Antigen-Presenting Cells in Essential Fatty Acid–Deficient Murine Epidermis: Keratinocytes Bearing Class II (Ia) Antigens May Potentiate the Accessory Cell Function of Langerhans Cells

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Essential fatty acid deficiency (EFAD) is a useful model for studying the role of (n-6) fatty acid metabolism in normal physiology. Because cutaneous manifestations are among the earliest signs of EFAD and because abnormalities in the distribution and function of tissue macrophages have been documented in EFAD rodents, we studied the distribution and function of Class II MHC (Ia) antigen-bearing cells in EFAD C57B1/6 mouse epidermis. Immunofluorescence studies revealed 1.9–9.6 (mean ± SEM = 5.2 ± 2.6) times more class II MHC (Ia) antigen-bearing epidermal cells in suspensions prepared from EFAD as compared to normal skin. Analysis of epidermal sheets demonstrated similar numbers of dendritic Ia^+ and $NLDC145^+$ cells in EFAD and normal epidermis, however. This discrepancy occurred because some keratinocytes in EFAD epidermal sheets expressed class II MHC (Ia) antigens, whereas keratinocytes in normal mouse epidermis did not. Two-color flow cytometry confirmed that all Ia^+ cells in normal epidermis are Langerhans (Ia^+ $NLDC145^+$) cells, whereas Ia^+ cells in EFAD epidermis are comprised of Langerhans cells and a subpopulation of keratinocytes (Ia^+ $NLDC145^-$). Similar levels of Ia antigens were expressed on EFAD and normal Langerhans cells.

EFAD and normal epidermal cells were also compared in several in vitro assays of accessory cell function. Epidermal cells prepared from EFAD C57B1/6 mice present the protein antigen DNP-Ova to primed helper T cells more effectively than epidermal cells prepared from normal animals. EFAD epidermal cells are also more potent stimulators of T cells in primary and secondary allogeneic mixed lymphocyte-epidermal cell reactions than normal epidermal cells. The functional differences between EFAD and normal epidermal cells do not appear to result from increased cytokine release or decreased prostaglandin production by EFAD epidermal cells. In view of these findings and the observation that the antigen-presenting cell activity of EFAD epidermal cells correlates with the number of Ia^+ keratinocytes in epidermal cell preparations, Ia^+ keratinocytes (in the presence of Langerhans cells) may potentiate cutaneous immune responses in vitro and perhaps in vivo as well. These results also suggest that (n-6) fatty acids or metabolites of (n-6) fatty acids are involved in regulating the expression of class II MHC (Ia) antigens by keratinocytes in vivo. *J Invest Dermatol 96:950–958, 1991*

Essential fatty acid deficiency (EFAD), induced in mammals by deprivation of (n-6) fatty acids, has proved to be a useful tool to probe the role of (n-6) fatty acids (especially arachidonic acid) in various pathophysiologic processes. One noteworthy effect of EFAD is its ability to ameliorate autoimmune disease [1,2]. In experimental immune-mediated glomerulonephritis in rats, this striking effect has been shown to result from the depletion of resident macrophages from the tissues of EFAD animals [3,4]. In addition to altering the susceptibility of a tissue to autoimmune injury, depletion of resident macrophages appears to alter tissue immunogenicity. Renal allografts from EFAD rats survive and function in normal MHC-mismatched recipients for prolonged periods in the absence of immunosuppressive agents [5]. Functional abnormalities in mac-

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Abbreviations:

APC: antigen-presenting cell
DEC: dendritic epidermal cell
DNP-Ova: dinitrophenyl-conjugated ovalbumin
EC: epidermal cell
EFAD: essential fatty acid deficiency (deficient)
E-PHA: erythroagglutinating phytohemagglutinin
ETAF: epidermal cell–derived thymocyte-activating factor
FITC: fluorescein isothiocyanate
IL: interleukin
KC: keratinocyte
LC: Langerhans cell
MHC: major histocompatibility complex
MLER: mixed lymphocyte epidermal cell reaction
PGE: prostaglandin (E series)
SKSD: streptokinase-streptodornase

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rophages harvested from EFAD rodents have also been demonstrated [6].

A number of cutaneous abnormalities have also been documented in EFAD animals and humans. Among these are a scaling dermatosis, histologic evidence of epidermal hyperplasia, and dramatically increased epidermal water loss (reviewed in [7]). Because abnormalities in the distribution and function of macrophages have been demonstrated in EFAD rodents and the skin is structurally and functionally altered in EFAD, we compared the distribution and function of antigen-presenting cells in EFAD and normal mouse epidermis. The results of these studies demonstrate that EFAD epidermis contains Class II MHC (Ia) antigen-bearing keratinocytes (KC), which appear to potentiate the function of Langerhans cells (LC, which are apparently not affected by the deficiency state). These observations suggest a role for (n-6) fatty acids in the regulation of class II MHC (Ia) antigen expression by epithelial cells in vivo and implicate Ia^+ keratinocytes as active participants in cutaneous immune responses. In addition, our results highlight the distinctions between cells of the macrophage and dendritic cell lineages.

MATERIALS AND METHODS

Mice Male C57B1/6J weanlings (4 weeks old) obtained from the Jackson Laboratory (Bar Harbor, ME) were maintained on a standard laboratory diet or a fat-free diet (Purina Test Diets, Richmond, IN) and water ad libitum for a minimum of 8 weeks before being used as a source of normal or EFAD EC [6]. Responding T cells were prepared exclusively from 10–14-week old C57B1/6SnJ mice bred at the St. Louis Jewish Hospital Animal Resource Facility (St. Louis, MO) and fed a standard diet. Thymocytes were obtained from Balb/c mice similarly bred and housed at the St. Louis Jewish Hospital Animal Resource Facility.

Documentation of EFA Deficiency Epidermal sheets were prepared from normal and EFAD mice as previously described [8]. Samples of liver were also obtained and tissue lipids were extracted from epidermis and liver by the method of Bligh and Dyer [9]. Extracted fatty acids were transmethylated by the sequential addition of 0.5 N NaOH in methanol (23°C for 10 min) and 6 N HCl, extracted with hexane : diethyl ether (1 : 1) and isolated by thin-layer chromatography (hexane : diethyl ether : acetic acid, 75 : 5 : 1). Fatty acid methyl esters were then separated and identified using a Hewlett-Packard 5380 gas chromatograph containing an SP-2380 capillary column (25 m, 0.32 mm internal diameter, Supelco, Houston, TX). The carrier gas was helium and column head pressure was 15 psi. The detector was maintained at 250°C and the flame ionization detector was maintained at 300°C. The column was operated isothermally at 175°C. Fatty acid methyl esters were identified by co-migration with authentic standards. Fatty acid methyl ester retention times were computed with a Hewlett-Packard 3320A integrator interfaced with the gas chromatograph.

Epidermal Cell Suspensions Epidermal cells were prepared from the ear skin of C57B1/6 normal and EFAD mice using a standard technique [10]. Lipid-poor BSA (0.1%, Behring Diagnostics, La Jolla, CA) was substituted for serum to avoid exposing EFAD cells to exogenous fatty acids. Viable cells (routinely 85–95% of the total) were counted with the aid of trypan blue.

Immunofluorescent Staining of EC in Suspension EC suspensions were prepared as described earlier and stained (with saturating amounts of antibody) for class II MHC (Ia) antigens with the mouse monoclonal antibodies 34–5.3 (anti-Ia^d [11], obtained from Ted Hansen, Washington University School of Medicine, St. Louis, MO) or 10–2.16 (anti-Ia^k, ATCC, Rockville, MD), or the rat anti-mouse dendritic cell antibody NLDC145 ([12] obtained from Stephen I. Katz, NCI, Bethesda, MD) as indicated. Second antibodies included FITC-conjugated affinity-purified $F(ab')_2$ goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), FITC-conjugated affinity-purified $F(ab')_2$ goat anti-rat IgG (Organon Technica-Cappel, West Chester, PA), and Texas Red-conjugated monoclonal mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Antibodies were diluted in PBS containing 5% supplemented calf serum (Hyclone, Logan, UT) and sodium azide (0.2%). At each step, cells were incubated for 20 min at 4°C and washed twice to remove extraneous antibody. Cells were lightly fixed with 2% paraformaldehyde and stored at 4°C in the dark until they were analyzed. Stained cells were visualized using a Nikon Optiphot microscope equipped for epifluorescence or detected by analytical flow cytometry as indicated. Flow cytometry was accomplished with a Becton-Dickenson FACS IV or a Coulter Epics 753 flow cytometer equipped with 5 W argon and rhodamine 590 dye lasers.

Visualization of LC and KC In Situ Epidermal sheets were prepared from the ears of EFAD and normal mice using the method of Tamaki et al [10] after epilation with NAIR (Carter Products, New York, NY). Epidermis was separated from dermis with 0.5 M ammonium thiocyanate and epidermal sheets were fixed in acetone prior to incubation with antibodies. Incubation conditions were the same as described above. Stained epidermal sheets were fixed in paraformaldehyde before mounting in 10% glycerol in PBS.

Helper T Cell Proliferative Responses DNA-Ova was prepared and characterized as previously described [13]. Antigen-primed lymph node cells were harvested from the axillary and inguinal lymph nodes of normal B6 mice that had been injected subcutaneously in each flank 7–10 days earlier with 30 μg of DNP-Ova emulsified in complete Freunds adjuvant. Cells were incubated with saturating concentrations of the mouse monoclonal anti-Ia^d antibody 34–5.3 and sIg^+ and Ia^+ cells were removed by panning (two cycles) on plastic petri dishes coated with affinity-purified rabbit anti-mouse IgG (1 μg/ml) (Organon Technica-Cappel) as described [14]. Nonadherent cells were incubated with saturating concentrations of rat anti-mouse L3T4 (clone GK 1.5, ATCC, Rockville, MD) and applied to petri dishes coated with affinity-purified goat anti-rat IgG (Organon Technica-Cappel). Nonadherent cells were removed by washing extensively and adherent helper T ($L3T4^+$) cells were recovered using a teflon cell scraper (Costar, Cambridge, MA). T cells prepared in this fashion did not respond to antigens or mitogens unless accessory cells were added (data not shown).

DNP-Ova–primed helper T cells were incubated for approximately 96 h at 10^6 per ml in flat-bottom 96-well plastic culture plates (Corning, Corning, NY) in a humidified 5% CO_2 atmosphere at 37°C. Tritiated thymidine (70–90 Ci/mmole, NEN, Boston, MA) was added (1 μCi/well) for the last 16 h of the culture period. Culture medium for this and all other proliferation assays consisted of RPMI-1640 (Washington University School of Medicine Tissue Culture Support Center) supplemented with 0.5% EFAD mouse serum (fresh or once frozen), 1 × glutamine, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), penicillin (100 μ/ml), streptomycin (100 μg/ml), and gentamycin (500 μg/ml). EC (5×10^6/ml) were pulsed with antigen during a 2-h incubation in EFAD-serum–supplemented media containing 0.2 mg/ml DNP-Ova. DNP-Ova–pulsed EC were washed extensively to remove exogenous antigen before addition to culture.

Primary Mixed Lymphocyte-Epidermal Cell Reactions (MLER) Spleen cells were prepared from Balb/c mice and erythrocytes removed by hypotonic lysis with 0.15 M NH_4Cl in 0.02 M Tris HCl (pH 7.4). Accessory cells (macrophages and B cells) were coated with anti-Ia monoclonal antibody and depleted by panning, as described above. Balb/c ($H\text{-}2^d$) splenic T cells were suspended in complete EFAD serum supplemented media (2×10^6/ml) and incubated with the indicated numbers of EFAD or normal C57B1/6 ($H\text{-}2^b$) EC for 144 h (final volume 0.2 ml/well). Proliferation was assessed by measuring thymidine incorporation.

Secondary Mixed Lymphocyte-Epidermal Cell Reactions Alloantigen-primed T cells were prepared using a method developed by Steinman and co-workers [15]. Briefly, B6 spleen cells were incubated in 24-well culture plates (Flow Laboratories, Mclean,

Table I. Comparison of the Fatty Acid Compositions of Normal and EFAD Murine Liver and Epidermis[a]

	Liver		Epidermis	
Fatty Acid	Normal[b]	EFAD[b]	Normal[b]	EFAD[c]
16:0	40.9 ± 0.8	24.8 ± 0.7	10.8 ± 1.8	10.3 ± 0.5
16:1	ND[d]	6.9 ± 0.7	15.0 ± 3.0	18.2 ± 4.6
18:0	14.8 ± 0.5	5.1 ± 0.9	3.9 ± 1.0	4.1 ± 1.3
18:1	20.1 ± 0.2	56.6 ± 1.4	45.3 ± 1.8	49.4 ± 3.0
18:2	12.8 ± 0.6	0.4 ± 0.2	12.8 ± 1.6	1.3 ± 0.4
20:3	ND	2.2 ± 0.4	ND	2.2 ± 0.4
20:4	7.2 ± 0.6	0.8 ± 0.2	2.7 ± 0.7	2.3 ± 0.4
20:3/20:4		3.2 ± 0.5		1.0 ± 0.1

[a] Samples of liver and epidermis were obtained from normal and EFAD B6 mice and total tissue fatty acid compositions were determined as described in *Materials and Methods*. Mole percentages of recovered fatty acids (± SEM as indicated) are presented.
[b] n = 3.
[c] n = 4.
[d] ND, none detected.

VA) in complete media supplemented with 10% FCS (Hyclone) for 3 h at 37°C (4–5 × 10^6 cells in 1 ml/well). Nonadherent cells were discarded and 5 × 10^6 Balb/c splenic T cells (prepared as described above) in 1 ml of media were added to the adherent fraction (macrophages and dendritic cells). After 48 h, clustered cells were separated from non-clustered cells by density gradient centrifugation [15] and clustered cells were incubated for an additional 48 h. Alloantigen primed T cells (which spontaneously dissociate from clusters during the second 48-h incubation) were subsequently recovered by flotation on a Ficoll-Hypaque density gradient and restimulated in a secondary culture. Balb/c anti-B6 T cells (5 × 10^4/well) were cocultured for 44 h with the indicated number of EFAD or normal B6 EC in complete media supplemented with EFAD mouse serum (final volume 0.2 ml/well), and thymidine incorporation was determined.

Measurement of EC-Derived Thymocyte-Activating Factor (ETAF) EC were prepared from normal and EFAD mice as described above and incubated in complete medium for 96 or 144 h. Culture supernatants were harvested, depleted of particulate material by centrifugation (5 min × 10,000 × *g*) and stored at −20°C. Supernatants were subsequently assayed for lymphokine activity in a standard thymocyte comitogenesis assay [16]. Briefly, Balb/c thymocytes were cultured for 72 h in the presence of E-PHA (1 μg/ml) (Wellcome Diagnostics, Research Triangle Park, NC) and graded amounts of EC supernantants and cellular proliferation was determined by tritiated thymidine incorporation. Human recombinant IL-1_β (Cistron, Pine Brook, NJ) and murine recombinant IL-1_α (a gift from David D. Chaplin, Washington University School of Medicine, St. Louis, MO) were used as standards.

Because several cytokines have bioactivity in the thymocyte comitogenesis assay, in some experiments purified hamster monoclonal anti-murine IL-1_α antibody 161.1 (obtained from David D. Chaplin) was included in the thymocyte assay culture medium (0.5 μg/ml) to assess the contribution of IL-1_α to the ETAF activity released by EFAD and normal EC. This antibody has previously been demonstrated to bind and inhibit the bioactivity of mouse IL-1_α selectively [17].

Assessment of PGE_2 Production PGE_2 produced by normal and EFAD EC was quantitated by radioimmunoassay as previously described [18]. Tritiated PGE_2 (100 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Unbound PGE_2 was separated from rabbit-antibody–associated PGE_2 by sedimentation with dextran-coated charcoal.

RESULTS

Alteration of Murine Liver and Epidermal Fatty Acid Composition by EFA Deprivation Representative samples of epidermis and liver were harvested from B6 mice fed normal and fat-free diets and tissue fatty acid compositions were determined. As shown in Table I, the profile of lipids isolated from liver is typical of that previously associated with EFA deficiency [19]. Fatty acids of the (n-6) series (linoleate (18:2) and arachidonate (20:4)) decreased, whereas those of the (n-9) series (oleate (18:1) and Mead acid (20:3)) increased. The 20:3 to 20:4 ratio was greater than 3. Values in excess of 0.4 are indicative of EFA deficiency [19]. Changes in hepatic fatty acid composition were not accentuated by increasing the duration of EFA deprivation (12 vs 28 weeks).

Changes in epidermal fatty acid composition in EFAD animals contrasted with those seen in liver (see Table I). In normal epidermis, linoleate (18:2) was a prominent constituent, whereas arachidonate (20:4) was present in modest amounts. Palmitoleic acid [16:1 (n-7)], which is not a major component of liver lipids, was also found in normal skin in substantial quantities. In EFAD epidermis, linoleate was significantly depleted and arachidonate was relatively conserved. Nonetheless, Mead acid (20:3) accumulated and the epidermal 20:3 to 20:4 ratio was greater than 0.4. These changes were not accentuated in animals maintained on the diet for prolonged periods.

Quantitation and Characterization of Class II (Ia) Antigen-Bearing Cells in Suspensions and Whole Mounts Prepared from EFAD and Normal Murine Epidermis Studies by Katz and Stingl and their co-workers have established that in normal (uninflamed) epidermis, Langerhans cells (LC) express class II MHC (Ia) antigens and present haptens, protein antigens, and alloantigens to T cells (reviewed in [20]). Because previous work demonstrated that macrophages are depleted from certain tissues in EFAD rodents [3], it was of interest to compare the numbers of LC in EFAD and normal mouse epidermis. Because keratinocytes also express class II MHC (Ia) antigens in certain situations [21–23], it was necessary to use criteria in addition to class II MHC (Ia) antigen expression to conclusively identify LC.

Class II MHC (Ia) antigen-bearing cells were initially identified in epidermal cell suspensions using single-color analytical flow cytometry. In every instance (n = 9), suspensions prepared from EFAD skin contained more Ia^+ cells (9.5 ± 5.3%) than suspensions prepared from normal epidermis (1.9 ± 0.6%). On average, EFAD epidermis contained approximately fivefold (5.2 ± 2.6-fold) more Ia^+ cells than normal epidermis. Single-color immunofluorescence studies performed on epidermal sheets prepared from the ears of EFAD and normal B6 mice confirmed and extended the flow cytometry data. Epidermal sheets from normal mice that had been stained with an appropriate monoclonal anti-Ia antibody and a FITC-conjugated developing reagent had the expected appearance. Intensely fluorescent dendritic cells (LC) were uniformly distributed over the surface of the epidermal sheets (see Fig 1*A*). No Ia^+ keratinocytes were identified. A similar pattern of fluorescence was seen in normal epidermal sheets stained with NLDC145 and the appropriate secondary antibody (data not shown). NLDC145 is a rat anti-mouse dendritic cell antibody that binds to a surface antigen present exclusively on Langerhans cells and dendritic cells in peripheral lymphoid organs [12]. Examination of epidermal sheets prepared from the ears of EFAD mice revealed significantly different

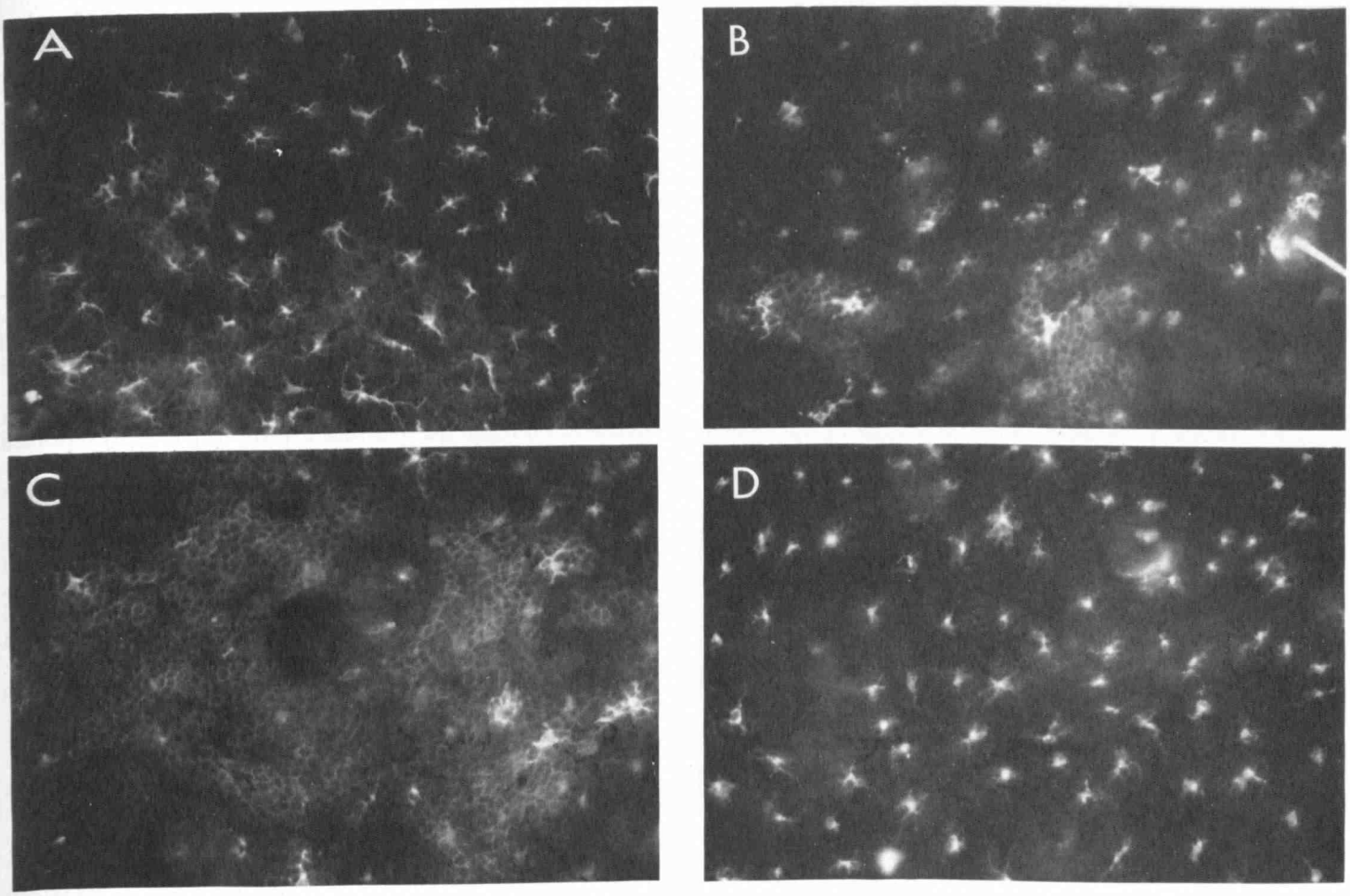

Figure 1. Localization of class II MHC (Ia) antigens on epidermal cells from EFAD and normal mice in situ. Epidermal sheets were prepared from EFAD and normal B6 ear skin using ammonium thiocyanate, fixed in acetone, and stained for Ia antigens with monoclonal antibody 34-5.3 and FITC-conjugated affinity-purified $F(ab')_2$ rabbit anti-mouse IgG as detailed in *Materials and Methods.* Specimens were photographed using a Leitz Optiphot photomicroscope equipped for epifluorescence (magnification ×250) and Fujichrome ASA 1600 film. *A,* Normal epidermis. *B–D,* Epidermis from EFAD mice.

results. EFAD epidermal sheets stained with NLDC145 were indistinguishable from NLCD145-stained normal sheets. EFAD epidermal sheets stained for class II MHC (Ia) antigen-bearing cells demonstrated the expected population of highly fluorescent dendritic cells (LC) and, in addition, a second population of less intensely stained cells, which had the morphology of keratinocytes (see Fig 1). These Ia^+ keratinocytes were distributed focally (Fig 1*B*) in some areas and more diffusely in others (Fig 1*C*). In some areas, KC-bearing class II MCH antigens were absent (Fig 1*D*). More Ia^+ keratinocytes were seen in epidermal sheets prepared from mice that had been maintained on the fat-free diet for a prolonged period (28 weeks) than in sheets from animals that had been deprived for a shorter period of time (12 weeks).

Table II. Enumeration of Langerhans Cells in EFAD and Normal Murine Epidermis In Situ[a]

	Ia^+ DEC	$NLDC145^+$ DEC
Normal	827 ± 94	840 ± 5
EFAD[b]	808 ± 35	892 ± 105
EFAD[c]	547 ± 47	741 ± 57

[a] Epidermal sheets were prepared and fixed as described in *Materials and Methods.* Sheets were incubated sequentially with the monoclonal anti-Ia antibody 34.5.3 and FITC-conjugated affinity-purified $F(ab')_2$ rabbit anti-mouse IgG or the rat monoclonal anti-dendritic cell antibody NLDC145 and FITC-conjugated affinity-purified $F(ab')_2$ goat anti-rat IgG as appropriate. Stained dendritic cells were enumerated in 5–10 non-overlapping grids of defined area in specimens prepared from 2–3 separate animals. Data are expressed as cell number per square millimeter (± SEM).

[b] Epidermal sheets were prepared from mice maintained on an EFAD diet for 12 weeks.

[c] Sheets were prepared from animals fed the EFAD diet for 28 weeks.

Table III. Characterization of Class II MHC (Ia)-Antigen-Bearing Cells in Epidermal Cell Suspensions Isolated From Normal and EFAD Mice[a]

	anti-Ia Ab	Ia^+	Ia^+ $NLDC145^+$	Ia^+ $NLDC145^-$
Experiment 1				
Normal	10-2.16	0.1	N.D.	N.D.
	34-5.3	1.1	1.0	0.1
EFAD	10-2.16	0.1	N.D.	N.D.
	34-5.3	8.1	1.4	6.7
Experiment 2				
Normal	34-5.3	1.6	1.3	0.3
EFAD[b]	34-5.3	4.7	1.8	3.2
EFAD[c]	34-5.3	15.3	1.4	13.9

[a] Epidermal cell suspensions were prepared from mice fed control and EFAD diets as described in *Materials and Methods.* Cells were incubated sequentially with mouse monoclonal anti-Ia antibody, FITC-conjugated affinity-purified $F(ab')_2$ rabbit anti-mouse IgG, the rat monoclonal anti-dendritic cell antibody NLDC145, and a Texas Red-conjugated mouse monoclonal anti-rat IgG. Stained cells were lightly fixed with 2% paraformaldehyde and analyzed using a Coulter Epics 753 flow cytometer equipped with a 5 W argon and a rhodamine 590 dye laser. Twenty-five-thousand cells were analyzed per sample. Data are expressed as percent positive.

[b] EC were prepared from mice fed an EFAD diet for 12 weeks.

[c] Donor mice had been EFA deprived for 28 weeks.

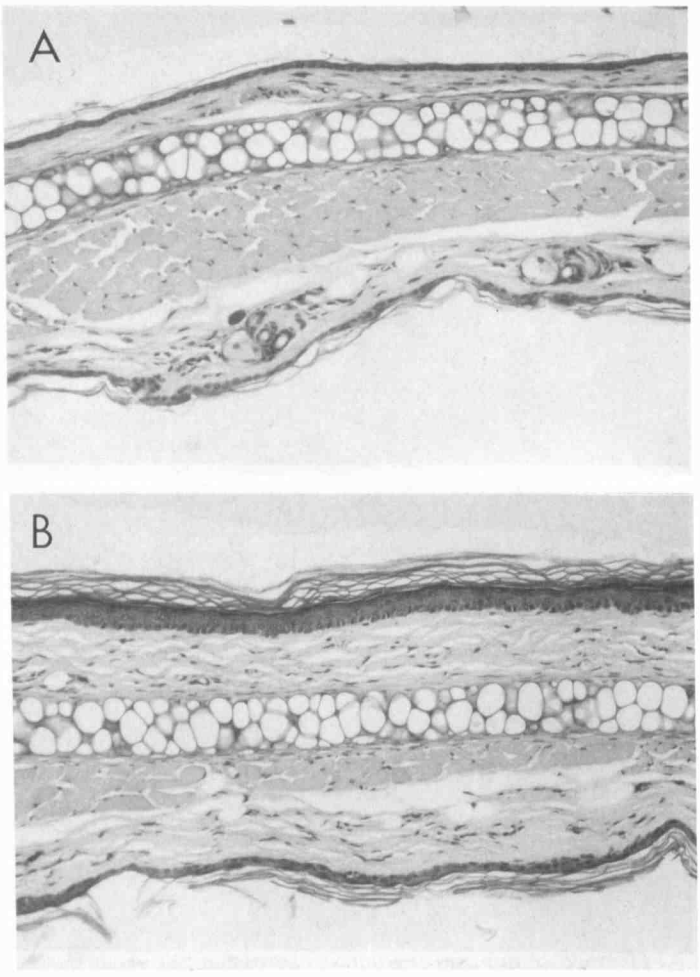

Figure 2. Microscopic anatomy of ear skin from EFAD and normal mice. Ears harvested from B6 EFAD and normal mice were fixed in neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Representative fields were photographed with a Leitz Optiphot photomicroscope (magnification ×250). *A,* Normal skin. *B,* EFAD ear skin.

Enumeration of Langerhans cells in epidermal sheets prepared from EFAD and normal animals confirmed the subjective impression that similar or identical numbers of dendritic Ia^+ and $NLDC145^+$ cells were present in EFAD and normal skin (Table II). This suggests that all Ia^+ dendritic cells are also $NLDC\ 145^+$ in both normal and EFAD epidermis. Macrophages (Ia^+ $NLDC^-$ dendritic cells, which could conceivably enter epidermis from the underlying dermis) were not detected in EFAD epidermis in significant numbers. The modest discrepency between the number of Ia^+ DEC (547 ± 47) and $NLDC145^+$ DEC (741 ± 57) in mice fed the EFAD diet for 28 weeks is of uncertain significance. Functional studies were performed exclusively using EC prepared from mice fed an EFAD diet for shorter periods of time, however.

Two-color fluorescence studies performed on EC in suspension allowed the simultaneous resolution of Ia^- $NLDC145^-$, Ia^+ $NLDC145^-$ and Ia^+ $NLDC145^+$ EC. Because the Texas Red-conjugated mouse anti-rat IgG utilized exhibited some nonspecific staining of keratinocytes, it was not possible to confirm or exclude the existence of an Ia^- $NLDC145^+$ population alluded to above. The data are presented in Table III. All Ia^+ EC in normal epidermis also stain with NLDC145, confirming that all Ia^+ cells in normal epidermis are Langerhans cells. In agreement with the results described earlier, more Ia^+ EC were present in suspensions isolated from EFAD mice than in those from normal animals. In addition, mice maintained on the fat-free diet for a prolonged period yielded more Ia^+ EC than animals deprived for a shorter period. The data also indicate that the number of Ia^+ $NLDC145^+$ EC (Langerhans cells) is not substantially changed from normal in EFAD, as predicted from the data in Table II. The appearance of class II MHC (Ia) antigen-bearing keratinocytes (Ia^+ $NLDC145^-$) in EFAD epidermis appears to account entirely for the increased number of Ia^+ EC isolated from EFAD skin. In addition, EFAD and normal LC express similar levels of class II MHC antigens, based on relative fluorescence intensity (data not shown).

Relationship of the Number of Ia^+ Keratinocytes in Epidermis to Inflammation Because Ia^+ keratinocytes have been identified in a number of inflammatory human skin disorders [21] and the skin of EFAD mice is somewhat scaly, we examined EFAD and normal skin in routine histologic sections for evidence of inflammation (see Fig 2). Hematoxylin and eosin stained cross-sections of mouse ears revealed the epidermal hyperplasia previously reported in EFAD skin [24,25] and a scant perivascular lymphocytic infiltrate in EFAD but not normal dermis. The lymphocytic infiltrate did not impinge on the epidermis in any of the sections examined. In addition, nonresident T cells could not be detected in epidermal sheets or EC suspensions prepared from EFAD and normal mice and stained with anti-L3T4 and anti-Ly 2,3 antibodies and the appropriate developing reagents (data not shown). The existence of small numbers of class II MHC-antigen–bearing leukocytes capable of antigen presentation (i.e., macrophages) in EFAD epidermis overlying focal areas of dermal perivascular inflammation is difficult to exclude with absolute certainty. Although antibodies that recognize surface antigens expressed on murine Langerhans cells but not macrophages (e.g., NLDC145) are available, antibodies that react exclusively with mouse macrophages are not. Nonetheless, data presented in Table II do not suggest that a significant number of Ia^+ $NLDC145^-$ leukocytes (which could easily be differentiated from Ia^+ $NLDC145^-$ keratinocytes in epidermal sheet preparations) are present in EFAD epidermis. In both normal and EFAD epidermis, the number of $NLDC145^+$ dendritic cells (Langerhans cells) is equal to (or perhaps slightly greater than) the number of Ia^+ dendritic cells (Langerhans cells or macrophages).

Protein Antigen Presentation by Cells from EFAD and Normal Mouse Epidermis Having demonstrated that epidermis from EFAD mice contained normal numbers of LC, we compared the functional activities of EFAD and normal EC. Stingl and coworkers have previously shown that EC preparations containing LC effectively present the protein antigen DNP-Ova to murine T cells in the absence of additional accessory cells [26]. DNP-Ova–primed lymph node helper T cells were prepared from normal C57B1/6 mice and cultured with varying numbers of EFAD or normal B6 antigen–pulsed mixed epidermal cells. Lymphocyte proliferation as assessed by tritiated thymidine incorporation was measured after a 96-h culture period. Culture media was supplemented with 0.5% EFAD mouse serum as the sole source of exogenous lipid to avoid artificially altering the fatty acid composition of the epidermal cells in vitro. Preliminary studies indicated that in most instances EFAD mouse serum was superior or equal to normal mouse serum with regard to its ability to support T-cell proliferation (data not shown). At every cell number tested, antigen-pulsed EFAD epidermal cells stimulated primed helper T-cell proliferation more than antigen-pulsed normal epidermal cells (see Fig 3*A*). In the experiment shown, EFAD EC were approximately 5 times more effective accessory cells than normal EC (i.e., 1×10^4 DNP-Ova–pulsed EFAD EC stimulated the same level of thymidine incorporation by T cells as did 5×10^4 DNP-Ova–pulsed normal EC). Similarly, EFAD EC were more effective presenters of DNP-Ova than normal EC when antigen, responder T cells, and epidermal cells were present together for the entire culture period (Fig 3*B*). BSA-pulsed EFAD EC do not cause DNP-Ova–primed T lymphocytes to incorporate significant amounts of thymidine, excluding a non-

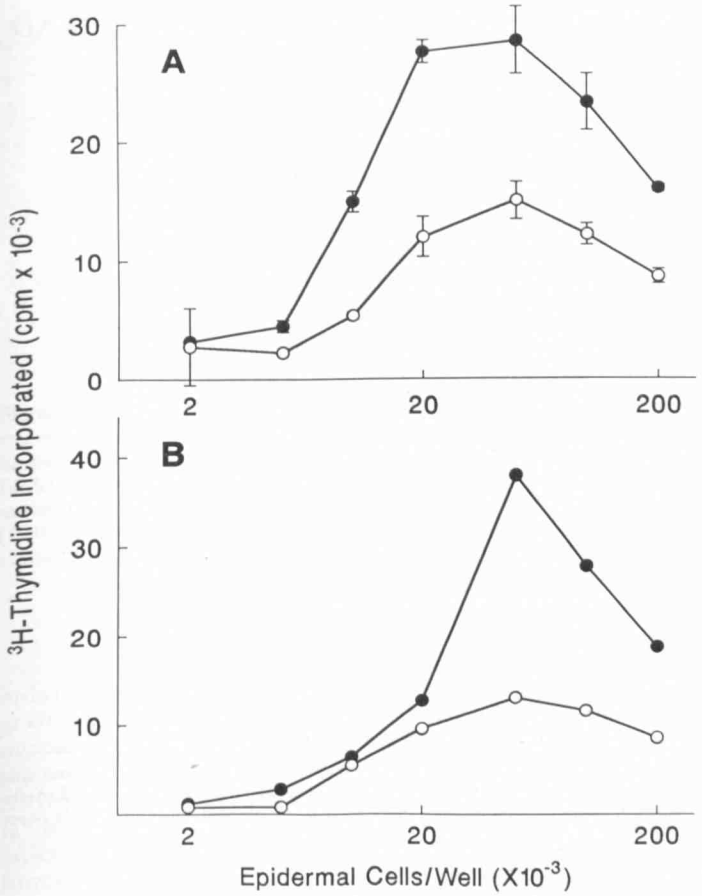

Figure 3. EC prepared from EFAD mice present protein antigens to primed helper T cells more effectively than EC from normal mice. *A*, C57B1/6SnJ DNP-Ova–primed L3T4$^+$ lymph node T lymphocytes (2×10^5) were co-cultured for 96 h with varying numbers of B6 EFAD (●) or normal EC (○) that had been previously incubated for 2 h with DNP-Ova and washed free of exogenous antigen. Tritiated thymidine was added for the final 18 h of the culture period. *B*, DNP-Ova–primed L3T4$^+$ cells were similarly cultured with B6 EFAD (●) or normal (○) EC in the presence of DNP-Ova (25 μg/ml). Mean values (± SEM) from a representative experiment are shown.

specific stimulatory effect of EFAD EC on lymphocyte proliferation (data not shown). In addition, the failure of BSA-pulsed EC to stimulate DNP-Ova–primed T cells suggests that EFAD LC do not have an enhanced ability to stimulate autologous responses relative to LC from normal mice. When the results of experiments performed on different days were compared, the ability of individual EC preparations to present protein antigens correlated directly with the proportion of KC expressing class II (Ia) antigens.

EFAD and Normal EC as Stimulators of Mixed Lymphocyte-Epidermal Cell Reactions (MLER) In addition to supporting protein antigen–specific T cell proliferative responses, EC prepared from normal mice induce primary and secondary allogeneic MLER [27]. Accessory cell–depleted Balb/c (H-2^d) splenic T cells were cultured in EFAD serum–supplemented media with B6 (H-2^b) EFAD or normal EC for 144 h. Lymphocyte proliferation was assayed by measuring thymidine incorporation. In two of four experiments, EFAD EC were better able to stimulate a primary allogenic MLER than normal EC (see Fig 4*A*). In the remaining experiments, EFAD and normal EC were functionally equivalent. In order to directly compare the stimulatory capacities of EFAD and normal EC in in vitro primary and secondary immune responses (e.g., primary and secondary MLER), Balb/c (H-2^d) splenic T cells were separated from B cells and macrophages and primed in vitro with B6 (H-2^b) splenic dendritic cells (see *Materials and Methods*). H-2^b–primed Balb/c T cells were then co-cultured for 44 h with varying numbers of EFAD or normal C57B1/6 EC and thymidine incorporation was measured. EFAD EC were invariably better stimulators of a secondary allogeneic (H-2^d anti–H-2^b) MLER than normal EC (see Fig 4*B*). In the experiment shown, EFAD EC were approximately 2 times more active than normal EC.

Quantitation and Characterization of ETAF Produced by EFAD and Normal EC Because EC produce a number of lymphokines (including IL-1, IL-6, and GM-CSF), which enhance T-cell proliferation in response to antigens and mitogens, we compared the ETAF activity produced by EFAD and normal EC. EFAD and normal EC released similar amounts of thymocyte-stimulating activity into 96-h and 144-h culture supernatants (see Table IV). In most instances, however, the amount of ETAF produced by EFAD EC was somewhat greater than that produced by normal cells. The majority (more than 70%) of the ETAF bioactivity released by EFAD and normal EC was inhibited by hamster monoclonal anti–murine IL-1$_\alpha$ antibody 161.1 (see Table V), indicating that IL-1$_\alpha$ represents the predominant T cell stimulating activity produced by both EFAD and normal EC.

Prostaglandin Production by EFAD and Normal EC Because EFAD and normal epidermal sheets contain similar amounts

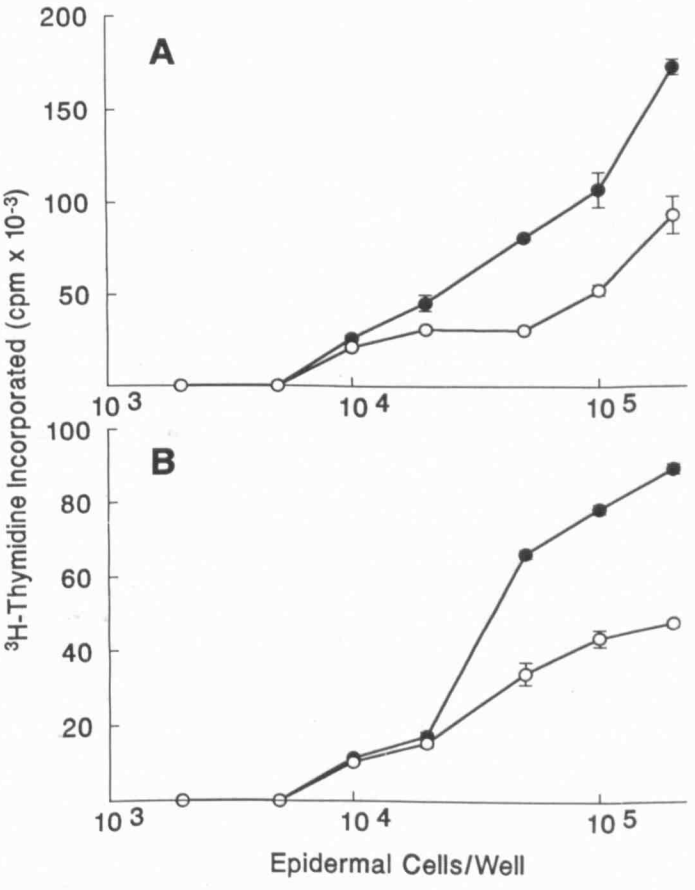

Figure 4. EC isolated from EFAD mice are more potent stimulators of allogeneic MLER than EC from normal mice. *A*, Primary MLER. Balb/c spleen cells were depleted of sIg- and Ia-bearing accessory cells and enriched for T lymphocytes by panning as described in *Materials and Methods*. Balb/c T cells (4×10^5) were co-cultured for 144 h with the indicated numbers of EFAD (●) or normal (○) B6 EC. Thymidine incorporation was determined as previously described. *B*, Secondary MLER. Balb/c spleen cells were depleted of accessory cells, enriched for T cells, and primed in vitro with B6 splenic dendritic cells as described in *Materials and Methods*. Balb/c anti-B6 alloblasts (5×10^4) were re-stimulated during a 44-h co-culture with EC prepared from EFAD (●) and normal (○) mice as indicated. Tritiated thymidine was included in the medium for the final 18 h of the culture period. Mean values (± SEM) from a representative experiment are shown.

Table IV. Thymocyte Activating Activity Produced by Normal and EFAD Epidermal Cells[a]

Incubation Conditions	ETAF (U/ml) Experiment 1	Experiment 2
Normal EC (96-h incubation)		
2×10^5	9.2 ± 2.8	3.5 ± 1.2
1×10^5	5.8 ± 1.4	2.4 ± 0.6
5×10^4	N.D.	1.8 ± 0.5
2×10^4	N.D.	0.4 ± 0.1
1×10^4	N.D.	0.2 ± 0.0
EFAD EC (96-h incubation)		
2×10^5	11.7 ± 1.5	8.5 ± 1.6
1×10^5	7.5 ± 1.1	7.2 ± 1.9
5×10^4	N.D.	3.3 ± 0.7
2×10^4	N.D.	0.7 ± 0.3
1×10^4	N.D.	<0.2
Normal EC (144-h incubation)		
2×10^5	9.2 ± 2.0	5.5 ± 2.4
1×10^5	6.4 ± 2.2	3.1 ± 0.7
5×10^4	2.0 ± 0.4	1.6 ± 0.5
2×10^4	0.2 ± 0.0	0.6 ± 0.2
1×10^4	<0.2	<0.35
EFAD EC (144-h incubation)		
2×10^5	8.8 ± 1.8	7.1 ± 1.3
1×10^5	8.0 ± 3.2	6.2 ± 0.9
5×10^4	3.8 ± 1.6	2.9 ± 0.7
2×10^4	<0.2	1.6 ± 0.9
1×10^4	<0.2	<0.35

[a] EC were prepared from normal and EFAD B6 mice as described previously and cultured as indicated in complete media supplemented with 0.5% EFAD serum. Culture supernatants were assayed for thymocyte-activating activity in a standard thymocyte comitogenesis assay. Balb/c thymocytes were used as indicator cells. Human recombinant IL-1_β was used as the standard.

Table VI. Prostaglandin Production by Normal and EFAD Epidermal Cells[a]

Incubation Conditions	PGE_2 (ng/ml)
Normal EC (96-h incubation)	
2×10^5	0.56
1×10^5	0.61
5×10^4	0.39
2×10^4	0.27
1×10^4	0.17
EFAD EC (96-h incubation)	
2×10^5	<0.10
1×10^5	<0.10
5×10^4	<0.10
2×10^4	<0.10
1×10^4	<0.10
Normal EC (144-h incubation)	
2×10^5	0.58
1×10^5	0.65
5×10^4	0.31
2×10^4	0.22
1×10^4	0.18
EFAD EC (144-h incubation)	
2×10^5	0.11
1×10^5	0.17
5×10^4	0.11
2×10^4	0.14
1×10^4	0.12

[a] EFAD and normal EC were isolated as described in *Materials and Methods* and cultured in complete media supplemented with 0.5% EFAD serum. Conditioned media was harvested at the end of the culture periods as indicated and PGE_2 levels were determined by radioimmunoassay.

of arachidonic acid and metabolites of arachidonic acid are known to influence in vitro immune responses, cell-free supernatants from cultures of EFAD and normal EC were assayed for PGE_2 content by radioimmunoassay. PGE_2 is the arachidonic acid metabolite produced in greatest quantities by keratinocytes [28]. Although cells were cultured in media supplemented with EFAD serum, EC from normal mice still produced a significant amount of PGE_2 (see Table VI). EFAD EC produced little if any PGE_2.

Effects of IL-1 and Indomethacin on Protein Antigen Presentation The increased accessory cell activity of EFAD EC as compared with normal EC could result from increased IL-1 or decreased PGE_2 production by EFAD EC. To address this question, we assessed the ability of EFAD and normal EC to present DNP-Ova to primed T cells in the presence of recombinant human IL-1_β or the cyclooxygenase inhibitor indomethacin. As shown in Fig 5, IL-1 and indomethacin augmented the ability of both normal and EFAD EC to induce antigen-specific T-cell proliferation. The enhanced ability of EFAD EC to present antigen persisted in the presence of IL-1 (100 ng/ml) and indomethacin (1 μg/ml), however. The amount of indomethacin added reduced prostaglandin production by EFAD and normal EC to barely detectable levels (data not shown). These results suggest that EFAD EC are not better antigen-presenting cells than normal EC simply because they produce more IL-1 or less PGE_2.

Table V. Inhibition of ETAF Produced by Normal and EFAD Epidermal Cells by Anti-IL-1

	ETAF (relative activity)[a] Anti-IL-1 Absent	Anti-IL-1 Present	Inhibition (%)
96-h Incubation			
Normal EC	1.0 ± 0.0	0.3 ± 0.1	73.8 ± 12.7
EFAD EC	2.5 ± 1.2	0.3 ± 0.2	88.9 ± 3.6
144-h Incubation			
Normal EC	1.5 ± 0.5	0.3 ± 0.1	77.7 ± 7.5
EFAD EC	2.8 ± 1.4	0.3 ± 0.1	85.7 ± 8.9

[a] Supernatants from EC (10^6/ml) were prepared on three separate occasions and assayed for ETAF activity as described in the legend of *Table VI*. Mouse recombinant IL-1_α was used as the standard. To correct for variations in the absolute amounts of ETAF produced and measured in each individual experiment, ETAF activity is expressed relative to that present in 96-h supernatants prepared from normal EC. Hamster monoclonal anti-murine IL-1_α antibody (161.1) was added into the thymocyte comitogenesis assay as indicated.

DISCUSSION

The studies reported here were undertaken because previous work had demonstrated abnormalities in the distribution and function of tissue macrophages in EFAD rodents [3,5,6]. Although epidermis from EFAD mice was clearly depleted of linoleic acid (18:2), LC number and morphology was entirely normal. EFAD LC were also functionally unimpaired as EFAD EC were able to reconstitute several in vitro accessory-cell-dependent systems. Although the explanation for this dichotomy is not certain, differential effects of EFA deprivation on resident macrophages and Langerhans cells may reflect their distinct ontogeny. Steinman and coworkers have proposed that LC are more closely related to the dendritic cells of peripheral lymphoid organs (spleen and others) than monocytes and macrophages [29]. It is, therefore, not surprising that tissue localization and function of LC and macrophages are regulated differently. A resident population of Ia^+ cells in murine pancreatic islets (which may be dendritic cells) is also not depleted in the setting of EFA deficiency [2].

In normal epidermis, class II MHC (Ia) antigens are expressed exclusively on LC. EFAD epidermis was remarkable in that significant numbers of keratinocytes also bore Ia antigens. Ia^+ keratinocytes have been previously identified in human skin in several disease states, most notably those with a T-lymphocyte-predominant

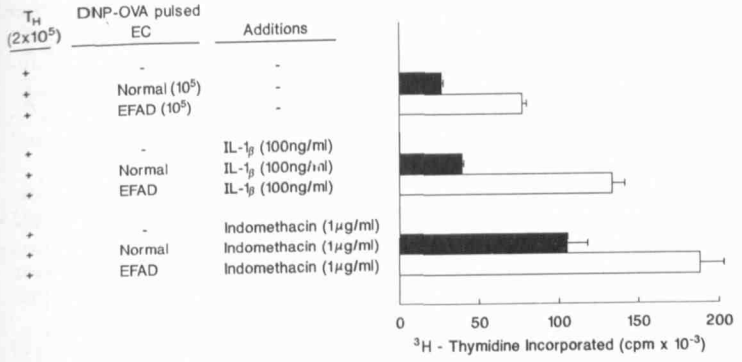

Figure 5. Increased antigen-presenting ability of EFAD EC does not result from increased IL-1 or decreased PGE_2 production. B6 DNP-Ova–primed helper T cells (2×10^5) were co-cultured for 96 h with antigen-pulsed EFAD or normal EC (10^5) as described earlier, and cell proliferation was measured by tritiated thymidine incorporation. Human recombinant IL-1_β (100 ng/ml) and indomethacin (1 μg/ml) were added as indicated. Mean values (± SEM) from a representative experiment are shown.

inflammatory infiltrate impinging on the epidermis (e.g., acute graft-versus-host disease, lupus erythematosus, lichen planus, and mycosis fungoides) [21], and in animal skin undergoing skin graft rejection [22] or participating in contact sensitivity reactions [23]. Although other explanations are possible, our results suggest that Ia^+ keratinocytes in EFAD epidermis have antigen-presenting capabilities in and of themselves or potentiate the antigen-presenting function of Langerhans cells. Gaspari and Katz have recently studied the functional capabilities of isolated class II MHC (Ia) antigen-bearing mouse keratinocytes in vitro [30] and have reported that Ia^+ KC (in the absence of LC) can stimulate alloantigen-specific helper T-cell clones, serve as targets for the appropriate Ia antigen–restricted cytolytic T cells, and present processed antigen (antigen fragments) to protein antigen–specific helper T cell hybridomas, but are unable to present native protein antigens and do not initiate primary allogeneic MLER. In a separate report [31], these authors have presented data that indicate that isolated Ia^+ KC have the capacity to tolerize cloned helper T cells in an antigen-specific and Ia-restricted fashion. They suggest that Ia^+ KC may have negative regulatory properties in vivo. These experiments may not bear directly on whether or not Ia^+ KC may have immunopotentiating properties in mixed EC preparations, as suggested by our results.

We propose that although isolated class II MHC antigen–bearing KC possess limited APC activity in isolation, they are able to augment T-cell proliferative responses when other accessory cells (e.g., Langerhans cells) are present. Of interest, Roberts and coworkers have previously noted a positive correlation between the intensity of Ia expression on KC and the degree of ear swelling in murine contact sensitivity responses [23]. Although the mechanism by which LC and Ia^+ KC cooperate is uncertain, the potential for these kinds of interactions has been explored by Geppert and Lipsky [32]. They reported that isolated interferon-gamma–treated human fibroblasts bearing Ia antigens were unable to stimulate naive allogeneic T cells or present the protein antigen SKSD to primed T cells, but were able to stimulate allogeneic T-cell lines. Ia^+ fibroblasts were able to stimulate allogeneic naive T cell proliferation when macrophages syngeneic to the responding cells were present, however. In addition, SKSD-pulsed paraformaldehyde-fixed Ia^+ fibroblasts stimulated primed T lymphocytes in the presence of syngeneic (or allogeneic) accessory cells [33]. Although recombination experiments such as these may seem somewhat artificial, they may have special relevence to the skin where traditional accessory cells (LC) and epithelial cells capable of expressing Ia antigens are normally juxtaposed. Class II MHC (Ia) antigen-bearing keratinocytes, in the setting of human skin diseases, may well augment immune responses initiated by LC, thereby promoting cutaneous inflammation rather than down-regulating it [31].

Because class II MHC (Ia) antigens play a critical role in accessory cell function, the identification of physiologic regulators of Ia expression by antigen-presenting cells (macrophages, dendritic cells, and LC) and epithelial cells is an area of obvious interest. The T-cell–derived cytokine interferon-gamma is the best studied inducer of Ia expression. Interferon-gamma induces Ia expression by Ia^- mouse peritoneal macrophages (34), $T6^+$ Ia^- human epidermal Langerhans cells [35], and several types of epithelial cells, including murine [36] and human [37] keratinocytes. The expression of Class II MHC antigens by keratinocytes in inflammatory skin diseases is thought to be mediated by interferon-gamma produced by T cells in the local environment. Although Menton has previously described dermal mononuclear cell inflammation in EFAD mouse skin [24,25], the present study suggests that this is not a prominent feature in EFAD CS7B1/6 mice. On the other hand, we cannot exclude the existence of small foci of inflammatory cells that escaped detection or a drastic change in the sensitivity of EFAD KC to small quantities of interferon-gamma produced locally. Also, the role that $Thy\ 1^+$ dendritic epidermal cells may play in the expression of Class II MHC antigens by EFAD keratinocytes has not been addressed.

Our results suggest that (n-6) fatty acids or their metabolites directly or indirectly inhibit the expression of class II MHC (Ia) antigens by KC. The relationship between class II MHC antigen expression by antigen-presenting cells and arachidonic acid metabolism has been best studied in murine peritoneal macrophages [34]. Prostaglandin E_2 and a stable analog of PGI_2 prevented or reversed the induction of Ia antigens by inteferon-gamma when added to macrophage cultures before, after, or simultaneously with lymphokine. Other arachidonic acid metabolites were without effect. Cyclooxygenase inhibitors produced a modest enhancement of interferon-gamma–induced Ia expression but had no effect alone, perhaps because very limited quantities of arachidonic acid metabolites are produced by unstimulated peritoneal macrophages in culture. The induction of class II MHC (Ia) antigens on human mucosal $T6^+$ Ia^- LC by interferon-gamma is, however, mimicked by indomethacin [35]. Keratinocytes produce both PGE_2 and $PGF_{2\alpha}$, which conceivably could be involved in the autoregulation of class II MHC antigen expression in vivo. Our results demonstrate that although arachidonic acid is present in small amounts in both normal and EFAD epidermis, cultured EFAD KC produce considerably less PGE_2 than normal KC. This situation is reminiscent of that occurring in renal cortex and heart muscle isolated from EFAD mice, where the rate of agonist-induced arachidonic acid metabolism decreases even though total tissue arachidonate is maintained or increases [38]. In these tissues, phosphatidyl inositol (the principal source of arachidonic acid released from phospholipid in response to agonists) is selectively depleted of arachidonic acid during EFA deprivation. Because EFAD and normal epidermal phospholipids were not fractionated prior to transmethylation, we cannot comment on changes in the arachidonate content of individual phospholipids. If keratinocytes mobilize arachidonic acid from phospholipid stores selectively, relevant changes in arachidonic acid distribution might not be detected at the tissue level. Thus, quantities of important bioactive metabolites of arachidonic acid may differ in the epidermis of EFAD and normal mice despite the apparent availability of similar amounts of substrate. Additional experiments will be required to determine the effects of EFA deficiency on the (n-6) fatty acid content of individual phospholipids. Future studies will also seek to determine the precise role that (n-6) fatty acids (or their metabolites) play in the regulation of Ia antigen expression by keratinocytes. It will also be necessary to purify EFAD Ia^+ KC and (if possible) EFAD LC and study their antigen-presenting capabilities in isolation and after recombination to conclusively determine the relevant contributions of each of these cells to the enhanced functional activity of EFAD EC noted here. These investigations should promote better understanding of the factors that regulate cutaneous inflammation in vivo.

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