exclusion, was greater than 90%. The labeled cells were examined with a fluorescence microscope (Zeiss), or with a FACS III (Becton Dickinson Electronic Laboratories, Mountain View, CA). The light source for the fluorescence microscope was a 50 w mercury burner. Excitation filters LP 455 plus SP 490 were used with a barrier filter of LP 520.

Electron Microscopy of Cultured Epidermal Cells

G-membranes bearing epidermal cells were removed from Petri dishes and fixed in 3% phosphate buffered glutaraldehyde for 1 hr, washed twice with phosphate buffer (pH 7.4), postfixed with 2% OsO_4 for 1 hr, washed and dehydrated in graded alcohols [25]. The G-membranes were embedded in Epon 812 and polymerized in a 58°C oven for several days before cutting sections for electron microscopy.

Epidermal Cell-Lymphocyte Reaction (ELR)

The ELR was performed as described earlier [17]. Routinely, 5×10^4 epidermal cells were coincubated with 5×10^4 allogeneic lymphocytes in complete RPMI 1640 medium in microtiter wells at 37°C in an atmosphere of 5% CO_2/95% air. On the 6th day of culture, 1 μCi of ^{3}H-T (New England Nuclear, Boston, MA) was added per well, and 18 hr later the cultures were harvested with a Mash II Apparatus (Microbiological Associates, Bethesda, MD). The incorporation of ^{3}H-T was measured in a liquid scintillation counter (Beckman, Irvine, CA).

To determine the ability of cultured epidermal cells to stimulate in the ELR, a single cell suspension was prepared as described above. An aliquot was removed and incubated with allogeneic lymphocytes in the ELR (day 0). The remaining epidermal cells were cultured and harvested at 24, 48 or 120 hr before use as stimulator cells.

Two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Epidermal cells were cultured for 12 days, harvested, maintained overnight in complete RPMI medium and labeled with ^{35}S-methionine as described [24]. Briefly, the cell membrane was disrupted with 0.5% Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL) and the insoluble material was removed by centrifugation at 15,000 ×*g* for 3 min. The detergent extract from 2.5×10^7 cells was incubated first with monoclonal anti-DR antibody and then with Cowan 1 strain of staphylococcus. The bound antigen complexes were eluted as described and analyzed by the method of 2D-PAGE [26]. In the first dimension, molecules were separated according to charge in nonequilibrium pH gradient electrophoresis. The second dimension was run in 10% acrylamide slab gels using a discontinuous Tris glycine/sodium dodecyl sulfate gel system. The slab gels were fixed, stained, dried, and exposed to Kodak NS-2T No-Screen x-ray film.

RESULTS

The Capacity of Cultured Epidermal Cells to Stimulate Allogeneic Lymphocytes in the ELR

Epidermal cells were cultured for varying time periods prior to harvest and used as stimulator cells in the ELR (Table I). Like fresh epidermal cells, epidermal cells cultured for up to 48 hr were capable of eliciting a vigorous response in the ELR. However, by day 5, the capacity of cultured cells to stimulate in the reaction had declined markedly and by day 8 cultured epidermal cells were incapable of stimulating allogeneic lymphocytes. The experiment in Table I is representative of over 20 experiments all of which demonstrated the inability of epidermal cells cultured for more than 7 days to stimulate allogeneic lymphocytes.

Subsequently, we examined the effect of culture conditions

TABLE I. *Capacity of cultured epidermal cells to stimulate allogeneic lymphocytes*[a]

Duration of culture (days)	Stimulation of allogeneic lymphocytes (cpm)	
	Donor A	Donor B
None	51,239 ± 5,334[b]	63,124 ± 6,251
1	48,627 ± 4,956	43,891 ± 4,115
2	46,450 ± 4,430	50,781 ± 5,487
5	14,213 ± 1,342	12,321 ± 1,363
8	290 ± 30	484 ± 56
15	819 ± 91	551 ± 63

[a] Cells were harvested from cadaver skin and resuspended in complete growth medium. An aliquot was removed, centrifuged, resuspended in complete RPMI medium and immediately coincubated with 50,000 allogeneic lymphocytes in microtiter wells (none). The remaining cells were seeded into collagen thin gel dishes, harvested at the time indicated, resuspended in complete RPMI medium and used as stimulator cells in the ELR. The epidermal cell concentration in the ELR was always 5×10^4 viable cells.

[b] ± standard error of the mean.

TABLE II. *Effect of type of culture and cell density on the capacity of epidermal cells to stimulate in ELR*[a]

Epidermal cell source	Type of culture	Days after seeding	Cell density	Stimulation index[b]
Human newborn foreskin	None[c]	—	—	35.0
	1st Passage	14	Preconfluent	1.2
	1st Passage	27	Confluent	1.4
Human adult abdomen	None[c]	—	—	29.0
	Primary	5	Preconfluent	.5
	Primary	7	Confluent	.9
	Primary	12	Confluent	1.3

[a] Epidermal cells were harvested from newborn foreskin obtained at circumcision or from the abdominal wall of adult cadavers at autopsy. The cells were either coincubated with allogeneic lymphocytes in microtiter wells (none) or seeded on collagen thin gel plates for the time interval indicated. The keratinocytes derived from adult skin were harvested from preconfluent cultures or confluent cultures. The foreskin cells were harvested after 2 weeks, reseeded on collagen thin gel plates, trypsinized at the indicated time points and coincubated with allogeneic lymphocytes in the ELR.

[b] Stimulation index is the cpm of ^{3}H-T incorporated into responder lymphocytes when coincubated with epidermal cells divided by the cpm ^{3}H-T incorporated into responder lymphocytes cultured alone.

[c] Cells were not cultured, but used immediately.

and the growth state of the cells at the time of harvest on their capacity to stimulate allogeneic lymphocytes (Table II). Preconfluent cells and confluent cells at the time of harvest both failed to elicit a response in the ELR. Further, the age of the skin donor and the site of origin of the skin did not appear to affect the cells' capacity to stimulate allogeneic lymphocytes.

Since routine culture medium contains 1.1×10^{-6} M cortisol and since corticosteroids are known to have a catabolic effect on some lymphocyte subpopulations as well as skin cells [27], we assessed the capacity of epidermal cells cultured in medium with and without cortisol to stimulate in the ELR. Epidermal cells cultured in the absence of corticosteroid also had lost the capacity to stimulate in the ELR (not shown).

Indirect Immunofluorescence (IIF) Light Microscopy and FACS Analysis

To determine if the failure of cultured epidermal cells to stimulate in the ELR was due to a reduction of HLA-DR antigens, cultured epidermal cells were trypsinized, maintained in medium for 18 hr, and stained with either monoclonal antibody to HLA-DR plus R/M FITC or anti β_2-microglobulin plus G/R FITC. After 12 days of culture, examination of harvested cells stained with anti HLA-DR antibody and R/M FITC under the fluorescence microscope revealed no cell membrane fluorescence (not shown), whereas cells stained with anti β_2-microglobulin and G/R FITC demonstrated fluorescence of many of the cells (not shown). These data were confirmed and quantitated by analysis of the stained cells in the FACS. Cells harvested after 8 days of culture and stained with antibody to HLA-DR plus R/M FITC showed no net fluorescence (Fig 1*A*) whereas over 90% of these cells were fluorescent with anti β_2-microglobulin plus G/R FITC (Fig 1*B*).

The possibility exists that trypsinization of cultured cells selectively removes HLA-DR antigen. Therefore, 7 and 14 day old cell cultures were labelled *in situ* with either antibody to HLA-DR or β_2-microglobulin plus the appropriate FITC and examined with the fluorescence microscope. These preparations were not detectably stained by HLA-DR antibody (Fig 2*A*). However, the cells did show membrane fluorescence with antibody to β_2-microglobulin (Fig. 2*B*).

Search for LC Among Cultured Epidermal Cells

Since the ATPase stain is specific for LC in the epidermis, epidermal cells cultured for 7 days and longer were treated with this stain. The cultures treated with complete incubation medium did not show more ATPase positive cells than did cultures treated with the control medium. Thus, staining for ATPase did not reveal significant numbers of ATPase positive LC in cultures 7 days or older. Further, in over 50 fields visualized with the electron microscope, only keratinocytes but no LC were seen. LC are the only cells in the epidermis which demonstrate Fc receptors and this property can be used to identify these cells [23]. Trypsinized, cultured epidermal cells incubated with antibody-coated BRBC did not show any rosetted cells. By contrast, freshly prepared epidermal cells (not cultured) showed about 2% rosetted cells.

2D-PAGE of Immunoprecipitated Proteins from Cultured Epidermal Cells

The 2D-PAGE pattern of HLA-DR immunoprecipitates from metabolically labeled (cultured) epidermal cells did not show spots in the range of 27,000 to 34,000 daltons (Fig 3*A*), the region in which labeled HLA-DR proteins are found in detergent extracts from dispersed, fresh (not cultured) skin cells (Fig 3*B*). Thus, by the method of 2D-PAGE, HLA-DR molecules were undetectable in detergent extracts of cultured epidermal cells.

Culture of Epidermal Cells on G-Membranes

Epidermal cells which do not express HLA-DR antigen may be useful for transplantation to unrelated recipients, especially if intact cell monolayers could be transferred to patients. Therefore, we investigated the possibility of culturing epidermal cells on membranes which could be removed intact from the culture dish. Epidermal cells attached and proliferated on the described G-membranes. Similar to cells grown on collagen coated Petri dishes, cells harvested on day 14 from a G-membrane had also lost the capacity to stimulate allogeneic lymphocytes in the ELR (not shown).

To ensure that the cultured cells were indeed keratinocytes, we harvested G-membranes on days 7, 14, and 21 of culture and processed them for electron microscopy. In fields chosen at random, all cells demonstrated tonofilaments and formed desmosomes with contiguous cell membranes (Fig 4). Thus, the vast majority of epidermal cells grown in culture were keratinocytes.

DISCUSSION

We have demonstrated that human keratinocytes cultured for 7 days or more lack the capacity to stimulate allogeneic lymphocytes in the ELR. FACS analysis of cultured cells incubated with anti-HLA-DR antibody showed no net fluorescence. However, this analysis was complicated by the fact that harvested cells cultured for 8 days or longer showed significant autofluorescence, presumably due to the autofluorescence of keratin [28]. Therefore, we examined antibody labeled, harvested cells with the fluorescence microscope. Using filters which eliminated autofluorescence, none of the cells showed specific fluorescence with anti-DR antibody.

Although the cells were maintained at room temperature overnight to allow reconstitution of cell surface structures before labeling with antibody [29], the failure of trypsinized cultured epidermal cells to express HLA-DR may be explained by proteolytic digestion of this glycoprotein by trypsin. Therefore, cultured cells were labeled *in situ* but these cells also failed to bind DR antibody whereas they were stained by antibody to β_2-microglobulin. Finally, 2D-PAGE analysis of labeled cell extracts from cells cultured for 12 days did not demonstrate "spots" in the regions where HLA-DR molecules synthesized by uncultured epidermal cells and B cells migrate [24]. On the basis of these results, we conclude that epidermal cells cultured for 7 days or longer neither synthesize nor express HLA-DR molecules in detectable quantity.

The explanation for the failure of cultured epidermal cells to express HLA-DR antigen is uncertain. Conceivably, subpopulations of HLA-DR bearing cells such as LC and endothelial cells which are present in the original cell suspension are lost during culture. Since no LC were found by electron microscopic examination of 50 random fields and since no LC were detected with the stain for ATPase or after rosetting with antibody-coated BRBC, it appears likely that few if any LC remain in epidermal cell cultures. Our inability to demonstrate LC after 1 week or more of culture are at variance with the observations of others who have reported that in epidermal cell cultures obtained from guinea pigs, LC proliferate and can be detected for 7 days or longer [30,31]. Possibly, guinea pig LC are cultured more easily than human or our conditions of culture are not optimal for LC. Thus, it is conceivable that the collagen coat used on the petri dishes prevents attachment or proliferation of LC. The layer of collagen on the Petri dish surface in our cultures may also explain the apparent absence of trypsin resistant macrophage observed in cultures of murine skin [32]. An alternative explanation for the absence of HLA-DR antigens on cultured epidermal cells is that those populations expressing HLA-DR lose this antigen (but not HLA-A, B and C antigens) during a differentiation or dedifferentiation process which may occur during culture. Whatever the mechanism, it is clear from these experiments that HLA-DR expressing and synthesizing cells are much reduced if not totally absent from cultured keratinocyte monolayers.

Although HLA antigens represent the major barriers for transplantation in man [33,34], at times, corneal allografts transplanted to unmatched recipients have been successful

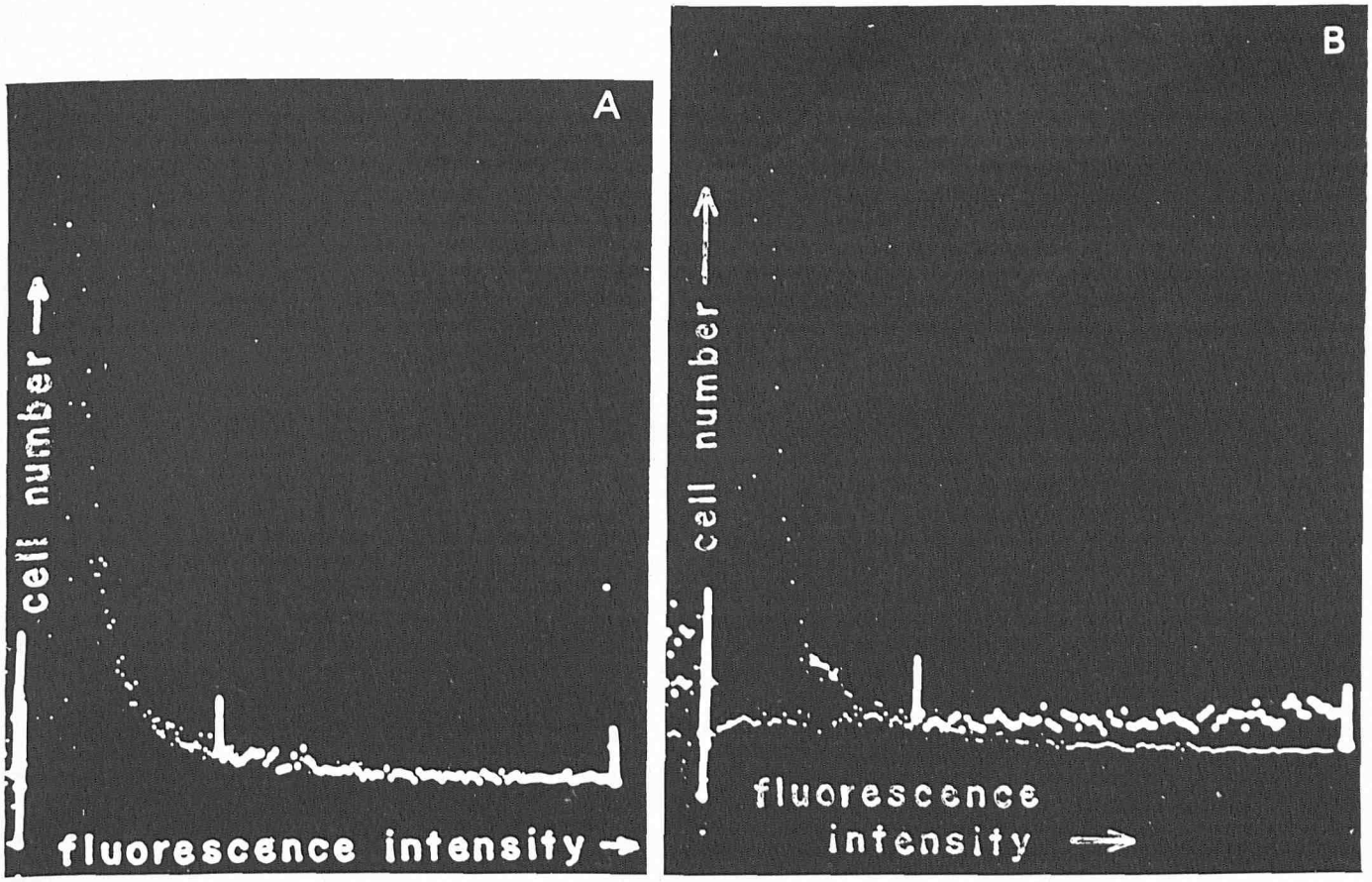

FIG 1. FACS analysis of antibody labeled epidermal cells. Epidermal cells were harvested from 8-day-old cultures, resuspended in complete RPMI and maintained for 18 hr at room temperature. The cells were labeled with either anti-DR antibody (*A*) or antibody to β_2-microglobulin (*B*) and the appropriate second step FITC and analyzed on the FACS. The FACS settings were scatter window 36-125 and fluorescence window 170-255. Each *curve* represents analysis of 10,000 cells. In both photographs the *lower curves* are of cells stained with the appropriate animal-FITC alone. In *panel A*, the 2 curves are virtually superimposable indicating that the cells lack DR antigen. In *panel B*, virtually all of the cells are stained, indicating the presence of β_2-microglobulin on these cells. The viability of cells, checked by trypan blue exclusion just before staining, was greater than 90%.

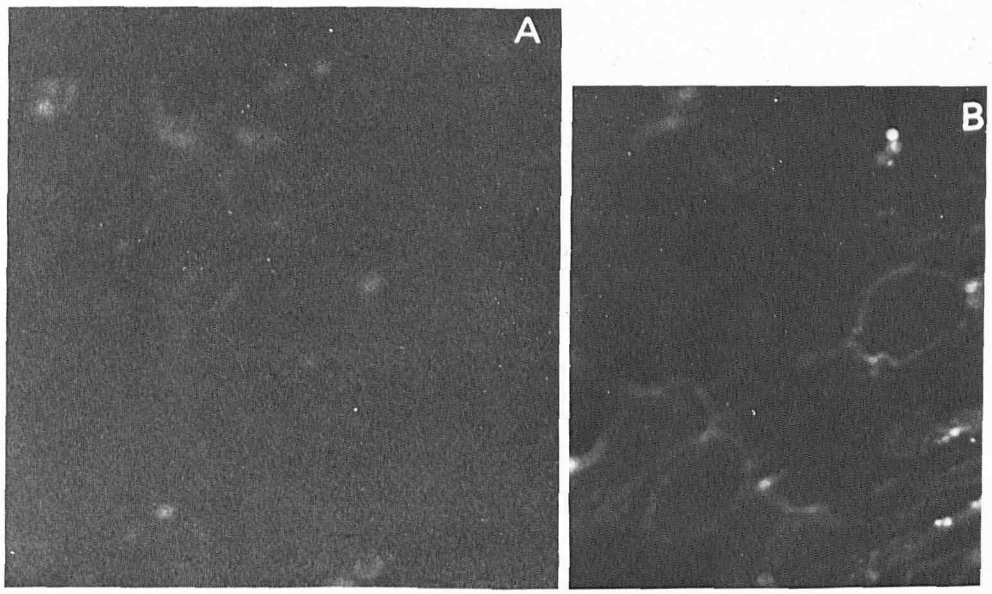

FIG 2. IIF light microscopy of cell cultures labeled *in situ*. Seven day cultures were washed twice with PBS, stained with either anti-DR antibody plus R/M-FITC (*A*) or antibody to β_2-microglobulin plus G/R-FITC (*B*) (×16). Similar fluorescence patterns were obtained in 14-day-old cultures (not shown).

[35]. The absence of Ia antigen bearing LC in mouse cornea may explain this surprising finding [6]. Moreover, the absence of LC in cornea would predict that in HLA-A, B, and C, but not D matched recipients, corneal allografts would show good survival and indeed this has been reported [36]. In an analogous situation, the absence of HLA-DR antigen from platelets has led to their successful use in transfusion to HLA-A, B matched, HLA-DR unmatched recipients [37]. A recent report using a

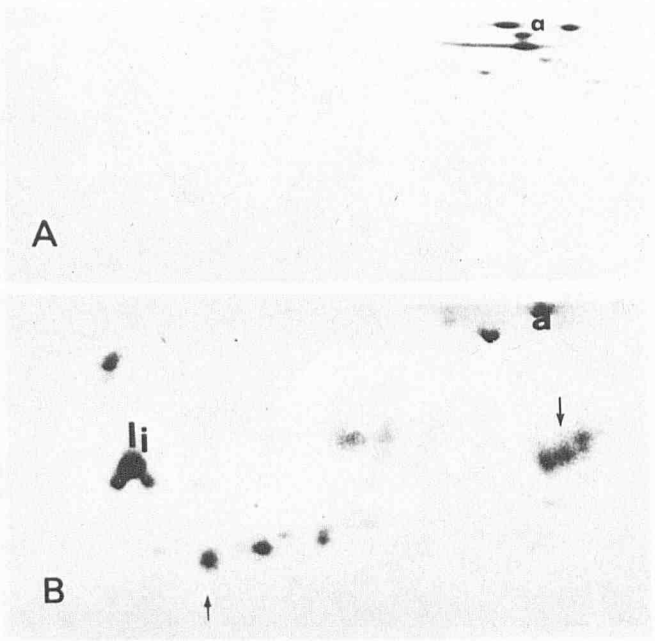

FIG 3. Analysis of epidermal cells by the method of 2D-PAGE. ^{35}S-methionine labeled cultured epidermal cells were extracted with Nonidet P-40 detergent, immunoprecipitated with monoclonal anti-HLA-DR antibody and analyzed by 2D-PAGE (*panel 3A*). The acidic end of the gel is on the right. No spots are present in the 34,000 to 27,000 dalton region of the gel. For comparison, a gel showing the HLA-DR molecular pattern obtained from freshly dispersed, (not cultured) epidermal cells is shown in panel 3*B*. (↓) 32,000 to 34,000 dalton acidic chain, (*Ii*) a 31,000 dalton invariant chain, (↑) a set of 27,000 to 28,000, s.c. dalton polypeptides; (*a*) actin is a marker molecule.

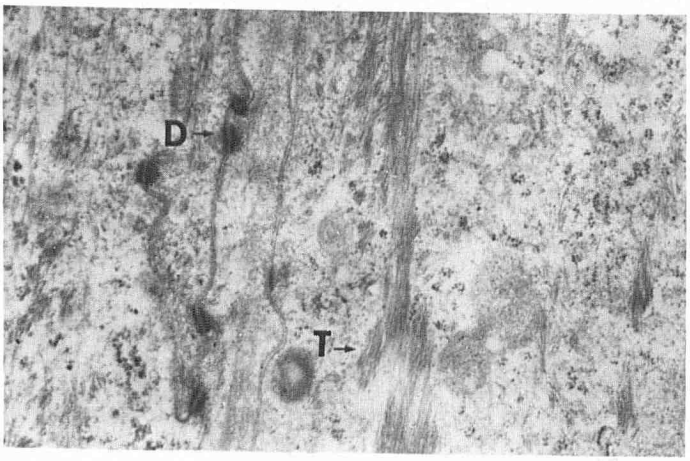

FIG 4. Electron micrograph of cultured keratinocytes. Cells were cultured on a G-membrane for 21 days. The G-membrane was removed from the Petri dish and processed for electron microscopy. (*T*) indicates tonofilaments, (*D*) indicates desmosomes, both of which are organelles characteristic of keratinocytes (reduced from ×22,000). G-membranes harvested on days 7 and 14 showed cells with similar ultrastructure (not shown).

system similar to the one described indicates that murine epidermal cell cultures implanted on an allogeneic host formed differentiating cell sheets and demonstrated prolonged survival [38]. Finally, maintenance of endocrine glands such as the thyroid in organ culture before transplantation to an allogeneic host markedly enhances allograft survival [39]. The availability of intact monolayers of cultured keratinocytes which appear to be free of HLA-DR antigens may, therefore, be useful for transplantation purposes.

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