Culture and Characterization of Murine Dendritic Thy-1$^+$ Epidermal Cells*

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Although numerous advances have been made in characterizing the phenotype, ontogeny, ultrastructure, and cytochemistry of the murine Thy-1$^+$ dendritic epidermal cell (Thy-1$^+$ EC), elucidation of its functional qualities has been hampered by the difficulty in preparing pure populations of these cells. We therefore sought to obtain expanded, purified populations of Thy-1$^+$ EC using culture techniques. Since Thy-1$^+$ EC are bone marrow-derived, density gradient enriched populations of freshly harvested epidermal cells (FH-EC) were placed in culture under conditions known or suspected to promote mitogenesis among leukocyte subsets. FH-EC prepared from truncal skin of C3H/HeN mice (Thy-1.2$^+$) were cultured at 37°C in 5% CO_2 in complete medium (CM) of Eagle's Hanks' amino acid with 10% fetal calf serum, nutrients, and antibiotics at 10^6 FH-EC/well in 24-well culture plates. CM was supplemented with one or more of the following: concanavalin A (Con-A), interleukin-1/epidermal cell-derived thymocyte-activating factor (IL-1/ETAF), IL-2, IL-3, γ interferon, indomethacin (IM), and anti-Thy-1.2 antibody. Media with appropriate supplements were changed every 2–3 days. Freshly isolated, enriched FH-EC contained 7–20% Thy-1$^+$ EC (defined as brightly fluorescing cells readily distinguishable from weakly fluorescing keratinocytes), which also stained with antibodies directed against asialo GM_1, Ly 5.1, and vimentin but did not stain with antibodies to other T cell-, B cell- or macrophage phenotypic markers. Analysis of 10 separate cultures revealed a 3- to 10-fold expansion of nonkeratinocyte Thy-1$^+$ cells after 21 $\pm$ 4 days in culture in CM supplemented with Con-A and IM, and 70–100% of viable cells after expansion were Thy-1$^+$. Phenotypic analysis of expanded cells revealed the emergence in 10 separate cultures of one of two mutually exclusive distinct populations: one Thy-1$^+$, asialo GM_1^+, L3T4$^-$ (natural killer phenotype) and the other Thy-1$^+$, asialo GM_1^-, L3T4$^+$ (T helper phenotype). Experiments designed to explain the emergence of an L3T4$^+$ population suggest that phenotypic modulation occurred in vitro. *J Invest Dermatol 86:615–624, 1986*

A population of Thy-1 antigen-bearing dendritic epidermal cells (Thy-1$^+$ EC) distinct from known subpopulations of epidermal cells (EC) including keratinocytes, Langerhans cells (LC), melanocytes, and Merkel cells, has recently been described in murine skin [1,2]. Dendritic Thy-1$^+$ EC, which constitute 0.8–2.7% of all EC, fluoresce intensely with anti-Thy-1 antibodies. Keratinocytes have also been shown to express Thy-1 antigen in immunofluorescence and cytotoxicity studies [1,3], but keratinocytes fluoresce less intensely with anti-Thy-1 antibodies and presumably express lesser quantitites of this antigen [1]. Since Thy-1 antigen is present on the surface of cells in a wide variety of rodent tissues [4–12], initial studies were directed toward defining the lineage of the dendritic Thy-1$^+$ EC. This cell expresses Ly-5 antigen, a membrane determinant present on bone marrow-derived leukocytes [1]. The bone marrow derivation of the dendritic Thy-1$^+$ EC has been confirmed in chimerization studies [13,14].

Though earlier studies suggested a limited distribution of Thy-1 antigen, expression of Thy-1 antigen by murine bone marrow cells is not restricted to prothymocytes; it is also present on pluripotential bone marrow stem cells, and on some B-cell, myeloid, and macrophage precursors [15,16]. However, dendritic Thy-1$^+$ EC do not express other antigens common to these various marrow-derived cells [17], nor do they express the conventional mu-

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Abbreviations
BSA: bovine serum albumin
CM: complete medium
Con-A: concanavalin A
EC: epidermal cells
ETAF: epidermal cell-derived thymocyte-activating factor
FACS: fluorescence-activated cell sorter
FCS: fetal calf serum
FH-EH: epidermal cells harvested from the interface of Ficoll-Hypaque density gradients
γ-IFN: gamma interferon
IL: interleukin
IM: indomethacin
LC: Langerhans cells
LNC: lymph node cells
MLR: 2-way mixed lymphocyte reaction
mAb: monoclonal antibody
NK: natural killer
PBS: phosphate-buffered saline
SC: spleen cells
Sup: supernatant
Thy-1$^+$ EC: dendritic epidermal cells bearing Thy-1 antigen
EHAA: Eagle's Hanks' amino acid

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Table I. Monoclonal Antibodies Directed Against Surface and Cytoplasmic Antigens

Antibody	Specificity	Source
Anti-Thy-1	Thymocytes, peripheral T cells, bone marrow hematopoietic precursors, central and peripheral neurones, fibroblasts, mammary myoepithelial cells, subsets of natural killer cells, epidermal cells	α-Thy 1.2: Becton Dickinson, Sunnyvale, CA; α-Thy 1.1 and α-Thy 1.2: New England Nuclear, Boston, MA
Anti-Ly-5.1 } Anti-Ly-5.2 }	Bone marrow-derived leukocytes	New England Nuclear
Anti-Lyt-1	Variably expressed on all peripheral T-cells	Becton Dickinson
Anti-Lyt-2	Suppressor and cytotoxic T cell subset	Becton Dickinson
H129.19	Rat antimurine helper T cell subset [20]	Julie Titus, NIH Bethesda, MD
GK1.5 (L3T4)	Rat antimurine helper T cell subset [21]	Ethan Shevach and Thomas Malek, NIH
MAR 18.5	Mouse antirat immunoglobulin [22]	Ethan Shevach and Thomas Malek, NIH
Mac-1	Human and murine monocyte antigen (C3bi receptor)	Hybritec Inc., San Diego, CA
10.2.16 (anti-Ia^k) } MKD.6 (anti-Ia^d) }	Class II alloantigens on murine Langerhans cells, B cells, monocytes, antigen-presenting cells [23,24]	Cells obtained from American Type Culture Collection, Rockville, MD
C82	Vimentin type intermediate filaments	S.I. Katz, NIH
C56	Keratin type intermediate filaments	S.I. Katz, NIH
2.4G2	Rat antimurine FcIgG receptor [25]	Julie Titus, NIH
Anti-Leu-8	Murine antihuman T cell	Becton Dickinson

rine T-cell markers Lyt-1, Lyt-2, or Lyt-3 and L3T4 [1,17], and their precursors do not exist in large numbers in the thymus [13]. Thus the lineage of Thy 1$^+$ EC among marrow-derived cells remains somewhat puzzling. However, dendritic Thy-1$^+$ EC, like murine natural killer (NK) cells [18,19] express membrane asialo GM$_1$ [17].

Although the phenotypic markers of the dendritic Thy-1$^+$ EC have been extensively characterized, the function of this newly recognized epidermal cell remains to be defined. Attempts at functional characterization of this cell have been hampered by the fact that dendritic Thy-1$^+$ EC constitute only a small percentage of total EC in unfractionated EC suspensions. Enrichment of EC suspensions for pure populations of dendritic Thy-1$^+$ EC by "panning" or flow cytometric sorting is technically complicated by the fact that keratinocytes also express Thy-1 antigen. We therefore sought to determine whether it was possible to grow dendritic Thy-1$^+$ EC in long-term cultures of EC suspensions under conditions which would select for these cells. Since dendritic Thy-1$^+$ EC are bone marrow-derived, we attempted to induce division and hence expansion of these cells by addition to the cultures of various substances known or suspected to promote mitogenesis of leukocyte subsets. We report here that addition of Concanavalin A (Con-A) to EC suspensions, which were partially enriched for dendritic Thy-1$^+$ EC by density gradient centrifugation, resulted in cultures that were enriched for nonkeratinocyte populations of cells which were either Thy-1$^+$, asialo GM$_1^-$, L3T4$^+$ (T helper phenotype) or Thy-1$^+$, asialo GM$_1^+$, L3T4$^-$ (NK phenotype).

MATERIALS AND METHODS

Animals Female C3H/HeN MTV$^-$ (H-2^k, Thy-1.2$^+$) and BALB/cAnN (H-2^d, Thy-1.2$^+$) mice were obtained from the Charles River Breeding Laboratory, Wilmington, Massachusetts. Female C3H/HeJ (H-2^k, Thy-1.2$^+$) and AKR/J (H-2^k, Thy-1.1$^+$) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. Female AKR/Cum (H-2^k, Thy-1.2$^+$) mice were obtained from Cumberland View Farms, Clinton, Tennessee. Male NZB mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility. All mice were used at 6–12 weeks of age.

Serologic Reagents and Their Specificities A battery of monoclonal antibodies (mAb) directed against murine cell surface and cytoplasmic antigens was employed; mAb were used in unconjugated and various fluorochrome-coupled forms (Table I). Other unconjugated and fluorochrome- or peroxidase-conjugated reagents and their specificities included: rabbit polyclonal anti-asialo GM$_1$: glycolipid on murine NK cells (Wako, Dallas, Texas); goat antimouse IgG (heavy and light chain specific; Tago, Burlingame, California); goat antimouse IgM (μ chain specific; Tago); goat antimouse IgG [F(ab')$_2$ fragment specific; Cappel Laboratories, Cochranville, Pennsylvania]; F(ab')$_2$ fragments of goat antimouse IgM (μ chain specific; Cappel); goat antirat IgG (heavy and light chain specific; Cappel); affinity-purified goat antirabbit IgG (heavy and light chain specific; Tago); biotinylated goat antirat IgG (Becton Dickinson, Sunnyvale, California); avidin phycoerythrin (Becton Dickinson); MOPC 210 (purified homogenous IgG$_1$) and TEPC 183 (purified homogenous IgM$_k$; Litton Bionetics, Kensington, Maryland). Mouse, rat, rabbit, and goat serum were obtained from normal preimmune animals. Control monoclonal supernatant was obtained from cultures of the non-antibody producing murine myeloma line, SP2/0, and control ascites from animals injected with SP 2/0 cells. Young rabbit serum was used as a source of complement.

Immunofluorescence Staining of Epidermal Sheets The phenotypic characteristics of dendritic Thy-1$^+$ EC were investigated by determining reactivity with antibodies directed against murine antigens using an indirect immunofluorescence staining technique on epidermal sheets as described [13].

Cell Preparation EC suspensions were prepared by trypsinization as previously described [26]. The viability of EC in these suspensions ranged between 75–85% as measured by trypan blue dye exclusion. In order to enrich for the subset of dendritic Thy-1$^+$ EC,[‡] fresh EC suspensions were subjected to fractionation on Ficoll-Hypaque density gradients. Preliminary experiments directed at enrichment of dendritic Thy-1$^+$ EC demonstrated that use of a mixture of 9% Ficoll (Ficoll 400, Pharmacia Fine Chemicals, Piscataway, New Jersey) and 50% Hypaque (sterile water-diluted Hypaque-M, 90%, Winthrop Laboratories, New York, New York) in a ratio of 3:2 yielded optimal recovery of these cells. EC (5×10^6/ml) in Puck's saline plus 10% fetal calf serum (FCS), were layered onto 3 ml of the above Ficoll-Hypaque mixture. EC at the interface (FH-EC) after centrifugation at 400 g for 30 min at 20°C were harvested and washed once in phosphate-buffered saline (PBS) and 3 times in Puck's saline with 10% FCS and were used in all culture experiments. The yield of viable FH-EC recovered ranged between 20–30% of total EC layered onto the gradient. The viability of these FH-EC ranged between 85–98%. Murine peripheral and mesenteric lymph node cells (LNC), thy-

‡EC in suspension fluorescing intensely with anti-Thy-1 mAb, clearly separable in C3H and AKR mice from the majority of keratinocytes fluorescing only weakly, correspond to dendritic Thy-1$^+$ EC seen in epidermal sheets and are henceforth referred to as "dendritic Thy-1$^+$ EC," though in suspension they are no longer markedly dendritic.

mocytes, and spleen cells (SC) were obtained and, in certain experiments, SC or LNC were depleted of Ia+ cells as previously described [13,27].

Fluorescence Flow Cytometric Analysis of Cell Suspensions (FACS) The presence of the effectiveness of depletion of specific subsets of EC, SC, and LNC were monitored by flow cytometric analysis using a FACS II Dual Laser cell sorter (Becton Dickinson FACS systems, Mountain View, California) as previously described [28] after 1-, 2-, or 3-step staining for specific populations. Fluorescein isothiocyanate (FITC) was excited at 488 nm and propidium iodide was excited at 568.2 nm. Data on individual cells were collected, stored, displayed, and analyzed using a PDP 11/24 computer (Digital Equipment Corporation, Maynard, Massachusetts) interfaced to the FACS II. Fluorescence data were collected using logarithmic amplification, provided by a 3-decade logarithmic amplifier constructed from an NIH-modified design of R. Hiebert, LASL (Los Alamos, New Mexico). One-color fluorescence data are displayed as cell frequency histograms or immunofluorescence profiles in which logarithmically increasing fluorescence intensity is plotted in 1024 channels on the x-axis and cell number is shown on the y-axis. Propidium iodide was added to stained preparations just prior to analysis to exclude false-positive fluorescence attributable to dead cells. Both unfractionated SC and purified T-cell suspensions, as well as trypsin- and DNAase-treated SC and T cells, were used for control staining in FACS analyses. At least 40,000 viable cells as determined by forward light scatter and propidium iodide exclusion were analyzed for specific staining, and in some instances $1–2 \times 10^5$ cells were studied when searching for the presence of low numbers of cells expressing certain antigens.

Immunofluorescence Staining of Cell Suspensions Epidermal cells, SC, LNC, or thymocytes ($1–10 \times 10^5$) in single cell suspensions were stained either by a one-step procedure using a fluorochrome-conjugated antibody or by an indirect immunofluorescence technique as described [29]. Single or first-step antibodies were used at concentrations of 0.5–2.0 μg/ml per 10^6 cells, depending upon the antibody. Flourochrome- or biotin-conjugated second- or third-step reagents were used at a 1:40 dilution and avidin phycoerythrin was used at 1:80. In certain experiments, a mAb directed against the murine FcIgG receptor (2.4G2) was utilized in a preincubation blocking procedure at 1 μg/ml per 10^6 cells to prevent nonspecific binding of reagents via their Fc portion. For double fluorescence staining with reciprocal fluorochromes, either a 2- or 3-step procedure was used as described [29]. For quantification of viable fluorescing cells using fluorescence microscopy, ethidium bromide (0.5 μg/ml final concentration) was added to EC suspensions just before mounting under a coverslip. Preparations were viewed with a Leitz orthoplan microscope equipped with epifluorescence and the percentage of positively stained cells was determined by counting at least 300 cells.

In certain experiments immunofluorescence staining was performed as described [29] on methanol-fixed cell preparations in order to detect intracytoplasmic as well as surface antigens. Controls consisted of the murine Pam 212 spontaneously transformed EC line and murine NIH 3T3 fibroblasts grown on glass coverslips. Following fixation, staining was performed as described above except that antibodies were diluted in 0.3 M PBS with 10% normal goat serum, 1% bovine serum albumin (BSA) (Sigma, St. Louis, Missouri), and 0.5% Triton X-100 (Sigma).

Transmission Electron Microscopy of EC Suspensions Epidermal cell suspensions were prepared for transmission electron microscopy as described [30]. Thin sections were cut, stained with lead citrate and uranyl acetate, and viewed with a Phillips 400 electron microscope.

Generation of Culture Supernatants Supernatant was obtained from a 2-way mixed lymphocyte reaction (MLR-Sup) performed by coculturing 1×10^6 BALB/c SC with 1×10^6 C3H/HeN SC for 72 h in complete medium (described below) and acted as a source of gamma interferon (γ-IFN). Culture supernatants were also obtained from WEHI-3 cells (as a source of interleukin-3, IL-3), Pam 212 cells (as a source of ETAF/IL-1), and L cells (supernatant nutritive in bone marrow culture systems) grown to confluence in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, New York) containing 10% FCS, 20 mM L-glutamine (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Epidermal Cell Culture C3H/HeN (Thy 1.2+) FH-EC (1×10^6/well) were plated in Eagle's Hanks' amino acid (EHAA) medium (National Institutes of Health Media Unit) with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 2 mM L-glutamine (GIBCO), and 2.5×10^{-5} 2-mercaptoethanol (complete medium) in 24-well tissue culture plates (Linbro, Flow Laboratories Inc., McLean, Virginia). In certain experiments, irradiated (10,000 rad from a ^{137}Cs source) Pam 212 cells or AKR/J (Thy 1.1+) SC or bone marrow cells (3300 rad) were added as feeder cells at a concentration of 5×10^5/well. In other experiments, the complete medium was supplemented by addition of one or more of the following substances at the final concentrations given: Con-A (Calbiochem Behring, San Diego, California) 0.1–20 μg/ml; IL-2 containing supernatant (rat T-cell clone) (Collaborative Research, Inc., Lexington, Massachusetts) 5–10%; anti-Thy-1.2 mAB (New England Nuclear) 1:500; indomethacin (IM) (Sigma) 1 μg/ml; MLR-Sup 10%; WEH1-3 supernatant 12.5–25%; L-cell supernatant 12.5–25%. Media containing appropriate additives were changed every 2–3 days. Aliquots of cells grown under the various culture conditions were harvested periodically for fluorescence microscopic, flow cytometric, ultrastructural, and functional studies. In some experiments, varying numbers of purified AKR/Cum (Thy-1.2+) T cells were added to cultures of AKR/J (Thy-1.1+) FH-EC in order to determine the potential rate of proliferation of any lymphocytes that might have been present in FH-EC preparations as a result of dermal contamination. Expansion of Thy-1+ EC was determined by a combination of parameters: phase microscopic appearance of cells in culture; periodic staining of cultured cells with anti-Thy-1 and other mAb; and after pooling all expanding cells, by determination of the absolute number by hemocytometer counting of viable Thy-1+ cells present after various days in culture and comparing that number with the number of Thy-1+ EC initially added.

Assay to Detect Con-A Stimulation of Freshly Harvested and Cultured Cells Freshly prepared C3H/HeN FH-EC, SC, and Thy-1+ EC in FH-EC preparations cultured for 21 days in the presence of Con-A were assayed for their proliferative response to Con-A. Cells were washed 3 times in EHAA containing antibiotics, L-glutamine, IM, and 1% NZB mouse serum in order to remove Con-A from the culture medium, and allowed to rest overnight. Percentages of Thy-1+ cells in each population were determined by fluorescence microscopy after staining with anti-Thy-1 mAb, and concentrations of cells were adjusted so that equivalent numbers of Thy-1+ cells could be placed in each well of a round-bottom microtiter plate. Cells were cultured in the presence of Con-A at optimum concentrations in 5% CO_2 at 37°C for 72 h; experiments were performed in triplicate. Controls consisted of the various populations cultured in the absence of added Con-A, as well as populations cultured in the presence of Con-A but preirradiated with 10,000 R. Thy 1+ EC in 21-day Con-A cultures of FH-EC were provided with Ia+ accessory cells by adding 10^4 irradiated (3300 R) syngeneic SC and/or EC. Proliferation after 72 h was determined by [^{3}H]thymidine uptake and liquid scintillation counting after adding 1 μCi/well 18 h prior to harvesting.

RESULTS

Phenotypic Characterization of Freshly Isolated Uncultured Thy-1+ EC Fluorescence microscopic analysis of single-

and double-stained preparations of epidermal sheets, newly harvested unfractionated EC and FH-EC suspensions, and methanol-fixed FH-EC suspensions confirmed that the dendritic Thy-1$^+$ EC are asialo $GM_1{}^+$, Ly-5$^+$, and vimentin$^+$ (C82) and do not express class II alloantigens, FcIgG receptors, Mac-1, the T-cell markers GK1.5 (L3T4), Lyt-1 and Lyt-2, or cytokeratins (C56).

Addition of Con-A and IM to FH-EC in Culture Promotes Expansion of Thy-1$^+$ Cells Serial sampling of cultures of FH-EC grown under various culture conditions (Table II) revealed increasing percentages of Thy-1$^+$ cells occurring when Con-A and IM (1 μg/ml) were added to complete medium. The concentration of solubilized Con-A that most consistently resulted in optimum expansion of Thy-1$^+$ EC was 0.5 μg/ml. The response to the presence of either Pam 212 or bone marrow feeder cells was not consistently better than that to Con-A and IM alone. Antibody to the Thy-1 antigen, IL-2, supernatants containing IL-1/ETAF (Pam cell-Sup), IL-3 (WEHI-3 cell-Sup), γ-IFN (MLR-Sup), or factors nutritive in bone marrow culture systems (L cell-Sup) did not result in increased percentages of Thy-1$^+$ cells when added to IM-supplemented FH-EC cultures; nor did addition of these factors to Con-A-stimulated cultures enhance the response when compared with Con-A and IM alone. When cultured cells were viewed by phase microscopy, expanding Thy-1$^+$ EC under optimum conditions took on a plump, sausage-like appearance similar to that seen in proliferating lymphoid cultures and easily distinguishable from expanding keratinocyte lines (Fig 1). Phenotypically distinct expanded cells were indistinguishable by phase microscopy. At optimum expansion, the vast majority of viable cells stained intensely with anti-Thy-1 antibodies (Fig 2). In 10 separate experiments, the percentage of Thy-1$^+$ cells increased to 70–100% of viable cells and the absolute number of Thy-1$^+$ cells was found to have expanded 3- to 10-fold (Table II). Though expansion did not occur in every experiment (10 of 20 separate cultures), in 10 cultures supplemented with Con-A optimum expansion was apparent by day 21 $\pm$ 4.

Phenotypic Characterization of Cultured Thy-1$^+$ Cells Aliquots of each culture were studied by fluorescence microscopy to determine surface and cytoplasmic phenotypic characteristics of the cells. At optimum expansion (21 $\pm$ 4 days), cells from visibly expanding cultures under a given culture condition were pooled, counted, and stained in combination of 1-, 2-, and 3-step procedures using appropriate positive and negative controls. The results of 4 representative separate culture experiments (Table III) revealed that the culture conditions promoted the growth of 1 of 2 phenotypically distinct Thy-1.2$^+$ cells: a Thy-1.2$^+$, asialo $GM_1{}^+$, L3T4$^-$ population and a Thy 1.2$^+$, asialo $GM_1{}^-$, L3T4$^+$ population. Both populations were positive for the bone marrow marker Ly-5.1 and for vimentin type intermediate filaments (C82), and did not stain with antibodies to Thy-1.1, Ly-5.2, cytokeratin (C56), the murine T-cell markers Lyt-1 and Lyt-2, the C3H/He class II alloantigen Ia^k, and the murine macrophage marker Mac-1. Despite identical cell preparation, original phenotypic expres-

Table II. Identification of Thy-1+ Cells in Cultures of FH-EC

Culture Conditions (FH-EC with Indomethacin 1 μg/ml)	Day 6–9 (% Thy-1$^+$)	Day 12–16 (% Thy-1$^+$)	Day 20–22 (% Thy-1$^+$)	Day 20–22 Expansion Ratio
Exp A				
+ 0 (day 0, % Thy-1$^+$ = 17.7)	9[a] (28)[b]	5 (45)	6 (32)	—
+ IL-2 (5% Sup)	11 (10)	0 (7)	QNS[c]	—
+ Con-A[d]	33 (30)	68 (69)	85 (61)	4.3-fold[e]
+ α-Thy-1.2 1:500	0 (61)	0 (43)	QNS	—
+ MLR-Sup (γ-IFN) 10%	6 (35)	2 (38)	QNS	—
Exp B				
+ 0 (day 0, % Thy-1$^+$ = 7.3%)	4 (41)	3 (40)	0 (22)	—
+ Con-A	14 (43)	56 (52)	71 (63)	8.2-fold
+ XR Pam	8 (38)	6 (47)	1 (21)	—
+ XR Pam + Con-A	35 (39)	82 (83)	95 (68)	10.0-fold
+ XR BM	11 (57)	13 (43)	6 (33)	—
+ XR BM + Con-A	44 (41)	62 (68)	68 (61)	7.8-fold
Exp C				
+ 0 (day 0, % Thy-1$^+$ = 13.3%)	8 (32)	6 (38)	3 (27)	—
+ Con-A	22 (38)	61 (51)	93 (68)	9.4-fold
+ XR PAM	9 (36)	10 (34)	6 (29)	—
+ XR PAM + Con-A	24 (30)	51 (46)	72 (54)	6.8-fold
+ XR BM	14 (43)	11 (41)	11 (33)	—
+ XR BM + Con-A	19 (37)	45 (50)	71 (58)	5.9-fold
+ IL-2 (5% Sup)	11 (38)	18 (29)	13 (24)	—
+ IL-2 (5% Sup) + Con-A	16 (48)	29 (53)	34 (41)	—
+ MLR-Sup (γ-IFN) 10%	13 (39)	11 (41)	10 (28)	—
+ MLR-Sup + Con-A	20 (41)	24 (37)	18 (31)	—
Exp D				
+ 0 (day 0, % Thy-1$^+$ = 10.8%)	4 (31)	3 (19)	QNS	—
+ Con-A	29 (48)	58 (51)	74 (59)	5.6-fold
+ PAM-Sup (ETAF/IL-1) 10%	14 (34)	18 (30)	8 (20)	—
+ PAM-Sup + Con-A	21 (41)	38 (40)	39 (39)	—
+ WEHI-3-Sup (IL-3) 25%	4 (20)	1 (18)	QNS	—
+ WEHI-3-Sup + Con-A	16 (28)	14 (32)	6 (29)	—
+ L-cell-Sup 25%	6 (28)	4 (24)	QNS	—
+ L-cell-Sup + Con-A	10 (29)	11 (33)	8 (20)	—

[a]Percentage of viable cells staining with fluorochrome labeled anti-Thy-1.2 antibody; control staining with anti-Thy-1.1 antibody was negative in all cultures.
[b]Overall viability of cell preparation as determined by ethidium bromide exclusion.
[c]Quantity of viable cells not sufficient for testing.
[d]Con-A concentration 0.5 μg/ml in all experiments.
[e]Ratio of expansion of Thy-1$^+$ cells determined by comparing the number of Thy-1$^+$ cells on day tested (20–22) with the total number of Thy-1$^+$ cells placed in culture on day 0.
Key: BM = bone marrow
XR = x-irradiated
— = no ratio calculated because no significant expansion noted.

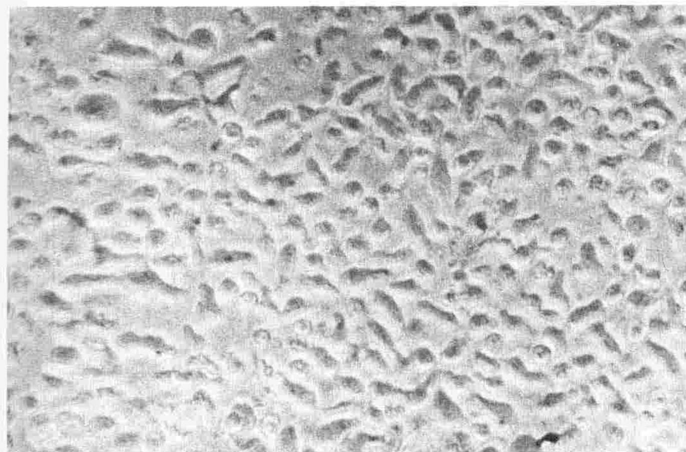
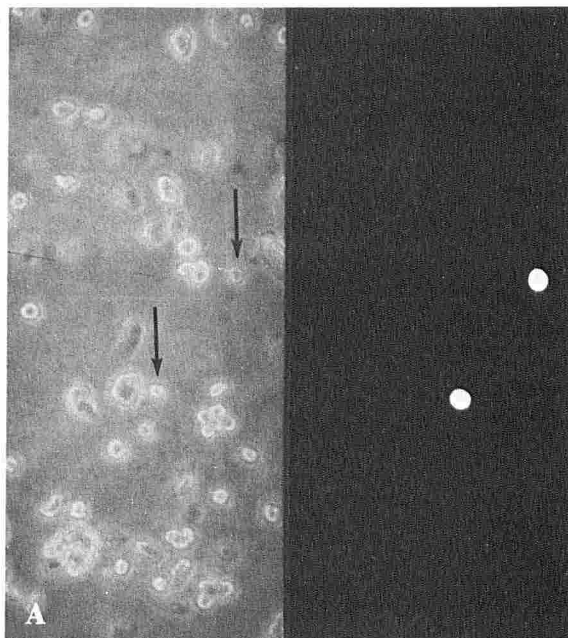


Figure 1. Phase microscopy of EC after 21 days culture in complete medium (CM), Con-A, and IM (× 360).

sion, and culture conditions, no culture experiment resulted in a mixed expansion of both populations, nor were there discernible differences in growth characteristics. Two- and 3-step reciprocal fluorochrome staining confirmed that all Thy-1.2$^+$ cells were vimentin-positive and cytokeratin-negative and that Thy-1.2$^+$ expanded cells were either L3T4-positive or asialo GM_1-positive, depending upon the culture. Phenotypic expression did not change with prolonged culture, though few cultural systems could be maintained beyond 6 weeks. Of the 10 separate cultures resulting in expansion of Thy-1$^+$ cells, in 4 the cells were asialo $GM_1{}^+$, L3T4$^-$ and in 6 the cells were asiaalo $GM_1{}^-$, L3T4$^+$.

Electron Microscopy of Cultured Thy-1$^+$ EC Cells studied after approximately 3 weeks in culture had a convoluted cell surface, a lobulated nucleus, and an active cytoplasm containing prominent light and dense granular structures which appear to be lysosomes and phagolysosomes, numerous mitochondria, Golgi, and abundant rough endoplasmic reticulum (Fig 3). While present, intermediate filament-like structures were not as prominent as in newly isolated Thy-1$^+$ EC, and the characteristic membrane-bound granule with electron-dense core as described by Tschachler et al [1] was only rarely observed. There were no striking differences among phenotypically distinct expanded populations, though not all cultures were studied ultrastructurally.

Coculture of Thy-1.1$^+$ EC with Thy-1.2$^+$ T Cells Experiments were designed to seek an explanation for the emergence of an L3T4$^+$ population of cells from culture of EC suspensions which were apparently L3T4$^-$ when freshly prepared; specifically, to determine whether the expanding Thy-1$^+$ population might result from contamination of the FH-EC by dermal or trafficking T cells. Cocultures of varying numbers per well of AKR/Cum lymph node T cells (H-2^k, Thy-1.2$^+$) added to 10^6 AKR/J EC (H-2^k, Thy-1.1$^+$) per well in complete medium supplemented with Con-A and IM were established to determine the minimum number of T cells needed to result in the observed expansion. AKR/Cum T cells, which were purified by G10 and nylon wool column passage followed by treatment with anti-Ia^k and complement, were 76% L3T4$^+$, 90% Thy-1.2$^+$, and 100% asialo $GM_1{}^-$. Serial phenotypic analysis of cocultures and controls (Fig 4) revealed that Thy-1.2$^+$ T cells at 10^5 (10% of total cells) and 10^4 (1% of total cells) per well containing 10^6 Thy-1.1$^+$ EC and Con-A rapidly expanded by 7 days, that Thy-1.2$^+$ T cells at 10^3 per well (0.1% of total cells) expanded by 14 days, but that Thy-1.2$^+$ T cells at 10^2 per well (0.01% of total cells) failed to expand under these conditions. In all situations, expanded Thy-1.2$^+$ cells were L3T4$^+$ and asialo $GM_1{}^-$, as were expanded control Thy 1.1$^+$ AKR/J EC cultured alone with Con-A. These studies



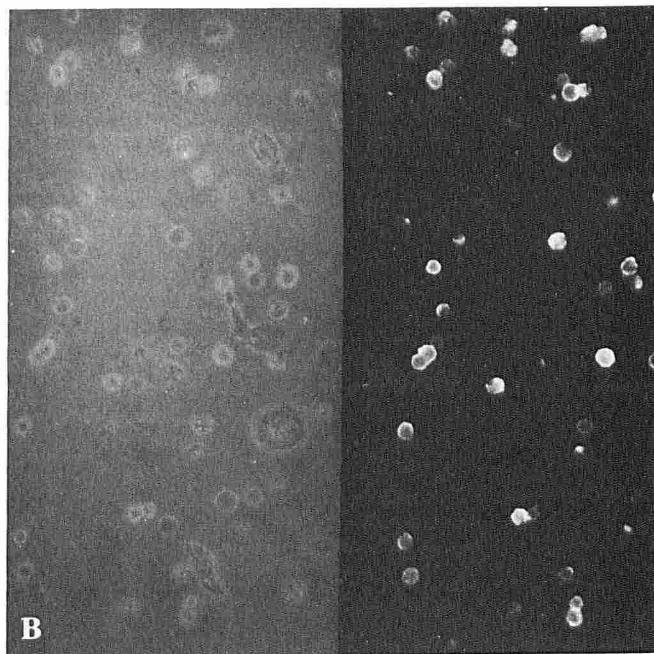


Figure 2. Identical fields under phase and fluorescence microscopy after staining aliquots of EC with anti-Thy-1.2 antibody. *A*, Fresh EC with <2% viable cells thy-1.2$^+$ (*arrows*) (× 225). B, Epidermal cells after 21 days culture in CM, Con-A, and IM with >95% viable cells Thy-1.2$^+$ (× 185).

suggest that expansion of a Thy-1$^+$, L3T4$^+$, asialo $GM_1{}^-$ population could occur in a situation where such cells were present at a 0.1% or greater concentration in the original cell preparation, but the time course of this T-cell expansion corresponded only with that observed for expansion of Thy 1$^+$ EC when T cells were present at a concentration of 0.1%.

Antigens L3T4 and Lyt-2 are Trypsin Sensitive Experiments consistently revealed the total absence of any L3T4$^+$ cells in freshly prepared unfractionated or fractionated EC suspensions. The possibilities therefore existed that this antigen was

Table III. Phenotypic Characteristics of Thy-1⁺ EC in Expanded FH-EC Cultures

Antibodies Directed Against	Exp 1 Day 0	Exp 1 Day 22	Exp 2 Day 0	Exp 2 Day 20	Exp 3 Day 0	Exp 3 Day 25	Exp 4 Day 0	Exp 4 Day 21
Thy-1.2	7.3%[a]	95%	19.1%	74%	7.5%	100%	13.8%	91%
Ly 5.1	NT[b]	97%	NT	78%	NT	100%	15.1%	96%
Asialo GM_1	7.4%	95%	18.6%	72%	7.8%	0%	13.1%	0%
Lyt 1	NT	0%	NT	0%	NT	0%	0%	0%
Lyt 2	NT	0%	NT	0%	0%	0%	0%	0%
L3T4	NT	NT	NT	0%	0%	100%	0%	89%
Thy-1.2 + C82 (vimentin)	NT	NT	NT	+[c]	NT	+	NT	NT
Thy-1.2 + C56 (cytokeratin)	NT	NT	NT	−	NT	−	NT	NT
Thy-1.2 + L3T4	NT	NT	NT	−	−	+	−	+
Thy-1.2 + asialo GM_1	NT	+	NT	+	+	−	+	−

[a]Percent of viable cells staining with indicated antibody.
[b]Not tested.
[c]Plus (+) denotes mutual coexpression of indicated antibodies; minus (−) denotes absence of mutual coexpression of antibodies indicated.

originally present but was trypsin sensitive, or that it emerged de novo over time in culture. The possibility that L3T4⁺ cells were originally present in any great number appeared to be extremely low since no such cells were seen in epidermal sheet preparations. To investigate further the growth of an L3T4⁺ population from EC in culture, freshly harvested FH-EC suspensions, untreated and trypsin-treated fresh SC suspensions, and identical populations placed in culture in complete medium with Con-A for various time periods were studied by flow cytometric analysis and fluorescence microscopy. Thy-1.2⁺ subpopulations were detected in each of the above cell suspensions (SC: Fig 5*A*,*B*). However, among freshly prepared preparations, L3T4⁺ and Lyt-2⁺ cells were detectable only in nontrypsinized SC. FH-EC and freshly prepared SC treated with trypsin in a manner identical to that used for preparation of EC failed to stain with antibodies to L3T4 (SC: Fig 5*C*,*D*) and Lyt 2. Thus, these antigens are trypsin sensitive and cannot possibly be detected in fresh, trypsin-prepared EC suspensions even if cells bearing these antigens in vivo are present. Since Lyt-2 antigen on thymocytes is again detectable within 24 h after enzymatic removal [28], FH-EC and trypsin-treated SC were placed in culture under conditions identical to those used for expansion of Thy-1⁺ EC and were analyzed by flow cytometric and fluorescence microscopic analysis 24, 48, and 72 h after trypsin exposure to determine the emergence or continued absence of trypsin-sensitive antigens. L3T4 was detectable on trypsinized SC after 24 h in culture and fully expressed after 72 h (Fig 6*A*,*B*). However, when compared with irrelevant mAb controls (anti-Leu-8), flow cytometric analysis of up to 2×10^5

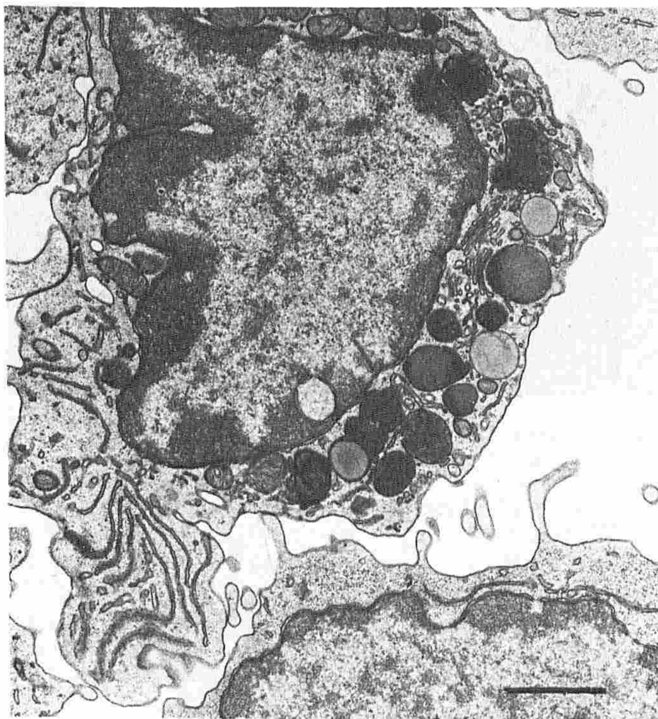


Figure 3. Electron microscopy of representative cell from FH-EC preparation after 20 days culture in the presence of Con-A, at which point >95% viable cells were Thy-1.2⁺, asialo GM_1⁺, and L3T4⁻. Cell displays a convoluted surface, lobulated nucleus, numerous electron-lucent and dense granular structures, mitrochondria, prominent rough endoplasmic reticulum, and Golgi apparatus. *Bar* = 1 μm.

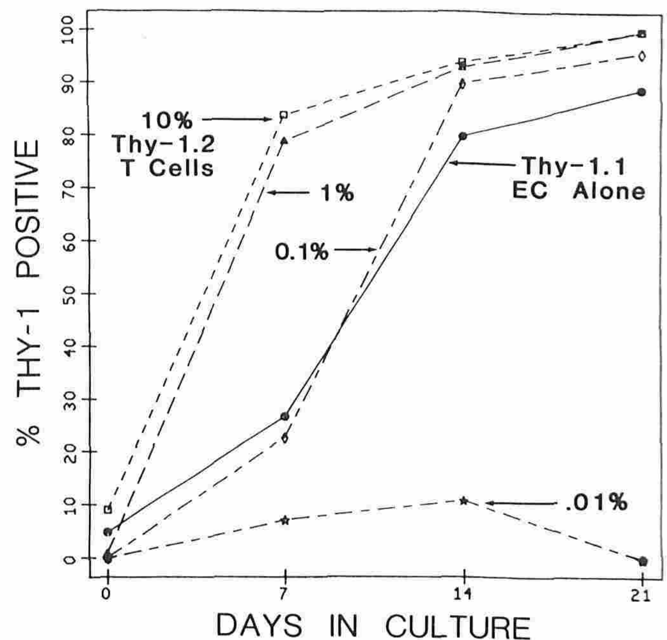


Figure 4. Serial quantitation of Thy-1⁺ cells in cultures of Thy-1.1⁺ FH-EC in CM, Con-A, and IM, to which varying numbers of congenic Thy-1.2⁺ T cells were added at day 0. At various time intervals, the numbers of T cells in expanding cocultures (identified by fluorescence microscopy after staining with anti-Thy-1.2 mAb: *dashed lines*) are compared with the numbers of Thy-1⁺ EC (identified by staining with anti-Thy-1.1 mAb: *solid line*) in expanding cultures of FH-EC alone. Results are expressed as a percentage of total viable cells. The ratios of Thy-1.2⁺ T cells to Thy-1.1⁺ EC at day 0 in different cocultures are indicated by designated percentage values and differing point symbols (*open square*, 10%; *open triangle*, 1.0%; *open diamond*, 0.1%; *star*, 0.01%).

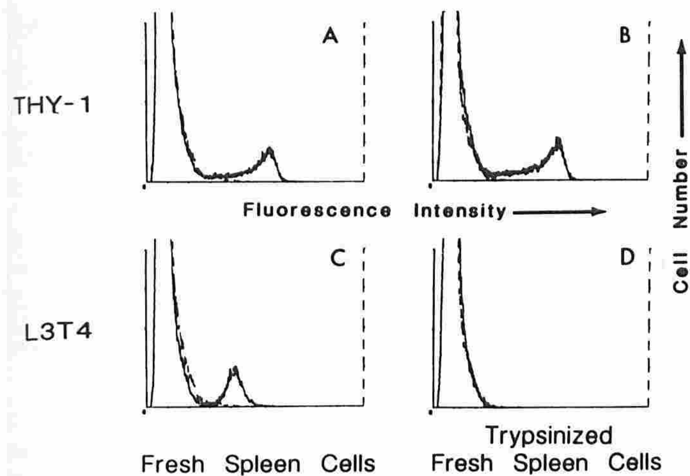


Figure 5. Flow cytometric analysis of freshly isolated SC and trypsinized freshly isolated SC stained with anti-Thy-1.2 mAb (*A and B*) and anti-L3T4 mAb (*C and D*). Logarithmically (3 decades) increasing green fluorescence intensity is plotted on the x axis vs cell number (linear units) on the y axis. Trypsinization has no effect upon detection of Thy-1 antigen (*B*), but renders detection of L3T4 antigen completely negative (*D*).

FH-EC failed to reveal any L3T4$^+$ population at either 24 or 72 h (Fig 6*C,D*), nor could any L3T4$^+$ cells be detected by fluorescence microscopy. When these same FH-EC were cultured for 21 days, however, L3T4 antigen was fully expressed on all viable cells by fluorescence microscopic analysis, and Lyt-2 remained negative. Though coculture experiments had demonstrated that 0.1% contaminating T cells could result in the expansion of an L3T4$^+$ line, it is unlikely that the emergence of an L3T4$^+$ population from cultured FH-EC is due to expansion of contaminating dermal or trafficking epidermal helper T cells. First, in coculture experiments, L3T4$^+$ T cells added at 0.1% were detectable by fluorescence microscopy from day 0 on; in the present experiment, no L3T4$^+$ cells were detected even after 72 h by either flow cytometric or fluorescence microscopic analysis. Second, if mature L3T4$^+$ T cells were present in FH-EC preparations, regeneration of the trypsin-sensitive L3T4 antigen on such T cells would be expected to occur much earlier in the course of the FH-EC culture, in a manner similar to that seen with cultured trypsinized SC; such a time course for regeneration of L3T4 antigen on a subpopulation of FH-EC was not observed. These studies suggest that in vitro modulation of phenotype, rather than selection of a minor contaminating population, might explain the expansion of a Thy-1$^+$, asialo GM_1^-, L3T4$^+$ population.

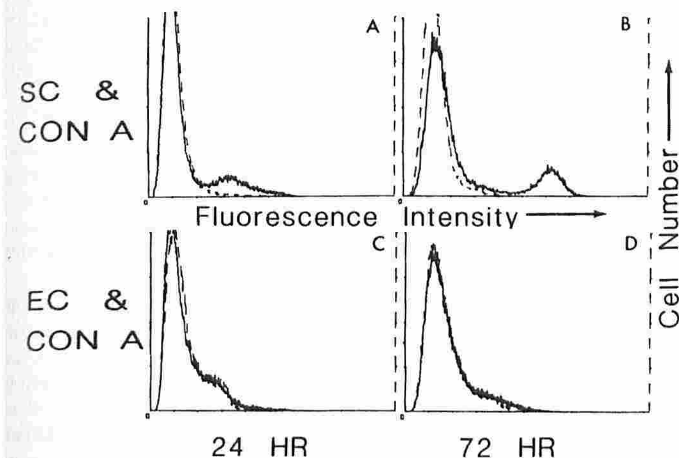


Figure 6. Flow cytometric analysis of trypsinized SC and EC showing regeneration of L3T4 antigen on SC (*A and B*) but not EC (*C and D*) after in vitro culture. Logarithmically (3 decades) increasing green fluorescence intensity is plotted on the x axis vs cell number (linear units) on the y axis. Spleen cells and FH-EC were cultured in the presence of Con-A 24 and 72 h after trypsinization, and the presence or absence of L3T4 antigen was determined by specific L3T4 staining (*solid lines*) compared with irrelevant mAb control staining (*dashed lines*). L3T4 antigen is clearly detected on SC 24 h after trypsinization (*A*) and fully expressed after 72 h (*B*). There is no identifiable L3T4$^+$ population among FH-EC after similar time periods in culture (*C and D*).

Concanavalin A Response of Expanded Thy-1$^+$ EC Varied with Phenotype Representative proliferative responses of various cell preparations stimulated with Con-A (Table IV) reveal that while SC proliferated vigorously to Con-A, freshly prepared Thy-1$^+$ EC proliferated to a very limited extent. The response of Thy-1$^+$ EC in FH-EC preparations expanded through 21 days of culture in the presence of Con-A varied with phenotype: Thy-1$^+$ EC that were asialo GM_1^+, L3T4$^-$ responded poorly, like freshly isolated Thy-1$^+$ EC; but Con-A stimulation of Thy-1$^+$ EC that were asialo GM_1^- and L3T4$^+$ resulted in proliferation similar to that seen with freshly isolated SC. The elevated background proliferation seen in cultured FH-EC preparations presumably reflects the fact that washing and overnight resting did not completely remove the effect of previous exposure to Con-A.

DISCUSSION

Our attempts at culturing Thy-1$^+$ EC have demonstrated the following: (1) Thy-1$^+$ EC can be expanded through tissue culture in the presence of Con-A; (2) phenotypically distinct populations of Thy-1$^+$ bone marrow-derived cells may emerge under the culture conditions utilized; and (3) the emergence of a Thy-1$^+$, asialo GM_1^-, L3T4$^+$ cell population from culture of Thy-1$^+$ EC may result from phenotypic modulation in vitro.

Since the intial description of a murine intraepidermal Thy-1-bearing dendritic cell distinct from LC [1,2], considerable attention in numerous laboratories has been given to this interesting cell [31]. Advances have been made in further characterizing its phenotype [17,32], in establishing its ontogeny and bone marrow derivation [13,14,33], and in detailing its ultrastructural, immunoultrastructural, and cytochemical characteristics [17]. Despite these numerous contributions, elucidation of the biologic role of Thy-1$^+$ EC is still in its infancy and is largely based upon phenotypic characteristics. Romani et al [17] have proposed a relationship of Thy-1$^+$ EC to NK cells based upon their mutual expression of surface asialo GM_1 and possession of cytoplasmic granules which stain with aryl sulfatase. They were unable, however, to demonstrate epidermal killing of an NK target cell line in preliminary studies. Bergstresser et al [14,34] have proposed that Thy-1$^+$ EC play a role in down regulation of the contact hypersensitivity response when LC-depleted, hapten-derivitized EC are used, and that the UV-resistant, anti-I-J antibody-sensitive cell that activates antigen-specific suppression [35] is the Thy-1$^+$ EC. However, Romani et al [17] were unable to kill Thy-1$^+$ EC with anti-I-J antibodies and complement.

Confirmation of these and other proposed functions has been hampered by the difficulty in obtaining large numbers of purified Thy-1$^+$ EC. To that purpose, Sullivan et al [36] have recently described density gradient separation and FACS techniques for obtaining purified Thy-1$^+$ EC from freshly harvested EC suspensions. However, in our hands the yield and efficiency are low in terms of resulting total numbers of Thy-1$^+$ EC utilizing these and similar methods. We therefore sought to obtain large populations of purified dendritic Thy-1$^+$ EC through culture techniques. We have demonstrated that Thy-1$^+$ EC can be expanded in culture in the presence of Con-A. Expansion of Thy-1$^+$ EC under the culture conditions described follows a time course considerably longer than that seen with equal numbers of mature T

Table IV. Proliferative Responses to Con-A of Freshly Isolated SC and FH-EC and FH-EC Cultured for 21 Days in the Presence of Con-A[a]

	Alone	+ Con-A	Stimulation Index
Freshly isolated			
SC[b]	2,082 ± 196	96,848 ± 8,240	46.5
FH-EC[c]	439 ± 31	1,781 ± 85	4.1
Con-A cultured FH-EC (Thy-1$^+$ EC)			
Asialo GM_1^-, L3T4^{+d}	5,496 ± 1,010	80,976 ± 7,545	14.7
Asialo GM_1^-, L3T4^{-e}	1,957 ± 221	6,643 ± 713	3.4

[a] Thy-1$^+$ cells (4 × 10^4) cultured in triplicate with or without Con-A for 72 h. Results expressed in CPM ± SEM of [^{3}H]thymidine incorporation. Twenty-one day cultured Thy-1$^+$ EC were provided with 10^4 syngeneic x-irradiated SC as a source of accessory cells when cultured both with and without Con-A. Stimulation indices for x-irradiated control populations + Con-A were ≤1.
[b] Twenty percent Thy-1$^+$ cells.
[c] Fourteen percent Thy-1$^+$ cells.
[d] Ninety percent Thy-1$^+$ cells after 21 days in culture in the presence of Con-A.
[e] Eighty-five percent Thy-1$^+$ cells after 21 days in culture in the presence of Con-A.

cells; and 3-day Con-A stimulation of freshly isolated epidermal cells, while producing modest proliferation, results in stimulation indices less than one-tenth that seen with mature T cells. Thus, mitogenic stimulation of Thy-1$^+$ EC cannot be construed as being identical to that of mature, thymus-derived lymphoid cells. Whether this Con-A-driven expansion of Thy-1$^+$ EC is I region antigen (Ia) dependent and IL-1 dependent as is conventionally held in the case of Con-A stimulation of splenic T cells [37,38] cannot be fully determined. Certainly Ia-positive LC can serve as accessory cells in Con-A-induced proliferation of T cells, and IL-1 can be provided by both LC and keratinocytes [39,40]. However, the addition of IL-1 (ETAF)-containing supernatants did not enhance or accelerate the response of Thy-1$^+$ EC to Con-A. Given the sometime emergence of an L3T4$^+$ population in our system, it is therefore of interest that Beckoff et al [41] have recently demonstrated the role of L3T4 antigen in Con-A stimulation of purified T cells in the presence of Ia-negative accessory cells.

The expansion of a Thy-1$^+$, L3T4$^+$, asialo GM_1^- cell line in some of our experiments raises the question of how such a phenotypically distinct line could emerge from culture of Thy-1$^+$ EC which were presumably asialo GM_1^+ and L3T4$^-$ initially. Multiple epidermal sheet preparations have repeatedly been negative for any cells of such a phenotype, and the dendritic Thy-1$^+$ EC is negative for L3T4 antigen in such preparations as well [17]. Possible explanations include: (1) expansion of contaminating dermal T cells; (2) expansion of T cells in transit in the epidermis; and (3) expansion of a resident epidermal bone marrow-derived cell which either phenotypically modulates or differentiates. We have demonstrated in coculture experiments of congenic EC and T cells that if L3T4$^+$ mature T cells were present in our EC suspensions, either as dermal contaminants or as in-transit intraepidermal cells, such a population could expand in our culture system; however, only if such T cells were present relative to EC at a ratio of 1:1000 would the time course of expansion approach that seen with EC plus Con-A alone. This possibility cannot be excluded. By demonstrating the trypsin sensitivity of L3T4 and Lyt-2 antigens, we have established that one cannot assume that FH-EC preparations obtained after trypsinization do not initially contain L3T4$^+$ or Lyt 2$^+$ contaminating lymphoid cells, even though no such cells are seen in epidermal sheet preparations that have not been subjected to trypsinization. However, it is clear that if mature T cells were present, regeneration of L3T4 antigen would be detectable within 24 h and fully expressed after 72 h of culture. No L3T4$^+$ cells were detectable by fluorescence flow cytometry or microscopy in our EC suspension under these conditions, even when large numbers of FH-EC were analyzed. Thus it is unlikely that the emergence of an L3T4$^+$ population in some of our Thy-1$^+$ EC cultures can be explained by contamination of FH-EC preparations with mature T cells.

Perhaps the emergence of T-helper phenotype as well as NK phenotype populations in our culture experiments provides more clues concerning the nature of Thy-1$^+$ EC than are initially apparent. While it is well recognized that asialo GM_1 can serve as an antigenic marker to identify NK cells, this antigen is not restricted in its distribution to these cells. Indeed, Habu et al [42] have demonstrated that anti-asialo GM_1 antibodies react with the majority of early embryonic thymocytes lacking characteristic T-cell markers. As gestation and differentiation progress, the proportion of thymocytes expressing asialo GM_1 decreases, and the proportion of thymus cells expressing Thy-1, Lyt-1, and Lyt-2 increases, suggesting that asialo GM_1 is present on embryonic thymocytes and is for the most part lost as T cells develop. Nevertheless, a small percentage of adult thymocytes express asialo GM_1, and cells expressing Thy-1 and asialo GM_1 and possessing NK activity have been grown from postnatal thymocyte cultures [43]. In addition, the T-cell lineage of some murine NK clones and a subset of fresh murine NK cells has recently been established with RNA hybridization techniques utilizing T-cell receptor β-chain probes and Southern blot analysis for rearrangement of genes encoding for the β-chain of the T-cell receptor [44]. Similar techniques have also recently established the T-cell lineage of selected human NK clones [45]. These studies have demonstrated that though NK cells may be of T-cell lineage, T-cell receptor expression in NK cells is in large part incomplete. Furthermore, incomplete expression and gene rearrangement of the T-cell antigen receptor has recently been described for a small population (3–4%) of immature thymocytes which, though expressing Thy-1 antigen, express neither Lyt-2 nor L3T4. These cells developmentally are intermediate between marrow-derived stem cells and mature T cells and are known as precursor thymocytes [46].

The results of our culture experiments and these data concerning T-cell antigen receptor expression and gene rearrangement in NK cells and precursor thymocytes provide ample ground for speculation concerning dendritic Thy-1$^+$ EC. Since expression of both asialo GM_1 and Thy-1 antigens have been identified as early events in T-cell maturation, perhaps Thy-1$^+$ EC are similar in both their ontogeny and their level of differentiation to precursor thymocytes. The relative similarities between skin and thymic epithelial environments are established well enough to propose migration of immature Thy-1$^+$, asialo GM_1^+ cells to the epidermis where, in the absence of appropriate signals, they undergo maturational arrest while those in the thymus continue to differentiate. This occurrence may explain why thymocytes did not appear to contribute to dendritic Thy-1$^+$ EC repopulation in congenic chimeric studies [13], since precursor thymocytes constitute a very small percentage of all thymus cells. It might also provide clues as to why it has proved difficult to demonstrate any function of freshly isolated dendritic Thy-1$^+$ EC: they are,

perhaps, functionally and phenotypically not fully differentiated. Under appropriate conditions, as is the case with both precursor thymocytes [46] and thymus-derived NK-like cell lines [43], Thy-1 + EC may be induced to differentiate along different phenotypic and functional avenues; hence the emergence of both asialo GM_1^+, L3T4− and asialo GM_1^-, L3T4+ cell lines in our experiments.

Studies are in progress to test this hypothesis. Nevertheless, the caveat applies that any functional studies of Thy-1+ EC expanded through culture must take into account the full phenotypic expression of cells so derived, and must recognize that the Thy-1+ cell studied after culture may not have the same phenotype or function as the dendritic Thy-1+ EC seen in vivo.

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