Brief Definitive Report

Interleukin 7 Is Produced by Murine and Human Keratinocytes

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Summary

Interleukin 7 (IL-7) was originally identified as a growth factor for B cell progenitors, and subsequently has been shown to exert proliferative effects on T cell progenitors and mature peripheral T cells as well. Constitutive IL-7 mRNA expression so far had been demonstrated in bone marrow stromal cell lines, thymus, spleen, and among nonlymphoid tissues in liver and kidney. Here we show that both murine and human keratinocytes express IL-7 mRNA and release IL-7 protein in biologically relevant amounts. The physiological or pathological relevance of keratinocyte-derived IL-7 is presently unknown. Our finding that keratinocytes can produce IL-7 in concert with reports that IL-7 is a growth factor for in vivo primed antigen-specific T cells, as well as for T lymphoma cells suggests, however, that keratinocyte-derived IL-7 is important in the pathogenesis of inflammatory skin diseases and cutaneous T cell lymphoma.

IL-7 was originally detected and isolated as a bone marrow stromal cell line–derived cytokine supporting the growth of B cell precursors (1, 2). Northern blotting analysis of various BALB/c tissues demonstrated that besides bone marrow stromal cells, IL-7 mRNA is constitutively expressed in liver, kidney, spleen, and, remarkably, also in thymic tissue (2), indicating a potential role for IL-7 in T cell development. Indeed, IL-7 was then shown to stimulate the growth of T cell progenitors as well (for a review see reference 3), thus emerging as a major regulator of early B and T cell development. More recently it has become evident that under certain conditions IL-7 can also stimulate the growth of mature peripheral T cells and T clones (4–8). This finding prompted us to follow up our incidental observation that murine keratinocytes seem to express IL-7 mRNA when studied by RT-PCR analysis (9). Here we demonstrate that both murine and human keratinocytes express IL-7 mRNA and release IL-7 protein. Our findings suggest a pathogenetic role of keratinocyte-derived IL-7 in inflammatory skin diseases, as well as cutaneous T cell lymphoma as antigen-specific T cells primed in vivo (5) and T lymphoma cells (10–13) have been shown to readily proliferate in response to IL-7.

Materials and Methods

Preparation of Murine Epidermal Cells and Keratinocytes. Specific pathogen-free BALB/c (H-2^d) mice (6–12-wk-old of both sexes) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). Epidermal cell (EC) suspensions were prepared from ear skin and treated with anti-Thy1-mAb (culture supernatant from clone 13.4, mouse IgM, TIB 99 from the American Type Culture Collection [ATCC], Rockville, MD) and rabbit C′ exactly as described (14), except that we included anti-I-$A^{b,d}$ mAb (clone B 21-2, rat IgG2b, TIB 229 from the ATCC) in most experiments to remove not only the Thy 1^+ TCR-γ/δ bearing dendritic epidermal T cells (15), but also the Ia^+ epidermal Langerhans cells. For further depletion, the resulting EC suspensions were then incubated with anti-I-$E^{k,d}$ (clone 14-4-4S, mouse IgG2a, HB32) as described (16) and anti-CD45 (clone M 1/9, rat IgG2, TIB 122 from the ATCC) mAbs, and then panned on petri dishes coated with nonspecies specific goat anti–mouse Ig exactly as described (14). The nonadherent cell fractions constituted virtually pure keratinocytes as evidenced by morphology and immunofluorescent staining of cytocentrifuge preparations with rabbit anti–cow keratin Ab (Dako, Glostrup, Denmark). They were used for further experiments right away or after 6–120 h of culture ($10–20 \times 10^6$ EC in 100-mm petri dishes (model 3003; Falcon Labware, Oxnard, CA) in 10 ml culture medium (RPMI 1640 supplemented with 10% FCS [56°C, 0.5 h; Seromed, Biochrom KG, Berlin, Germany], 1 mM L-glutamine, 5×10^{-5} M 2-ME, and 50 μg/ml gentamycin sulfate). 0.5–96 h before the end of culture we usually removed the nonadherent cells and added fresh medium with or without LPS (1 μg/ml) plus PMA (5 ng/ml). At the end of culture the supernatants and/or adherent cells were harvested and processed for detection of IL-7 protein and mRNA, respectively. Besides murine keratinocytes, we also examined the widely used spontaneously transformed BALB/c keratinocyte cell line PAM 212.

Preparation of Human Epidermal Cells and Keratinocytes. Normal human, female breast skin was obtained from corrective plastic surgery. To obtain single EC suspensions split-thickness skin was ex-

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posed for 16 h at 4°C to 0.25% trypsin (Flow Laboratories, Irvine, Scotland) in PBS as described previously (17). In most experiments, EC were then treated with anti-HLA-DR/DQ mAb (clone 9.3F10, kindly provided by Dr. R. M. Steinman, The Rockefeller University, New York), anti-CD3 (Becton Dickinson & Co., Mountain View, CA) and rabbit C′ to remove epidermal Langerhans and T cells. 15–20 × 10^6 EC were then plated in 10 ml culture medium in 100-mm petri dishes that had been coated with human collagen type IV (Collaborative Research Inc., Bedford, MA) to increase plating efficiency, and cultured for up to 40 d. Alternatively, we cultured EC on 3T3 feeder exactly as described (18), and used them for our experiments as secondary cultures which consisted almost exclusively of keratinocytes (18). Human (subconfluent to confluent) cultures were processed like murine ones (see above). Results obtained with primary cultures of freshly isolated EC fractions and secondary 3T3 feeder layer EC cultures were identical.

Preparation of Epidermal Sheets. Epidermal sheets were peeled from the epidermis either after short-term incubation in dispase type II (Boehringer-Mannheim, Mannheim, FRG) (19) or after heat exposure (55°C for ~30 s).

Preparation of RNA and cDNA. Total cellular RNA was isolated by the guanidinium isothiocyanate/cesium chloride method as described (16), and poly(A)$^+$ RNA by using the mRNA isolation kit (Stratagene Cloning Systems, La Jolla, CA). For PCR, 5 μg of total cellular RNA was reverse transcribed using a cDNA cycle kit (Invitrogen, San Diego, CA) as described (14).

Detection of IL-7 mRNA by RT-PCR. PCR conditions (30 cycles) were exactly as described (14). PCR primer pairs were purchased from Clontech (Palo Alto, CA) except for keratin (5′ primer: AGGCAGCGGCCCACTGAGATC-3′; 3′ primer: 5′-CATCTC-CACATTGACGTCTCC-3′; amplified fragment: 360 bp). Primers for murine IL-7 were: 5′ primer: 5′-GCCTGTCACATCATCTGA-GTGCC-3′; 3′ primer: 5′-CAGGAGGCATCCAGGAACTTCTG-3′; amplified fragment: 496 bp. Primers for human IL-7 were: 5′ primer: 5′-ATGTTCCATGTTTCTTTTAGGTATATCT-3′; 3′ primer: 5′-TGCATTTCTCAAATGCCCTAATCCG-3′; amplified fragment: 681 bp. PCR reaction products were separated as described (14) at 60 V for 2 h in 1% agarose in 1× TBE buffer containing 0.5 μg/ml ethidium bromide. 1 μg of a 1-kb and a 123-bp DNA ladder (Gibco BRL, Gaithersburg, MD) were run in parallel as molecular weight markers. The specificity of the amplified bands was validated by their predicted size and restriction enzyme digests giving appropriately sized fragments. Negative controls were included with each experiment (no cDNA, no cDNA but primers added; no RT, RNA reverse transcribed in the absence of reverse transcriptase).

Detection of IL-7 mRNA by Northern Blotting. To confirm the results obtained by PCR we performed Northern blotting as described (14) (20 μg total cytoplasmic RNA or 6 μg mRNA per lane) using a murine IL-7 cDNA probe (2) labeled with α-[^{32}P]dATP (Amersham International, Amersham, UK) using the oligoprimer procedure.

Detection of IL-7 mRNA by In Situ Hybridization. Human and murine keratinocytes grown on glass coverslips and 7-μm cryosections of normal and diseased (atopic dermatitis) human skin were investigated using digoxigenin-labeled probes and an in situ detection kit essentially according to the manufacturer (British Biotechnology Products Ltd., Abigdon, Oxon, UK). Probes used to detect IL-7 mRNA, and (as a positive control) IL-1α and actin mRNA consisted each of a cocktail of three exon-specific antisense oligonucleotides of ~30 bases in length to maximize sensitivity. The IL-7 probes were tested by dot-blot hybridization technique using murine and human IL-7 cDNAs (2, 20). As a negative control probe we used a digoxigenin-labeled synthetic 30-mer oligonucleotide (GGCGACGCGCCGTATTTATAATTCATTATG) that has a low homology to known cDNAs and the same GC content (48%) and melting temperature (86°C) as the other probes used. Bound digoxigenin-labeled probes were visualized according to the manufacturer's instructions using an antidigoxigenin Fab fragment conjugated to alkaline phosphatase and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) as a substrate, which yields a purple/black precipitate.

Detection of IL-7 Protein. Supernatants of keratinocyte cultures were used right away, or were lyophilized and reconstituted in 20% of the original volume. Supernatants were tested using a cell line (IxN/2bx) that proliferates in response to IL-7 but neither to any other cytokine nor to LPS or PMA (detection limit ~5 pg/ml) (10; Namen, A. E., unpublished results). The IL-7 content of human keratinocyte supernatants was also measured using a commercially available ELISA (British Biotechnology Products Ltd.; detection limit 4.5 pg/ml) that uses a neutralizing mAb to capture biologically active IL-7.

Results and Discussion

Murine and Human Keratinocytes Express IL-7 mRNA In Vitro. Freshly isolated murine BALB/c EC (Fig. 1) as well as EC that had been depleted of epidermal Langerhans cells and TCR-γ/δ bearing dendritic epidermal T cells (data not shown) and constituted virtually pure keratinocytes (see Material and Methods), expressed IL-7 mRNA, although not invariably, as demonstrated by RT-PCR. When examined after 6, 12, 24, 36, or 48 h of culture (with or without stimulation with LPS [1 μg/ml], PMA [5 ng/ml], or LPS plus

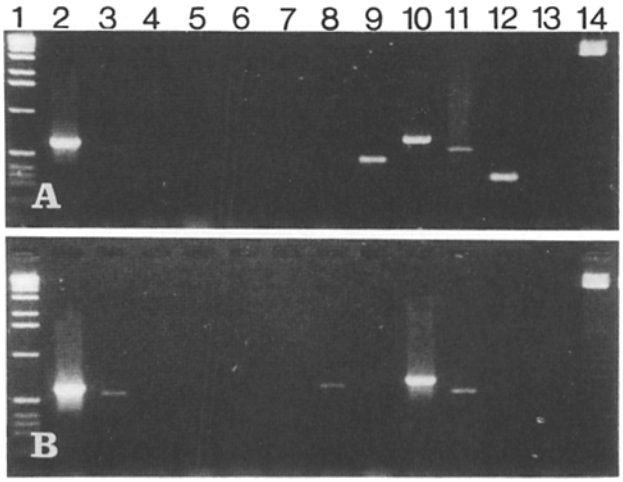

Figure 1. PCR analysis of cytokine gene expression by unfractionated EC suspensions prepared from murine ear skin by trypsinization (see Materials and Methods). Lanes *1–14*: 1-kb DNA ladder, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, TNF-α, TNF-β, GM-CSF, IFNα, 123-bp DNA ladder. Results obtained with two EC samples (*A* and *B*) are shown to demonstrate the usual variability of cytokine mRNA expression we observed. Note that bands specific for IL-7 (lane *9*) and GM-CSF (lane *12*) are present in *A*, but absent in *B*. IL-6 mRNA is detectable only in sample *B*. Both EC suspensions yield bands specific for IL-1α mRNA (lane *2*) (which is produced by keratinocytes [14] but not Langerhans cells), IL-1β (which is produced by Langerhans cells but not keratinocytes [14]) (faint bands in lane *2*), TNF-α (lane *10*), and TNF-β (lane *11*).

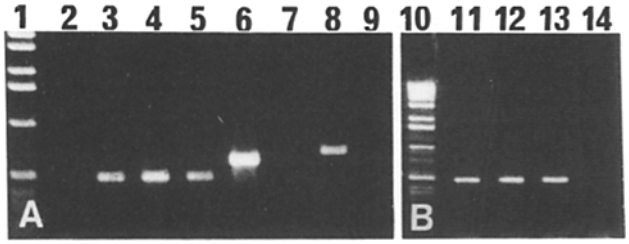

Figure 2. PCR analysis demonstrates IL-7 mRNA expression in cultured murine keratinocytes (*A*) (cultured for 6 h, lanes *2* and *3*; cultured for 24 h with LPS/PMA, lane *4*; without stimulation, lanes *5–9*) and the keratinocyte cell line PAM 212 (*B*). Lane *1*, 1-kb DNA ladder; lane *2*, no RT control (see Material and Methods); lanes *3–5*, IL-7; lane *6*, TNF-α; lane *7*, IL-1β; lane *8*, TNF-α; lane *9*, TNF-β; lane *10*, 1-kb DNA ladder; lane *11–13*, IL-7, lane *14*, no RT control.

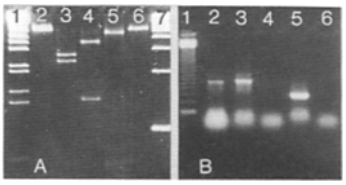

Figure 3. (*A*) Restriction enzyme analysis of amplified DNA fragments generated from reverse transcriptase PCR of murine keratinocyte RNA verifies the specificity for IL-7. Lane *1*, 1-kb DNA ladder; lane *2*, uncut; lane *3*, EcoRI digestion (yields 236- and 233-bp fragments); lane *4*, DdeI digestion (yields 341- and 140-bp fragments, and a 15-bp fragment not included on the gel); lane *5*, Sau3AI digestion (yields a 430-bp fragment and a barely visible 66-bp fragment); lane *6*, uncut; lane *7*, 100-bp DNA ladder. (*B*) PCR analysis demonstrates IL-7 mRNA expression in cultured human keratinocytes. Lane *1*, 123-bp DNA ladder; lanes *2* and *3*, IL-7; lane *4*, no cDNA control; lane *5*, β_2-microglobulin; lane *6*, no RT control. The specificity of the IL-7 amplified fragments was validated by restriction enzyme digestion with TaqI and ApyI (data not shown).

for the last 0.5–24 h) keratinocytes uniformly expressed IL-7–specific bands (see Fig. 2 *A*). The specificity of the amplified bands was validated by their predicted size and by restriction enzyme digests giving appropriately sized fragments (Fig. 3 *A*). Northern blot analysis confirmed that murine keratinocytes express IL-7 mRNA (Fig. 4). Interestingly, IL-7 mRNA did not appear to be upregulated by LPS/PMA stimulation, although the release of IL-7 protein rose sharply (see below, and Table 1). We also used in situ hybridization, which yielded only a barely detectable or no signal at all (data not shown).

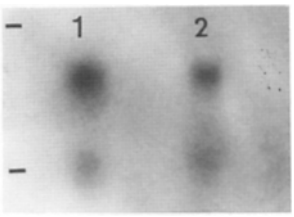

Figure 4. Northern blot analysis of IL-7 mRNA expression in murine keratinocytes. 6 μg of poly(A)$^+$ RNA derived from 16-h cultured subconfluent keratinocytes (*1*, unstimulated, *2*, stimulated by LPS [1 μg/ml] plus PMA [5 ng/ml] for 4 h) was hybridized with a ^{32}P-labeled IL-7 cDNA probe (1-d film exposure). (*Horizontal bars*) 18 and 28S rRNA.

Table 1. *IL-7 Bioactivity of Conditioned Media from Murine Epidermal Keratinocyte Cultures*

Expt.	Keratinocyte culture: Period of medium conditioning	Stimulation	IL-7 bioactivity
			pg/ml
1	Day 2 subconfluent → day 3 confluent	None	12
	Day 2 subconfluent → day 3 confluent	LPS plus PMA	192
2	Day 2 subconfluent → day 3 confluent LC and T cell depleted	None	10
	Day 2 subconfluent → day 3 confluent LC and T cell depleted	LPS plus PMA	96
3	Day 0, fresh cells → day 1 subconfluent LC and T cell depleted	None	5
	Day 1 subconfluent → day 2 subconfluent LC and T cell depleted	None	24
4	Day 0, fresh cells → day 3 confluent	None	24
5	Day 0, fresh cells → day 2 confluent	None	48

Bioactivity was assayed by measuring the proliferation of the IL-7–dependent indicator cell line IxN/2bx. This cell line neither responds to any known cytokine nor to LPS or PMA (13; Namen, A.E., unpublished observations). Culture supernatants were added to the indicator cells in a series of twofold dilutions. Bioactivity in pg/ml was calculated from a standard curve established with human recombinant IL-7. LPS was used at a final concentration of 1 μg/ml; PMA at 5 ng/ml.

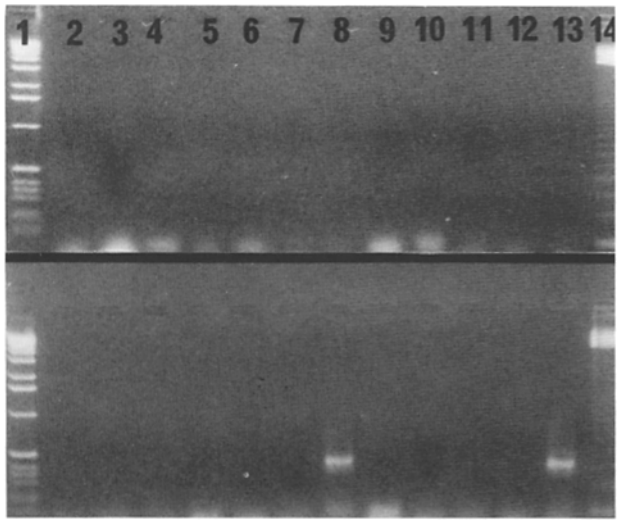

Figure 5. PCR analysis of cytokine gene expression by murine epidermal sheets prepared by heat separation (55°C for about 30 s) alone. Lanes *1–14* (*top*): 1-kb DNA ladder, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, TNF-α, TNF-β, GM-CSF, IFN-α, 123-bp DNA ladder; Lanes *1–14* (*bottom*): 1-kb DNA ladder, IFN-β, IFN-γ, IL-2R, TGF-β, CD4, CD8, β_2-microglobulin, macrophage inflammatory protein (MIP) 1α, MIP-1β, MIP-2, β-actin, keratin, 123-bp DNA ladder. Note that cytokine mRNAs are not readily detectable although β_2-microglobulin (lane *8*) and keratin (lane *13*) mRNAs are.

The in situ hybridization experiments were, however, nevertheless useful in that they excluded that only a minor contaminating cell population strongly expressed IL-7 mRNA, thus mimicking IL-7 mRNA expression by the keratinocytes. Our finding that the BALB/c-derived keratinocyte line PAM 212 also produces IL-7 mRNA (Fig. 2 *B*, lanes *11–13*) further underscored that murine keratinocytes can indeed transcribe IL-7 mRNA. Studying human keratinocyte cultures by RT-PCR analysis revealed that human keratinocytes, just like murine ones, constitutively express IL-7 mRNA in vitro (Fig. 3 *B*).

Murine and Human Keratinocytes Release IL-2 Protein In Vitro. In pilot experiments employing the IL-7–induced proliferation of pre-B cells grown in Whitlock-Witte bone marrow cultures as an IL-7 bioassay system (1), putative IL-7 activity (i.e., proliferation of pre-B cells that could be blocked by anti-IL-7 mAb) was detected only in one out of seven supernatants of stimulated (LPS plus PMA) murine keratinocytes, and not at all in supernatants from unstimulated keratinocytes (data not shown). Using an IL-7–dependent cell line that is more sensitive to IL-7 and responds neither to any other known cytokine nor to LPS and PMA (10; Namen, A. E., unpublished results), we could usually detect IL-7 in supernatants, particularly after stimulation of murine keratinocytes with LPS and PMA (Table 1). We could not unequivocally demonstrate IL-7 activity in human keratinocyte supernatants by using the IL-7–dependent cell line, yet were able to detect low amounts of IL-7 protein by an ELISA assay (Table 2). The fact that keratinocytes release only little IL-7 protein does not mean that their production of IL-7 is biologically irrelevant, as all the cells and cell lines that have been found to produce IL-7 do so only at low levels. The case is the most extreme in the human cell lines as it was even difficult to detect IL-7 production by the SK-Hep cell line (Namen, A. E., unpublished results), which was used to clone the cDNA for the human IL-7 (20).

IL-7 mRNA Is Expressed in Diseased Murine and Human Epidermis. To get information as to whether IL-7 mRNA is constitutively expressed in murine epidermis in situ, we studied epidermal sheets that had been prepared by heat separation (55°C for 30 s) rather than by conventional methods that require prolonged incubation (e.g., in dispase). We usually did not detect cytokine mRNAs by PCR analysis, although control β_2-microglobulin mRNA was readily de-

Table 2. *IL-7 Immunoactivity of Conditioned Media from Human Epidermal Keratinocyte Cultures as Determined by ELISA*

	IL-7 secreted into the culture medium										
Experiment	Day 0	3	4	5	6	7	8	9	10	13	36–40
				pg/ml							
1				2.7			7.1				
2		nd		2.2		6.0				7.8	23.0
3			nd					6.0			
4		nd			1.1						
5			1.1			16.0			13.0		
6*										4.2	

Culture supernatants were collected at the different time points, concentrated five times, and assayed by means of a commercial ELISA kit. The standard curve was established using recombinant human IL-7. Note that the amount of immunoreactive IL-7 increases with the time in culture.

* Secondary keratinocyte cultures obtained by the 3T3 feeder layer technique (see Materials and Methods); supernatants of 3T3 cells were consistently negative.

nd, not detectable.

tectable (Fig. 5). The expression of IL-7 mRNA by keratinocytes after their isolation from the skin is thus likely induced relative to the normal epidermis in situ (compare Figs. 1 and 5). In the course of allergic contact dermatitis (elicited by haptens like dinitrochlorobenzene), however, IL-7 mRNA is expressed in murine epidermis (data not shown), presumably by keratinocytes. Similarly, we found IL-7 mRNA in the epidermis of human diseased skin (e.g., atopic dermatitis), whereas IL-7 mRNA was often not readily detectable in nonlesional skin (data not shown). So far, we have not been able to unequivocally detect IL-7 mRNA by in situ hybridization in such diseased epidermis, although IL-7 mRNA was clearly present in rare stellate cells in the reticular dermis (data not shown).

Possible Relevance of Keratinocyte-derived IL-7. The range of cell types that express IL-7 mRNA and produce IL-7 is not yet well established. Previous studies have, for example, shown that spleen and thymus cells express IL-7 mRNA, although biologically active IL-7 could not be detected in respective supernatants (2). Our finding that keratinocytes can express IL-7 mRNA and release IL-7 in biologically relevant amounts is, therefore, certainly noteworthy. The physiological relevance of our finding escapes us at present, particularly as little or no IL-7 mRNA seems to be present in the adult, normal epidermis under steady-state conditions. One interesting possibility, at least in murine epidermis, is that keratinocyte-derived IL-7 acts on epidermal T cells. We have observed that highly enriched TCR-γ/δ bearing murine dendritic epidermal T cells exhibit a modest proliferative response, although not invariably, in response to IL-7 (with or without Con A, PHA, or PMA added) (Elbe, A., E. Payer, G. Schuler, and G. Stingl, unpublished observations). Furthermore, it has recently been demonstrated that IL-7 can induce TCR-γ gene expression by pre-T cells (21) in fetal liver and that TCR-γ/δ expressing dendritic epidermal T cells develop from Thy1$^+$, CD3$^-$, TCR$^-$ precursors in fetal skin (22). It is thus conceivable that IL-7 is constitutively expressed in vivo by normal murine keratinocytes at certain stages during ontogeny and critical to the development of TCR-γ/δ bearing T cells in murine epidermis. To get further hints as to a potential physiological role of keratinocyte-derived IL-7 it will, of course, be interesting to study whether other epidermal symbionts such as Langerhans cells or cells in the dermis respond to IL-7. Our finding that IL-7 mRNA is expressed in diseased epidermis (probably by keratinocytes) raises the issue of a potential role of IL-7 in skin diseases. The observation that IL-7 has costimulatory activity on purified mature T cells and is a T cell growth factor for in vivo primed antigen-specific T cells (4–8) suggests that keratinocyte-derived IL-7 might be pathogenetically significant in a variety of inflammatory cutaneous diseases. IL-7 might, however, also be an important progression factor in cutaneous T cell lymphoma as it has been shown to be a growth factor for various lymphoma cells, including those of cutaneous T cell lymphoma (10–13). One might even hypothesize that keratinocyte-derived IL-7 is relevant for the generation of cutaneous T cell lymphomas. Remarkably, cutaneous lymphoproliferation and lymphomas have recently been described in IL-7 transgenic mice (23).

The finding that keratinocytes can produce IL-7 raises several interesting issues for further studies on the biology and pathology of skin. Our studies also demonstrate that cytokine mRNAs are upregulated during enzyme digestion procedures usually employed to prepare epidermal cells. This observation underscores the importance of considering alternative techniques such as heat separation for preparing epidermal sheets for RNA preparation or in situ hybridization to obtain information that is truly relevant for the in vivo situation.

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