Identification of proliferative subpopulations within normal and lesional psoriatic epidermis and intralesional T lymphocyte activation as a mediator of psoriatic epidermal hyperplasia

Zsuzsanna Bata, M.D.

Ph.D. dissertation

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List of publications

I. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Flow cytometric identification of distinct proliferative populations within human epidermis: Keratinocyte hyperproliferation in psoriasis results from increased proliferation in the basal stem cell population. J Exp Med, 178:1271-1281, 1993.

II. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Kinetics and regulation of human keratinocyte stem cell growth in short term primary ex vivo culture; cooperative growth factors from psoriatic lesional T lymphocytes stimulate proliferation among psoriatic uninvolved but, not normal, stem keratinocytes. J Clin Invest, 95:317-327, 1995.

III. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Intralesional T lymphocyte activation as a mediator of psoriatic epidermal hyperplasia. J Invest Dermatol, 105:89S-94S, 1995

IV. Hammerberg C, Bata-Csorgo Zs, Voorhees JJ, Cooper KD: Differential IL-1 and IL-1ra protein levels in basal and suprabasal compartments of normal and psoriatic epidermis. J Invest Dermatol, 98:571, 1992 (A).

V. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Identification and quantification of heterogeneous proliferative populations in the epidermis; Localization of the primary hyperproliferative population in psoriasis. J Invest Dermatol, 98:637, 1992 (A).

VI. Hammerberg C, Bata-Csorgo Zs, Cooper KD: Concordant quantitative analysis of keratinocyte IL-1 and IL-1Ra by ELISA on whole culture extracts and by flow cytometry on individual cells. J Invest Dermatol, 100:580, 1993 (A).

VII. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Lesional psoriatic T cells stimulate growth of psoriatic uninvolved keratinocyte stem cells; increased responsiveness of psoriatic stem cells relative to normals. J Invest Dermatol, 100:498, 1993 (A).

VIII. Bata-Csorgo Zs, Hammerberg C, Voorhees JJ, Cooper KD: Lesional T cell stimulation of psoriatic keratinocyte stem cell hyperproliferation is multifactoria: gamma-IFN appears to be a critical component for growth stimulation, but requires the presence of other growth factors (GM-CSF, IL-3). J Invest Dermatol, 102:531, 1994 (A).

Introduction

The epidermis is a self-renewing tissue that traditionally has been divided into three functionally different compartments: germinative, differentiated and cornified. Through the well orchestrated processes of continuous proliferation, differentiation, and scaling the normal epidermis maintains a constant structure under physiological conditions. In many dermatological diseases there are secondary changes in this structure, and there is a well defined group of skin disorders in which abnormal proliferation, differentiation, and scaling of keratinocytes appear to be the primary pathological features. The most prominent example of this group is psoriasis, a chronic inflammatory disease with very distinct hyperproliferation of the epidermis. Dynamic assessment and physical identification of in vivo proliferative compartment subsets within human epidermis has been difficult due to the limitations of injecting radiolabeled nucleotides into subjects and the lack of a precise definition of the epidermal proliferative cell compartments. In order to identify pathological changes of epidermal proliferation in hyperproliferative sking conditions such as psoriasis it was first critical to directly quantitate proliferation in the morphologically and functionally different cell compartments of in vivo epidermis (1). Methods using radiolabeled nucleotides have been unable to directly measure proliferation in the different cell compartments of the epidermis. Previous flow cytometric approaches have also not defined the proliferative compartment subsets being examined (2). Although there is agreement that epidermal proliferation is elevated in psoriasis (3) difficulties in measurement have led to controversy as to the localization and mechanism (decreased cell cycle time vs increased growth fraction) of the elevated DNA synthesis in psoriasis (4,5).

We used four-five parameter flow cytometric analysis to study proliferation among in vivo epidermal subsets. In addition to quantitating cell size and cytoplasmic complexity, keratinocyte proliferation can be measured through the use of DNA dyes (6). DNA dyes circumvent the problem of cell metabolism of thymidine which can influence proliferative data acquired by radiolabelled thymidine incorporation (7,8). Two groups of antigens are closely related to microanatomic location and stage of differentiation in the epidermis: adhesion molecules (9-18) and keratins (19-25). Using antibodies to the β1 integrin (the entire proliferative basal compartments), and to the K1/K10 keratin pair (differentiating suprabasal compartment), we separated the epidermal cells according to their expression of these specific antigens. By combining such staining with optical light scatter characteristics, a fairly precise

definition of cell compartments was obtained. Subsequent analysis of DNA content and PCNA expression then allowed quantitation of the proliferative status of each subset in normal and psoriatic skin. With this approach, we were able to provide physical evidence that adult human interfollicular (and infundibular) epidermis harbors a primitive, slow cycling population and a rapidly cycling population which appears capable of transient amplification prior to terminal differentiation (26,27). Furthermore, we localized the upregulation in psoriasis to occur only in the normally slow-cycling, less differentiated subset.

Because epidermal hyperplasia is a key pathologic phenomenon in psoriasis, uncovering its triggering mechanisms is fundamental to research on pathomechanisms and new therapeutic strategies in the disease. An early cellular event in the development of psoriatic lesions is the infiltration of the target tissue by macrophages and activated T lymphocytes (28-30). Lesional psoriatic skin contains activated memory T lymphocytes (30-35) whose production of mRNA for lymphokines such as IL-2, γ-IFN and TNF-α is elevated relative to normal or uninvolved psoriatic epidermis (36). That the T cell activation and soluble lymphokine production plays a crucial role in the maintenance of epidermal hyperplasia in the psoriatic lesion is indicated by the beneficial clinical effect of such immunosuppressive agents as cyclosporin A, FK 506, anti-CD3, and anti-CD4 in the treatment of psoriasis (37-42). Psoriatic keratinocytes do appear to have been modulated by T cell lymphokines in vivo, because they abnormally express class II MHC and IP-10, molecules uniquely induced on keratinocytes by the T cell product γ-IFN (43-47). Indeed, T cells producing γ-IFN have been cloned from psoriatic lesions (48) and they appear to be able to induce keratinocyte class II MHC and ICAM expression (49) and growth of keratinocytes which have been multiply passaged in vitro (50). Thus, T cells are a likely source of signals for keratinocyte stem cell activation, either directly or indirectly.

Testing the responsiveness of human epidermal stem cells to putative growth regulatory signals released by T lymphocytes or other cells required an understanding of the behavior of stem cells in ex vivo culture. Although much information was available regarding growth regulation of long term, passaged keratinocytes, such cells are clearly modulated by the conditions associated with repeated subculture. Thus, passaged keratinocytes differ from in vivo epidermal cells in their expression of cytoskeletal proteins, membrane composition, cytokines and surface integrins (51-55). In addition, passaged keratinocyte cultures differ from in vivo epidermis in their composition of proliferative compartment cells, because cell cycle quiescent (G_0) stem cells are underrepresented and highly cycling clonogenic stem cells and transient amplifying cells are overrepresented (56). We utilized flow cytometric methods to demonstrate the kinetics of normal

stem cell behavior during establishment of primary in vitro human epidermal cell cultures. By quantitating their number, cell cycle status and differentiation state in the first few days of primary culture, human epidermal stem cells could thus be examined for their responsiveness to modulation in their fresh ex vivo state prior to modulation by repeated subcultures.

Using T cells cloned from lesional psoriatic skin, we showed that lesional psoriatic T cells activated by immobilized anti-CD3 plus fibronectin produced factors that induced increased keratinocyte colony formation as well as increased cell cycle entry by the normally quiescent stem cell population. Furthermore, the β1 integrin$^+$ K1/K10$^-$ keratinocyte stem cells in psoriatic uninvolved epidermis were significantly hyperresponsive to the growth stimulatory lymphokine milieu relative to β1 integrin$^+$ K1/K10$^-$ normal keratinocytes. Neutralization experiments suggested that γ-IFN, although growth inhibitory on its own, acts cooperatively with other T cell produced growth factors to cause growth induction. These data provide direct evidence that human epidermal stem cells are responsive to growth-stimulatory signals released by T lymphocytes, such as those in psoriatic lesions, and provide a mechanism by which keratinocyte stem cells can increase their proliferation in response to local stimuli.

Materials and Methods

Human subjects

Keratome biopsies were taken from the buttock area of normal volunteers and psoriasis vulgaris patients during the morning hours. Oral medication was not allowed in either group within 1 month prior to the time when specimens were taken. In addition, in the psoriatic group, external treatment was not allowed 2 weeks prior to the procedure. All lesional tissue represented inflamed but relatively stable psoriatic plaques. For tissue staining, 4 mm punch biopsies were taken from normal and lesional skin of the volunteers and psoriatic patients.

Tissue staining

Frozen sections (2-4 μ) in O.C.T.(Tissue Tec II, Miles Laboratories, Elkart, IN) were fixed in 70% cold ethanol for 10 min at -20 ^{0}C. Indirect immunofluorescence staining was performed with anti-β1 integrin (CD29), anti-K1/K10 antibodies and isotype controls, followed by FITC or rhodamine-conjugated second antibodies, as previously described (57).

Epidermal cell suspension

After overnight treatment of keratome biopsies with Dispase (Collaborative Biomedical Products, Bedford, MA) at 4 ^{0}C, epidermis was removed from dermis. The epidermal sheet was

placed into 0.25% trypsin (Sigma, St.Louis, MO) and incubated for 20 min at 37 ^{0}C. A single cell suspension was then prepared in the presence of 0.01% DNase (Sigma) and 10% FBS (Hyclone, Logen, UT) by gentle teasing. The suspension was filtered through a 112 micron nylon mesh and washed. After the final wash, cells were resuspended in 1 ml Hanks' Balanced Salt Solution without Phenol Red, Ca^{++} and Mg^{++} (Irvine Scientific, Santa Anna, CA) and slowly pipetted into 30 ml 70% cold ethanol. Samples were kept at -20 ^{0}C in ethanol until staining and flow cytometric analysis.

Staining procedure

The ethanol-permeabilized cells were centrifuged (10 min, 3000 rpm) and resuspended in Hanks' solution. The suspension was syringed through a 30 G needle before staining. Monoclonal antibodies used were: anti-β1 integrin (CD29, 4B4,Coulter Immunology, Hialeah, FL) 1:20 dilution, anti-K1/K10 (AE2, ICN, Costa Mesa, CA) 1:50 dilution, anti-PCNA 1:100 dilution (Boeringer, Indianapolis, IN) and isotype controls, which included purified mouse IgG1 (Sigma), and purified mouse IgG2a (Pharmingen, San Diego, CA). 10% normal goat serum (Cedarlane Laboratories, Hornby, Ontario) was used to block nonspecific binding. Goat anti-mouse IgG1FITC and IgG2aPE (Boeringer) was used as secondary antibodies at 1:100 and 1:80 dilutions respectively. Double staining with anti-β1 integrin and anti-K1/K10 was carried out in four steps. After primary staining with anti-K1/K10 and second step staining with goat anti mouse (FITC), the cells were incubated with 10% normal mouse serum (Accurate Chemical, Westbury, NY) for 30 minutes on ice and stained in the fourth step with PE conjugated anti-β1 integrin. The cells were then resuspended either in propidium iodide (PI, 50 μg/ml, Sigma) with RNase A (100 U/ml, Sigma) or 7 amino-actinomycin D (7AAD, 25 μg/ml, Calbiochem, San Diego, CA). Samples were analyzed within 24 hours by flow cytometer.

Flow cytometry

Flow cytometry was performed using an Epics Elite Flow Cytometer (Coulter Cytometry, Hialeah, Florida). Light scatter, forward and ninety degrees, was used for gating out debris and lymphocytes in the lesional psoriatic samples (a distinct small, low granularity cell population of β1 integrin$^+$CD45$^+$ cells). Cell aggregates were eliminated from the DNA analysis based on the ratio of integrated to peak fluorescence of PI or 7AAD. Listmode data was analyzed using Coulter Elite Software, and for cell cycle analysis, Multicycle software from Phoenix Flow Systems was used.

Flow cytometric identification of proliferative keratinocyte subsets in primary culture

Epidermal cell suspensions were prepared from normal deep keratomes by treatment with dispase (Collaborative Biomedical Products, Bedford, MA) (overnight 4 ^{0}C) followed by removal of the dermis then treatment of the epidermis with 0.25% trypsin (United States Biochemical Corporation, Cleveland, OH) (15-20 min, 37 ^{0}C). Cells were plated into six-well culture plates ($1x10^6$ cells/ml, 2 ml/well) and cultured in keratinocyte basal media (KBM, Keratinocyte-SFM media without bovine pituitary extract, BPE, and epidermal growth factor, EGF, Gibco, Grand Island,NY) supplemented with 1% fetal bovine serum (FBS, Hyclone, Logen, UT). At different time points after initiation of the cultures the cells were briefly trypsinized (0.025% trypsin+0.01% EDTA), collected and fixed in 70% cold ethanol at -20 ^{0}C and stained with the following monoclonal antibodies: anti-β1 integrin (4B4, 4B4-RD1 (anti β1 integrin antibody directly conjugated to phycoerythrin (PE), Coulter Immunology, Hialeah,FL) 1:20 dilution, anti-K1/K10 (AE2, ICN, Costa Mesa, CA) 1:50 dilution, anti-PCNA (Boeringer,Indianapolis, IN) 1:100 dilution and isotype control (purified mouse IgG1 and IgG2a, Sigma, St. Louis, MO). Goat anti-mouse IgG1 and IgG2a FITC (Boeringer) was used as secondary antibody at 1:100 dilution and 10% normal goat serum (Cedarlane Laboratories, Hornby, Ontario,Canada) to block nonspecific binding. After monoclonal antibody staining, the cells were resuspended in propidium iodide (50 μg/ml,Sigma) with RNase A (100 U/ml, Sigma) and incubated at room temperature for 30 minutes. Samples were stored at 4 ^{0}C and analyzed within 24 hours by flow cytometer. In one experiment cells from a 7-day culture were harvested and stained with anti β1-integrin before ethanol fixation to determine whether the bright staining was due to intracellular or surface β1 integrin. Flow cytometry was performed using an Epics Elite Flow cytometer (Coulter Cytometry, Hialeah, FL). Cell aggregates were eliminated from DNA analysis based on the ratio of integrated to peak fluorescence of PI. Data (an average of 30000 events from each sample) was collected on a logarithmic scale for FITC and on a linear scale for PI, and stored in Listmode. Data was analyzed using Coulter Elite Software (Coulter Cytometry).

Initiation of psoriatic T cell clones

The criteria for punch biopsy of lesional psoriasis for cloning T cells was that the lesion be expanding, small, inflammatory, psoriatic papules of patients who had not received internal medication 1 month prior, and topical treatment 2 weeks prior, to the biopsy. The dermis was cut away and the remaining epidermis cut into several small pieces which were then placed into

a 24-well tissue culture dish in RPMI 1640 media (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% AB serum (North American Biological, Miami, FL) and a low dose (50 U/ml) of recombinant human IL-2 (Collaborative Biomedical Products) to selectively propagate in vivo activated T cells expressing high affinity IL-2 receptors. After a 24-hour incubation (37 ^{0}C, 5% CO_2) the pieces were transferred to a second 24-well plate for another 24-hour incubation. Cells were then harvested from the dishes, combined, washed 3 times and replated in the above media. Next day the cells were cloned by limiting dilution and plated into 96-well v-bottom plates (0.3 cells/well) with allogeneic gamma irradiated (5000 rad) ficoll-hypaque (Sigma) purified peripheral blood mononuclear cells (MNC, $0.5x10^6$/well) and 1 μg/ml phytohemagglutinin (PHA) (Burroughs Wellcome, Greenville, NC). Cloning wells containing positive growth were expanded into v-bottom 96-well plates, then 24-well-plates, frozen in 90% FBS and 10% DMSO and stored in liquid-N_2. Cloned psoriatic lesional T cells were immunophenotyped with anti-CD3, anti-CD4, anti-CD8, anti-TCR γ/δ and anti-TCR α/β antibodies, using appropriate isotype controls (Beckton-Dickinson, San Jose, CA) and analyzed by flow cytometry.

T cell activation for generation of supernatants for stimulation of epidermal cells

Frozen T cells from the different clones were thawed and placed in AIM V (Gibco, Grand Island, NY) + 10%FBS (Hyclone) + 50 U/ml rIL-2 (Collaborative Biomedical Products) media in 24 well plates and then stimulated with PHA (1 μg/ml) and allogeneic MNCs ($0.5x10^6$/well). After 48 hours the MNCs were removed from the cultures by ficoll-hypaque (Sigma) density gradient centrifugation. 10-12 days were required for the T cells to reach resting state after PHA plus MNC activation, at which time they were then plated on fibronectin and anti-CD3 coated culture dishes in serum-free and IL-2-free AIM V media. Prior to cell plating the culture plates were coated initially with anti-CD3 (1 μg/ml) overnight 4 ^{0}C, washed with PBS (Gibco) and then coated with fibronectin (Sigma) (20 μg/ml) overnight 4 ^{0}C and washed with PBS, as previously described (58). Supernatants were collected 48 hours after plating, sterile filtered and stored at -70 ^{0}C. Lymphokine production by activated lesional psoriatic T cells was determined by using ELISA kits for GM-CSF, IL-3, IL-4 (R&D Systems, Minneapolis, MN) , γ-interferon (Genzyme, Boston,MA) and TNF-α (Pharmingen, San Diego,CA).

Clonal growth assay

Epidermal cell suspensions were prepared from normal and uninvolved psoriatic keratomes as described above. Cells were plated into six-well culture plates ($1x10^6$ cells/ml, 2 ml/well) in KBM (Gibco) media supplemented with 1%FBS. Activated T cell supernatants and AIM V media, as control, were diluted at 1:10 with KBM and added at the time of culture initiation to the cultures (200 µl/well). The media was changed on every 3rd day, each time supplemented with activated T cell supernatant or AIM V media as described. After 2 weeks plates were fixed with 1% formalin and stained with Rhodamine B (Sigma) (59).

Short term epidermal proliferation assay

Freshly isolated psoriatic uninvolved (n=7) or normal (n=6) epidermal cells were plated in six-well culture plates at $1x10^6$ cells/ml, 2 ml/well, and cultured for 72 hours in the presence of activated psoriatic T cell supernatant (4F4) or AIM V medium diluted 1:100 in KBM+1% FBS. Cells were then fixed in 70% ethanol and stained with anti-β1 integrin, anti-K1/K10 and anti-vimentin as above. DNA content was determined by staining with propidium iodide. Samples were analyzed by flow cytometry.

Neutralization experiments

In some experiments, lymphokines in the psoriatic activated T cell supernatants were neutralized with monoclonal antibodies. The following neutralizing antibodies were separately added to the epidermal cell cultures prior to addition of the activated T cell supernatant (4F4): mouse anti-human IFN-γ (Genzyme), 4.5 µg/ml, goat anti-human IL-3 (R&D Systems, Minneapolis, MN), 3 µg/ml, goat anti-human GM-CSF (R&D Systems) 20 µg/ml, mouse anti-human TNF-α (Boehringer), 10 µg/ml. Isotype controls were mouse IgG2a and IgG1 (Sigma) and goat IgG (R&D Systems), at identical concentrations. The concentrations were calculated to achieve a 100 fold excess of neutralizing activity based upon the known amounts of lymphokines in the supernatants and the neutralizing activity of each antibodies. The above described short term epidermal cell proliferation assay was used to test the effect of human recombinant γ-IFN (Collaborative Biomedical Products, at 10 and 100 U/ml) on keratinocyte stem cells.

Statistical analysis

Pairwise Student's t-test was used to compare keratinocyte stem cell growth in the presence of T cell supernatant and control media in the short term proliferation assays.

Results

Anti-β1 integrin and anti-K1/K10 antibodies distinguish basal from suprabasal epidermal cells in tissue sections

In order to utilize flow cytometry to quantitate proliferative compartments in the epidermis, antibodies that preferentially react with the proliferative or non-proliferative compartments of epidermal cells were identified. We verified by indirect immunofluorescence whether integrin and differentiation keratin antibodies detect distinct cellular compartments in in vivo human epidermis. In normal human skin a monoclonal antibody to the β1 subunit of the CD49/CD29 integrin complex stained the basal layer and occasional second layer cells in the epidermis (Fig. 1a). Anti K1/K10 antibody staining, on the other hand, was limited to upper layers of the epidermis, generally, but not exclusively, sparing the basal layer, (Fig. 1b).

The same overall pattern of K1/K10 expression was observed in lesional psoriasis skin sections as in normals, in that K1/K10 expression was localized mainly above the β1 integrin^{+} layers (Fig. 2b). However, psoriatic skin differed from normal skin in β1 integrin expression, in that β1 integrin was expressed on the first 2-3 rows of lower cells in psoriatic epidermis (Fig. 2a).

Figure 1. Immunostaining of normal skin

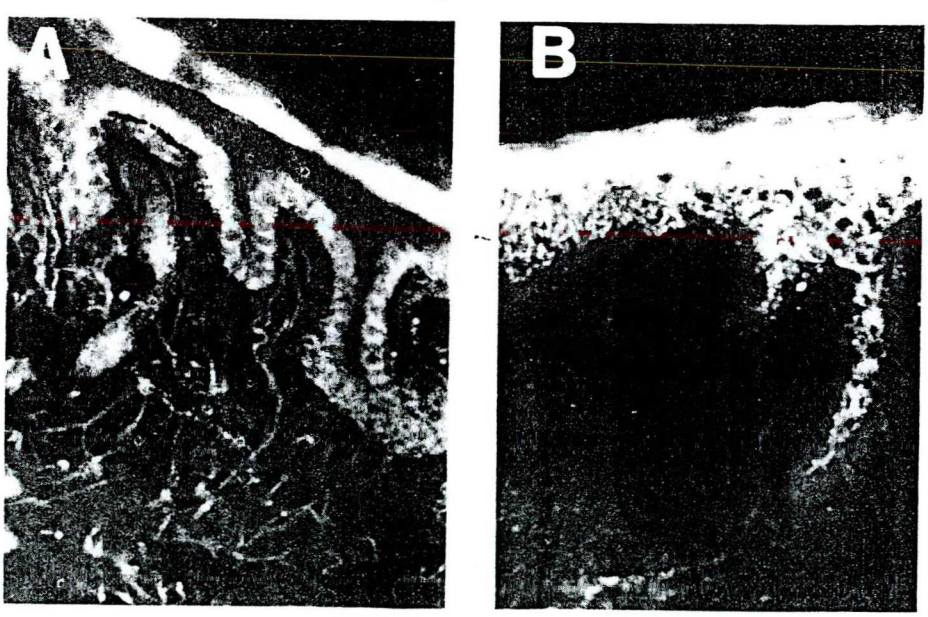
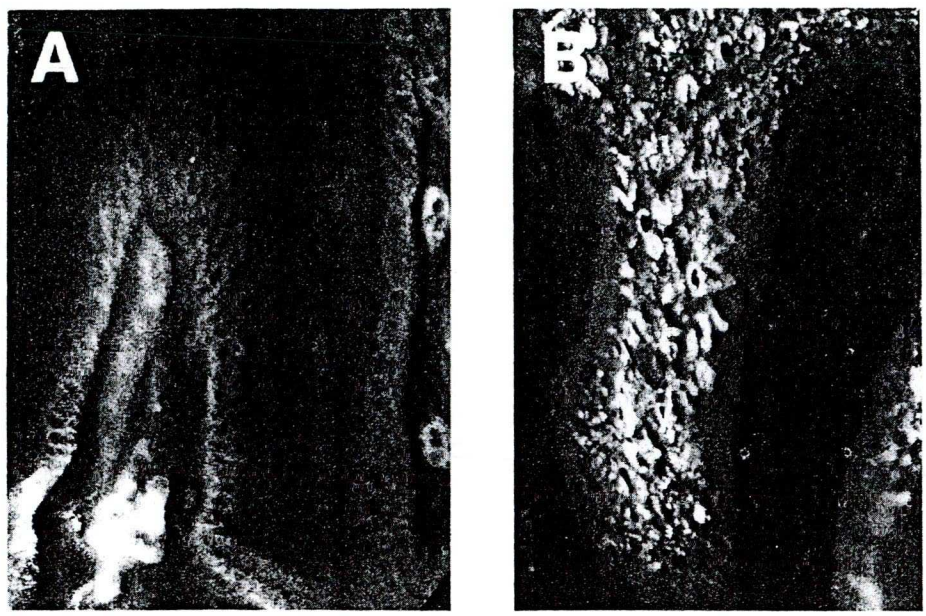


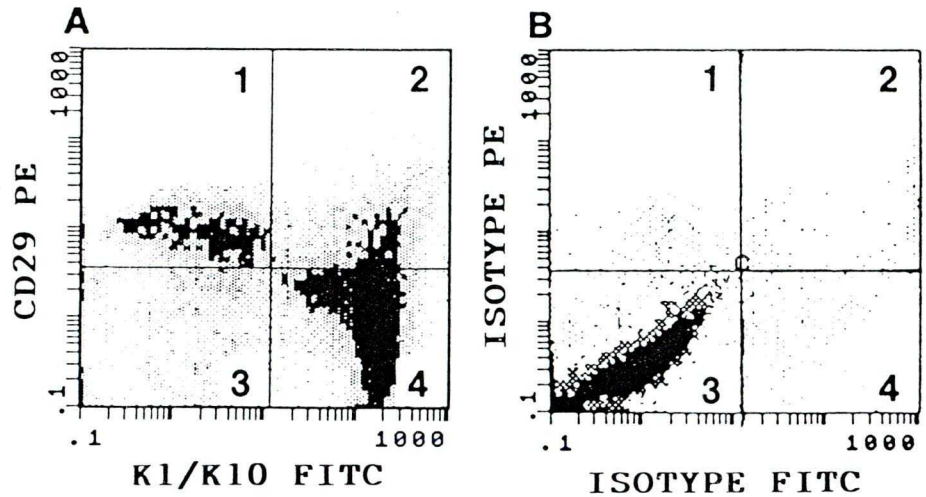
Figure 2. Immunostaining of psoriatic lesional skin



Quantitation of basal and suprabasal cell compartments in normal epidermis by flow cytometry.

Epidermal cell suspensions prepared from normal skin keratome biopsies were stained with antibodies against β1 integrin and K1/K10 keratins or isotype controls and analyzed by flow cytometry. Consistent with the in vivo staining pattern described above, three major populations comprising 89.2±7.1% (n=5) of the normal epidermal cells, were detected by flow cytometry. Predominantly basal cells were defined as β1 integrin$^+$K1/K10$^-$ and consisted of 20.7±1.6% of the stained epidermal cells (Quadrant 1, Fig. 3a). The suprabasal cells were identified based upon their positive expression of K1/K10 and could be divided into two populations depending upon their coexpression of β1 integrin. The majority of the epidermal cells were found in the β1 integrin$^-$K1/K10$^+$ population (Fig. 3a, Quadrant 4). Another population of epidermal cells, which coexpressed β1 integrin and K1/K10 (β1 integrin$^+$ K1/K10$^+$ population) comprised 13.8±3.2% of stained epidermal cells (Fig. 3a, Quadrant 2). The β1 integrin$^+$K1/K10$^+$ cell type has not previously been described, although its existence can be inferred based upon immunoelectron microscopic demonstration of K1/K10$^+$ cells in the basal layer (60) and reports of occasional β1 integrin$^+$cells in the second layer of the epidermis (14).

Figure 3. Identification of normal human epidermal cell populations by flow cytometry

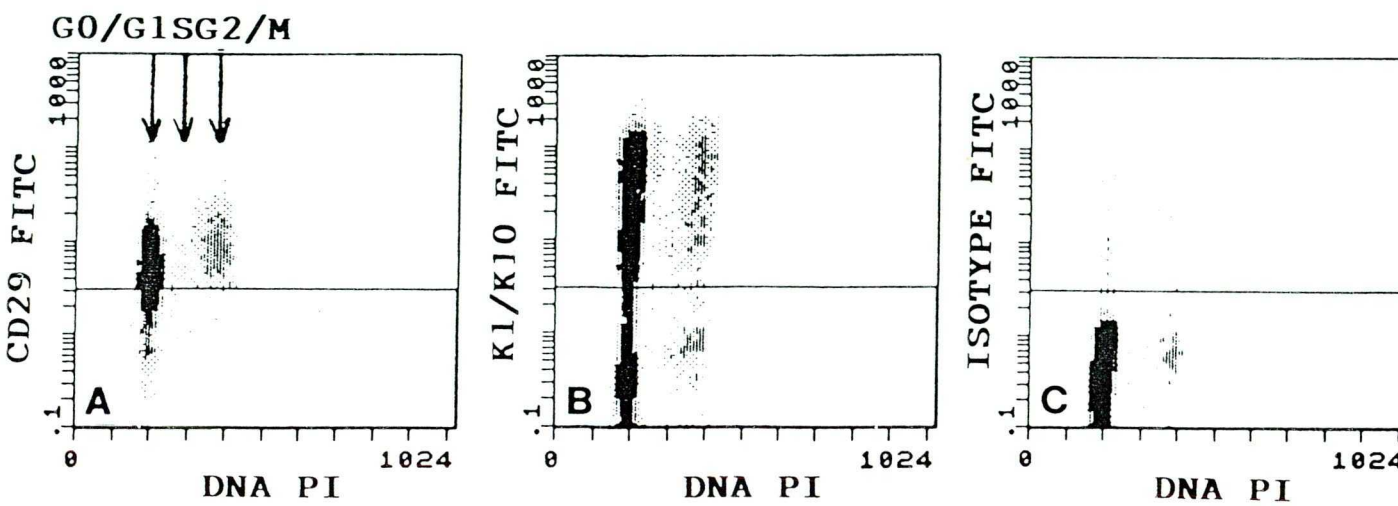


Two parameter scatter plot diagrams of double-stained (anti-β1 integrin PE and anti- K1/K10 FITC) epidermal cells from normal human skin. Cursors represent background fluorescence intensity based on isotype control staining (B). Single positive cells fall into quadrants 1(PE) and 4(FITC), double positive cells fall into quadrant 2 (A).

β1 integrin expression distinguishes between proliferative and non-proliferative compartments in normal epidermis.

To define the epidermal proliferative compartment, epidermal cells were simultaneously stained with anti-β1 integrin, anti-K1/K10 or isotype control antibody in combination with DNA dyes such as propidium iodide and 7AAD. DNA staining in combination with specific antibody permitted determination of cycling ($S/G_2/M$ phase cells) and non-cycling (G_0/G_1) states of specific epidermal compartments (Fig. 4). With this approach, it is clear that the entire pool of proliferating cells ($S/G_2/M$) are β1 integrin$^+$ (Fig. 4a). Using anti-K1/K10, the proliferating β1 integrin$^+$ cell population can be subdivided into two main subsets (Fig. 4b). One lacked K1/K10 and the other coexpressed K1/K10.

Figure 4. DNA content of epidermal cell subsets



In studies of keratinocytes cultured long term in vitro, two populations of proliferating keratinocytes have been observed (61). Small cells were found to be slowly cycling while the more rapidly proliferating cell population consisted of large cells. It has been suggested that the slowly cycling small population had stem cell properties and the larger cells represented transiently amplifying keratinocytes. We asked whether similar mitotic properties would be exhibited by human epidermal cells in vivo. Therefore, the DNA content of the small β1 integrin$^+$ and large β1 integrin$^+$ cells were analyzed separately. Cell size was determined by light scatter characteristics. Among the small β1 integrin$^+$ cells, 5.16±1.8% cells were found in S/G_2/M phase, whereas 16.6±4.54% of the large β1 integrin$^+$ cells were in cycle (n=5) (not shown).

Separation of proliferative compartment of normal epidermis into slow cycling β1 integrin$^+$K1/K10$^-$ cells and rapidly cycling β1 integrin$^+$K1K10$^+$ cells

Because there is a continuity in size and granularity among the β1 integrin$^+$ epidermal cells, especially in the psoriatic epidermis, separation of the cells into a small and large group for further analysis would be somewhat arbitrary. Therefore, K1/K10 expression was used to separate the proliferative β1 integrin$^+$ cells into subpopulations, and triple color analysis utilized to simultaneously quantitate DNA content with another DNA dye, 7AAD. Indeed, the DNA analysis revealed a great difference in mitotic activity between β1 integrin$^+$K1/K10$^-$ (4.53±1.1%) and β1 integrin$^+$K1/K10$^+$cells (48.8±18.92%) (n=3).

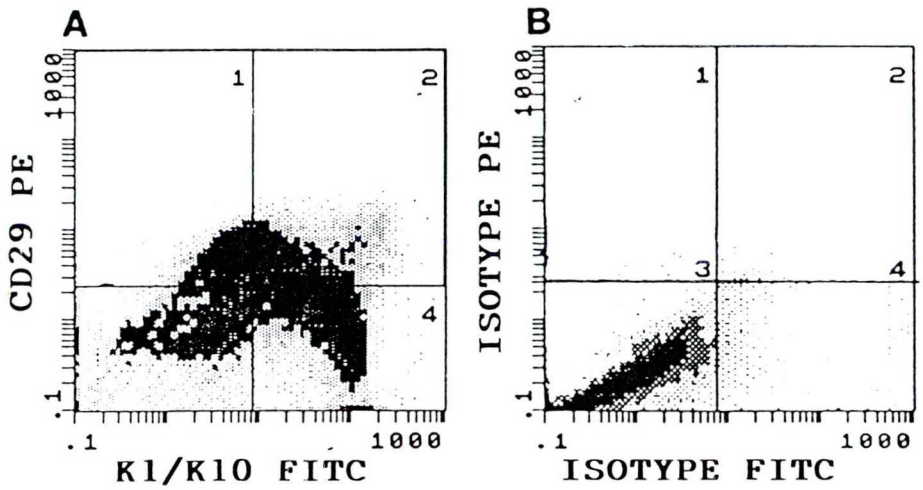
These data provided, for the first time, a direct demonstration that the proliferative compartment of in vivo human epidermis is characterized by β1 integrin expression and consists of at least two morphologically and functionally different compartments. One compartment mainly contains slowly cycling β1 integrin$^+$K1/K10$^-$ small cells with a relatively simple internal structure. The other proliferative compartment contains rapidly proliferating β1 integrin$^+$K1/K10$^+$ larger cells with more complex cytoplasmic organization.

β1 integrin$^+$K1/K10$^-$ keratinocytes are responsible for hyperproliferation in lesional psoriatic epidermis

Cytometric methods for analysis of freshly isolated human epidermal cells were applied to quantitate and localize alterations in the proliferative compartments of lesional psoriatic epidermal cells. Similar subpopulations were found in the lesional epidermal cells stained with anti-β1 integrin and anti-K1/K10 as were found in normal: β1 integrin$^+$K1/K10$^-$ (Fig. 5a, Quadrant 1), β1 integrin$^+$K1/K10$^+$ (Fig 5a, Quadrant 2) and β1 integrin$^-$K1/K10$^+$ (Fig 5a, Quadrant 4), but the overall pattern differed somewhat, in that there were more cells found in the rightmost portion of

Quadrant 1 and the leftmost portion of Quadrant 2 (Fig 5a). Although the cells in Quadrant 1 must be considered to be K1/K10$^-$ based on the isotype staining (Fig 5b), the cells in the leftmost portion of Quadrant 2 (Fig 5a) can be considered to be K1/K10^{dim}, indicating a transitional state of early K1/K10 expression.

Figure 5. Identification of psoriatic epidermal cell subsets by flow cytometry



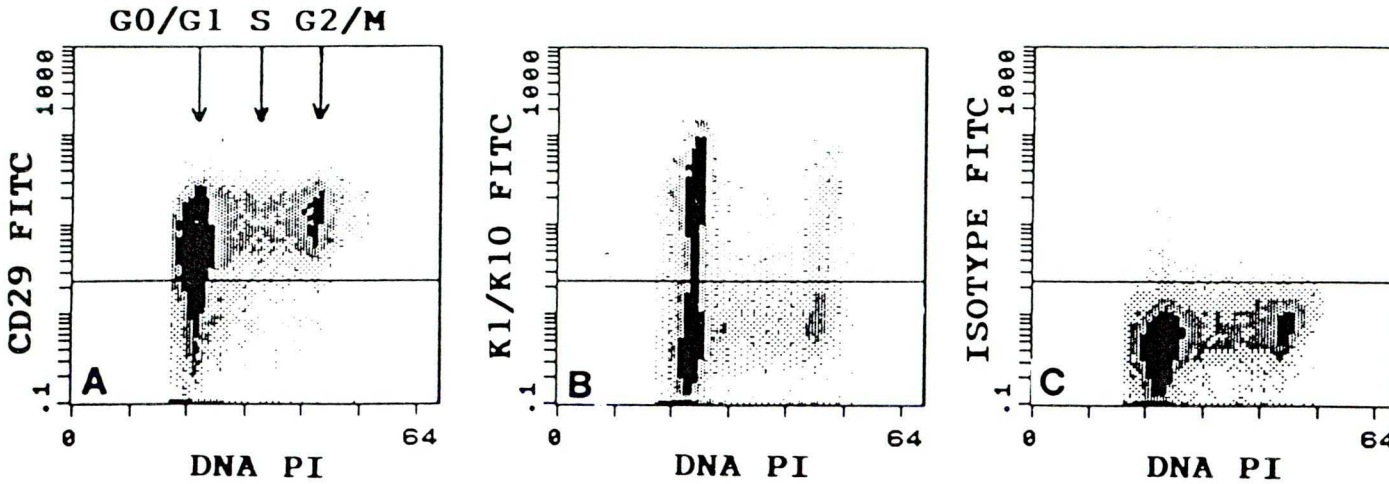
Two-parameter scatter plot of lesional psoriatic epidermal cells costained with antibodies against β1 integrin and K1/K10. Cursors are set based on double isotype staining (B). Cells expressing both antigens fall into quadrant 2, single positive cells are in quadrants 1 and 4 (A).

As in normal epidermis, the proliferative compartment ($S/G_2/M$) of the psoriatic epidermis was entirely comprised of β1 integrin$^-$ cells (Fig. 6a, cells above the horizontal cursor for β1 integrin$^+$ cells defined by the isotype control (Fig 6c). One difference, however, was the presence of a β1 integrin$^-$K1/K10^{dim} "S" phase cell population, with much variability in size (ranging from 0.7% to 9% of total epidermal cells in individual samples) in the psoriatic samples (Fig. 6, cells with >2n DNA below the horizontal cursor). This population is never present in normal samples.

In psoriasis the vast majority of the proliferating cells are clearly K1/K10$^-$ (Fig 6b, $S/G_2/M$ cells below the horizontal cursor), based on the isotype staining (Fig 6c). Of interest is the slightly increased presence of a cluster of G_2/M cells just above the horizontal cursor in psoriasis (Fig 6b) relative to normals (Fig 4b), again consistent with an increase in transitional cells (K1/K10^{dim}).

The two proliferating populations in the normal and lesional psoriatic epidermis exhibit the same relative proportions (Table 1) suggesting a parallel expansion of these two populations in lesional psoriatic skin.

Figure 6. Proliferative state of epidermal cell subsets in lesional psoriatic epidermis determied by DNA content and antibody staining.



Two parameter scatter plot diagrams show double stainings with an antibody, anti-β1 integrin (A), anti-K1/K10 (B) and propidium iodide, that staines DNA. Background fluorescence is determined be isotype staining (C).

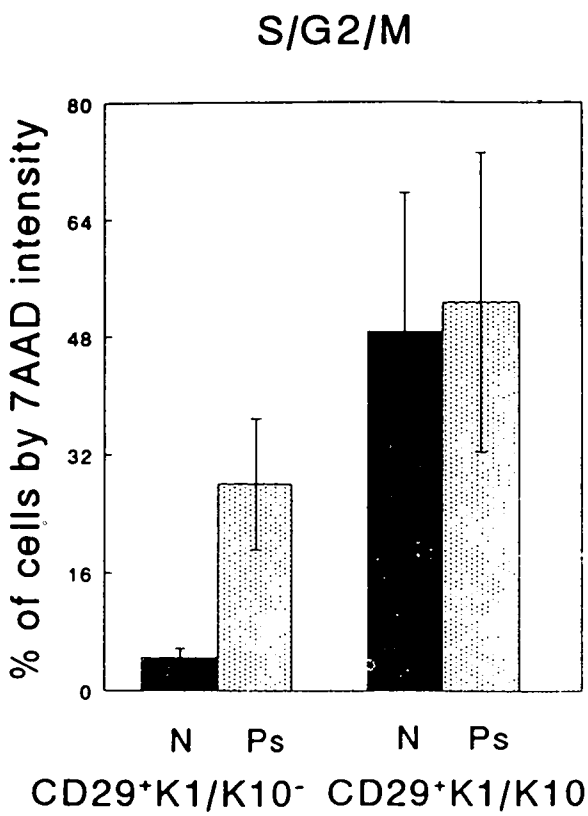
Table I. The relative proportions of the β1 integrin$^+$ proliferative cells compartments are the same in normal and psoriatic epidermis.

	β1 integrin$^+$ K1/K10$^-$	β1 integrin$^+$ K1/K10$^+$
Normal	65.4±4.8	34.6±4.8
Psoriatic	65.4±9.3	34.6±9.3

Percentages were calculated in 5 independent double staining experiments, values expressed as averages±SE in each group.

However, comparison of the mitotic activity of proliferative compartment subsets in normal and in diseased epidermis revealed a fundamental and robust difference between normal and psoriatic skin (Fig. 7). On the one hand, a major difference in the number of S/G_2/M phase cells among the β1 integrin$^+$K1/K10$^-$ cells was detected in lesional psoriatic epidermis upon comparison to normal (28.06±8.9% in lesional psoriasis vs 4.53±1.31% in normal, $p<0.05$). On the other hand, the cycling pool (S/G_2/M phase cells) within the β1 integrin$^+$K1/K10$^+$ differentiating transiently amplifying cells was almost the same in normals and psoriatics.

Figure 7. Cell cycle analysis localizes the hyperproliferative defect in lesional psoriatic skin to be within the β1 integrin(CD29)⁺ K1/K10⁻ cell compartment



Epidermal cells from normal (n=3) (solid bars) and lesional psoriatic (n=3) (stippled bars) skin were costained with anti-β1 integrin PE, anti-K1/K10 FITC, and the DNA dye 7AAD. Based on PE and FITC fluorescence cells were electronically separated into β1 ntegrin⁺K1/K10⁻ and β1 integrin⁺K1/K10⁺ populations and the DNA distribution based on the 7AAD staining was analysed by the Multicycle software package (Phoenix Flow Systems).

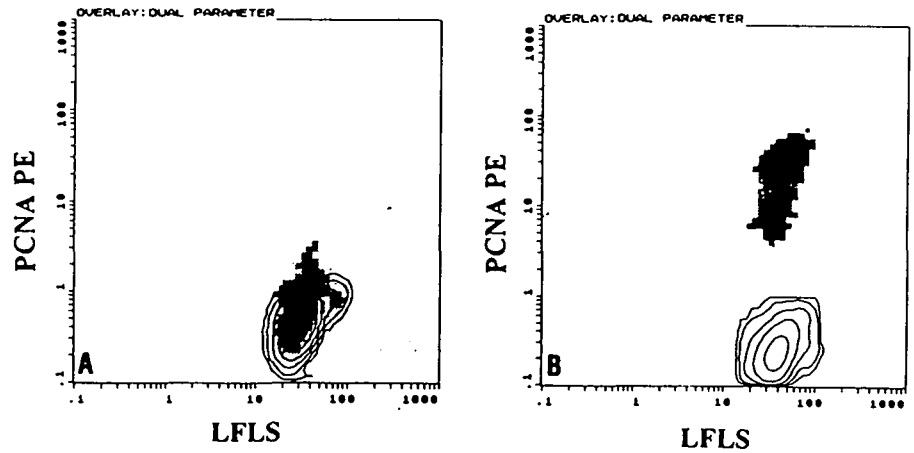
The finding that the same mitotic activity is present in the normal and lesional psoriatic β1 integrin⁺K1/K10⁺ transient amplifying cells indicates that there is no inherent change in proliferation among the transiently amplifying keratinocytes in lesional epidermis. In addition, the proportion of β1 integrin⁺K1/K10⁻ cells that differentiate to express K1/K10 is the same for both normal and psoriatic β1 integrin⁺K1/K10⁻ cells.

Hyperproliferation in the psoriatic epidermis is due to activation of the normally quiescent cells in the β1 integrin⁺K1/K10⁻ basal cell compartment

Altough stem cells must be contained within the β1 integrin⁺K1/K10⁻ cell population, they do not comprise the entire subset. To determine whether all of the cells in this compartment are affected by the hyperproliferative signal, we combined K1/K10 staining with PCNA staining. PCNA begins to accumulate during the G_1 phase of the cell cycle, is most abundant during S phase, and declines during G_2/M phase (62). In normal epidermis the vast majority (95.5%) of the K1/K10⁻ cells were PCNA negative, indicating quiescent state (Fig. 8a), consistent with a large component of slow-cycling cells. By contrast, in psoriatic epidermis, all of the K1/K10⁻ cells

showed PCNA positivity (Fig. 8b, upward shift of dark dot plot relative to isotype-stained cells represented by counter plot).

Figure 8. PCNA and K1/K10 double staining of normal and psoriatic epidermis.



K1/K10$^-$ cells are electronically gated according to FITC fluorescence (K1/K10 staining) in normal (A) and psoriatic involved (B) epidermis. Two-parameter diagrams show PCNA PE staining (dot plots, y axes) and isotype control staining (counter plots, y axes) versus forward angle light scatter (x axes).

These data suggest that the entire β1 integrin$^+$K1/K10$^-$ cell population has recently entered cell cycle in psoriatic epidermis, indicating that stem cells, which must be contained in this population are affected by the hyperproliferative signal(s).

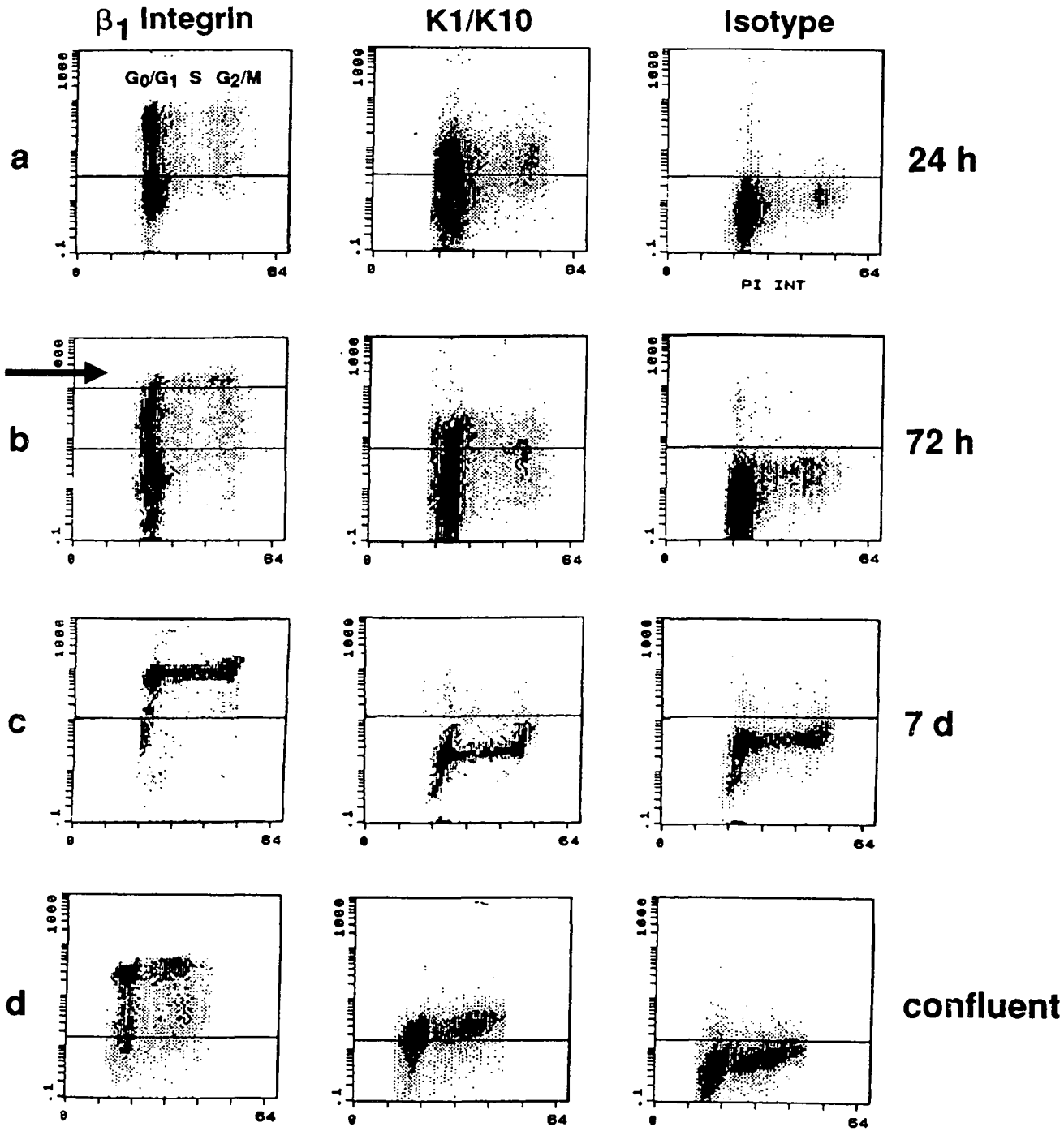
The β1 integrin$^+$ K1/K10$^-$ stem cell containing keratinocyte subset is responsible for establishing colonies in primary in vitro cultures of human epidermal keratinocytes

Normal fresh ex vivo epidermal cells were plated in keratinocyte basal media (KBM) +1%FBS, and at different time points the cells were harvested and stained with anti-β1 integrin, anti-K1/K10 and propidium iodide (PI).

At 24 hours, the cultured cells exhibited characteristics by flow cytometry identical to freshly isolated cells (Fig. 9a) (63). As compared to the isotype control-stained preparation (Fig. 9a, third column), essentially all proliferating cells were β1 integrin$^+$ (Fig. 9a, first column, y axis) ($S/G_2/M$ phase is indicated along the x axis by cells with greater than diploid DNA content by PI intensity). These β1 integrin$^+$ cells at 24 hours can be divided into two proliferating cell populations by K1/K10 expression (Fig. 9a, second column, y axis); 1) K1/K10$^-$ cells containing the cycling compartment of the stem cell population (a minor $S/G_2/M$ population that lies below the horizontal cursor denoting positive/negative fluorescence) and 2) K1/K10$^+$ cells representing the transient amplifying cell population which has committed to

differentiation in vivo but is still capable of proliferation (major population of cells in $S/G_2/M$ phase above the cursor).

Figure 9. Flow cytometric identification of keratinocyte subsets in in vitro primary cultures.



Red fluorescence intensity (PI, shown on linear scale, x axes) quantitates whether the cell is diploid (G_0/G_1) or has entered cell cycle ($S/G_2/M$). FITC fluorescence intensity (y axes) characterizes β1 integrin or K1/K10 positivity. Positive cells are represented by dots above the horizontal cursors, defined by nonspecific isotype staining.

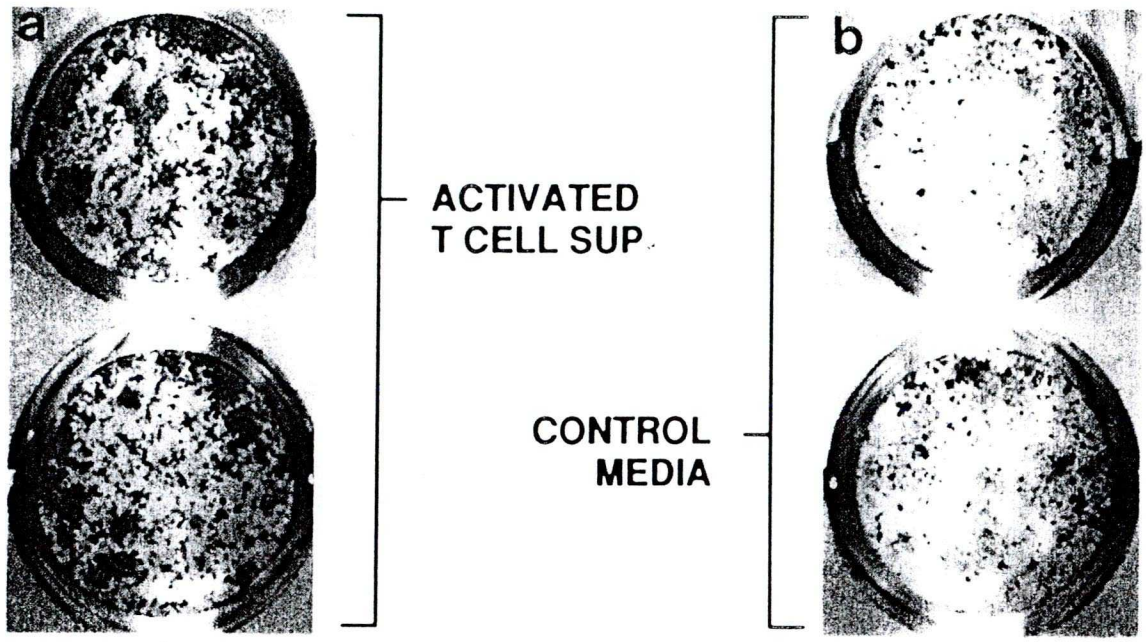
After 72 hours of culture, a distinct proliferating cell population begins to appear in the cultures, which has very bright β1 integrin expression (Fig. 9b, first column, arrow) while a few K1/K10$^+$ cells of moderate β1 integrin expression with greater than diploid DNA content are still present (Fig. 9b, second column, above the cursor). In contrast to fresh ex vivo cells, some cells in S/G_2/M phase are β1 integrin$^-$ at 72 hour in the cultures (Fig. 9b, column 1). The β1 integrin^{bright} population (arrow) contains a higher cycling pool than the β1 integrin ^{dim} or $^-$ cells in the culture, as determined by the number of cells with greater than diploid DNA content in each population (32.5% of β1 integrin^{bright} cells are in S/G_2/M phase, whereas only 5.1% of β1 integrin^{dim} or $^-$ cells are in cycle (Fig. 9b, column 1)).

After 7 days of culture, almost all the cells are β1 integrin^{bright} K1/K10$^-$ (Fig. 9c, left and middle columns) and are still characterized by a high cycling pool (S/G_2/M: 40.3%). At this time, small colonies (5-6 cells) of monolayered cells can be seen in the cultures. (Cells were also stained at 7 days with and without prior ethanol fixation and similar staining pattern was observed in both cases, indicating that the bright β1 integrin expression is related to the cell surface, data not shown.) At culture confluency, K1/K10 expression appears on the majority of the cells (Fig. 9d, column 2) and cells with lower expression of β1 integrin are also present. 33.2% of the cells are in S/G_2/M phase, indicating that the proliferation of the cells in the confluent culture is slowing, either as a result of cells becoming too differentiated to cycle or as a result of having a longer cell cycle time. Very few cells are β1 integrin$^-$ in the culture (6.9%), but the number of K1/K10$^-$ cells is substantial (18.5%) indicating that a β1 integrin$^+$ K1/K10$^-$ cell population (stem cell phenotype) remained present throughout the primary culture period. These cells are likely to represent the β1 integrin^{bright} cells responsible for establishing colonies in subcultures (55).

Keratinocyte colony formation in vitro can be regulated by the soluble products of T lymphocytes; growth regulatory effect correlates with the lymphokine profile of cloned psoriatic lesional T cell subsets.

Early psoriatic lesions were cultured in low concentration of IL-2 to propagate and clone in vivo- activated T cells expressing high affinity IL-2 receptors. After cloning T cells were plated on anti-CD3 and fibronectin coated tissue culture dishes in serum free media. In initial experiments, activated T cell supernatants from 12 different lesional T cell clones were screened by measuring their effect on freshly isolated epidermal cell colony formation in vitro (Figure 10.).

Figure 10. Keratinocyte clonal growth is induced by soluble factors produced by lesional T cells.



Psoriatic non-lesional epidermal cells were cultured in the presence of activated lesional T cell produced supernatant (a) or in medium alone (b).

At two weeks of culture, when the colony formation is clear but still sufficiently subconfluent that individual colonies can be counted and measured, the cultures were fixed, stained and assessed with regard to average colony size and density. Relative to cultures incubated with control media (a serum free lymphocyte medium: AIM V) supernatants from five T cell clones showed a clear growth-stimulatory effect both on psoriatic uninvolved and normal keratinocytes. This occurred in repeated experiments using different donor keratinocytes (Table II, upper group). In addition, three clones exhibited an inhibitory effect on keratinocyte clonal growth (Table II, lower group) and four clones showed no effect and either a positive response or no response on normal and psoriatic uninvolved keratinocyte growth (Table II, middle group).

Table II. Cloned psoriatic T cell effects on colony formation of freshly obtained psoriatic uninvolved and normal epidermal cells.

Cloned T cells‡	Psoriatic uninvolved	Normal
4F1	+ (1/1)*	+ (3/3)
4F4	+ (7/7)	+ (3/3)
1C11	+ (3/3)	+ (1/2)
1D2	+ (3/3)	+ (1/1)
4E2	+ (3/3)	+ (1/1)
3D11	± (3/6)	0 (0/2)
1H8	± (6/8)	± (3/4)
1D3	± (2/4)	± (3/4)
1D11	± (3/6)	NT
1C4	− (0/1)	NT
2G10	− (0/1)	NT
1B3	− (0/2)	− (0/3)

* No. of cultures showing a positive response/total No. of individuals tested. ‡ T cells derived by limiting dilution from an expanding psoriatic lesion. Supernatants were generated by activation of clones on immobilized anti-CD3 and fibronectin. +, consistently increased clonal growth by the T cell supernatant over the control media (AIM V) from succesive subjects; ±, supernatants induced both a positive response and no response in successive cultures; 0, no effect of the supernatant on clonal growth; −, inhibition of keratinocyte clonal growth; NT, not tested.

Thus, heterogeneity of T cell populations can be demonstrated at the clonal level in lesional psoriasis (35,49), and the nature of the heterogeneity includes the capacity to be either growth-promoting or growth-inhibitory for keratinocytes. Interestingly, 75% of clones exhibited a positive growth promoting effect on at least 50% of psoriatic donors. (Table II), suggesting that growth promoting clones are common among T cells undergoing intralesional activation , although preferential selection in vitro under low IL-2 concentrations for a reason other than high affinity IL-2 receptor expression shortly after activation is conceivably possible.

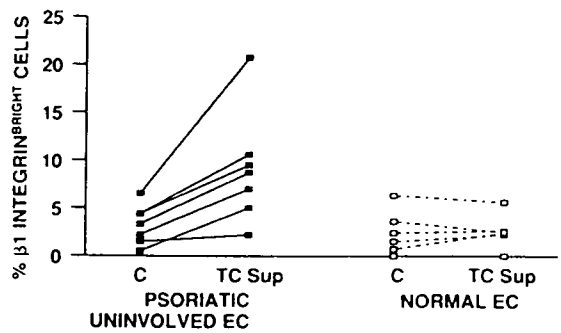
Quantitation of β1 integrin$^+$ K1/K10$^-$ stem cell containing epidermal cell subset transition to cell cycle reveals that growth of psoriatic uninvolved, but not normal stem cells can be stimulated by psoriatic lesional T cell supernatants.

Because in the clonal growth assay, cells were cultured for 2 weeks and restimulated with T cell supernatants every third day, the observed growth stimulation of T cell lymphokines can be attributed to either growth stimulation of the β1 integrin$^+$ K1/K10$^-$ subset or growth stimulation of the β1 integrin$^+$ K1/K10$^+$ subset which is committed to terminal differentiation, but which undergoes transient amplification. Because the primary hyperproliferative subset in lesional psoriatic epidermis is the stem cell population, we tested whether the colony growth-

stimulatory T cell supernatant (4F4) could stimulate the growth of β1 integrin^{bright} K1/K10^{-} cells early (first 72 hours of culture) during primary ex vivo culture.

Psoriatic uninvolved epidermal cells were obtained from seven patients and, in all but one case, lesional T cell (clone 4F4) supernatant induced substantially higher numbers of β1 integrin^{bright} cells to appear after 72 hours of primary culture (TC Sup) relative to cultures of uninvolved epidermal cells plated in control media (C). The percent β1 integrin^{bright} cells rose from 3.2±0.7 in control media cultures to 9.1±2.2 in lesional T cell supernatant-stimulated cultures (Fig. 11, p=0.009).

Figure 11. β1 integrin bright cells in psoriatic uninvolved and normal primary cultures.



T cell clone 4F1, another clone which was stimulatory in the clonal growth assay, also induced stem cell growth in primary culture (10.3±0.6% β1 integrin^{bright} cells in 4F1 cultures and 4.7±0.2% β1 integrin^{bright} cells in control cultures, n=2).

Although conditions of T cell activation in the skin can result in epidermal hyperplasia in non-psoriatic individuals (i.e. chronic dermatitis or tuberculoid leprosy (64)), the hyperplasia in psoriatic subjects is more intense and sustained, suggesting a hyperresponsive state. We therefore compared the hyperplastic response of psoriatic uninvolved keratinocyte stem cells to epidermal stem cells from normals. In contrast to the increased stem cell numbers appearing in cultures of psoriatic uninvolved cells, we could not detect lesional T cell induction of increased numbers of β1 integrin^{bright} cell population in epidermal cell cultures from normal individuals (Fig. 11, p=0.76). Increased numbers of β1 integrin^{bright} cells in cultures provided T cell lymphokines is likely due to proliferation of this subset, but could also represent preferential non-proliferative survival in the treated culture. We therefore asked if increased cell division among these cells was responsible for the preferential increase in numbers of β1 integrin^{bright} cells. Quantitation of the DNA content of β1 integrin^{bright} cells revealed that the proportion of

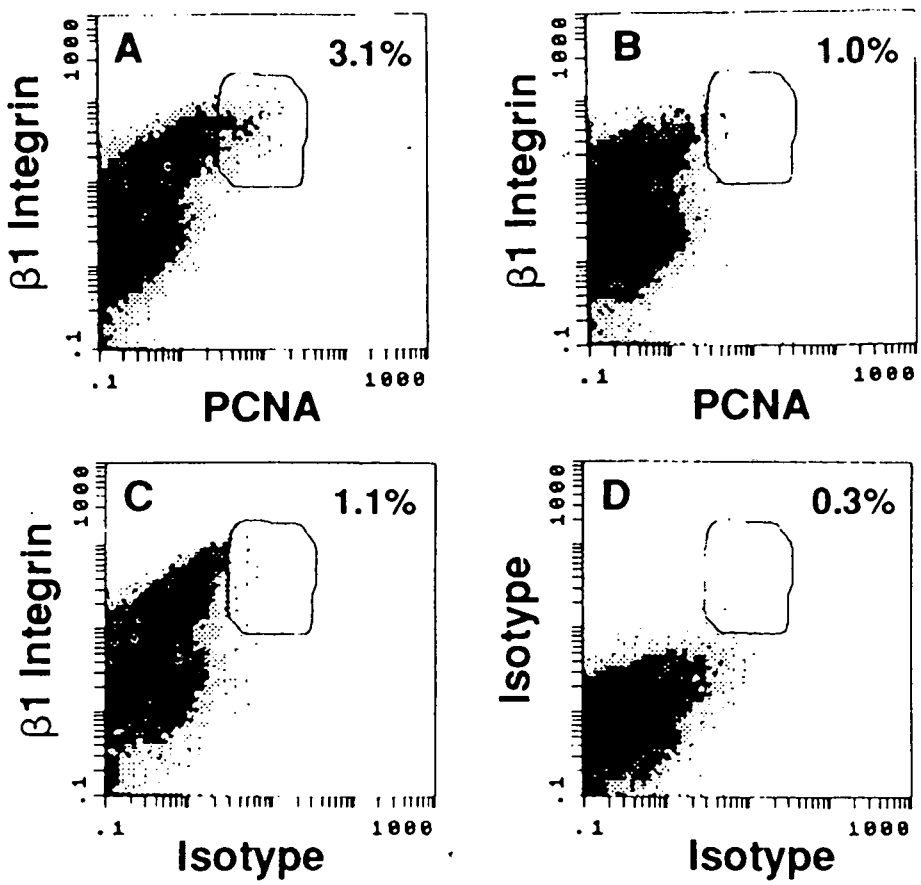
β1 integrin^{bright} cells that were hyperdiploid, that is, in S/G_2/M phase of the cell cycle, was indeed significantly enhanced by the addition of activated T cell supernatant (p=0.01). The percentage of β1 integrin^{bright} cells in S/G_2/M rose from 1.08±0.2% in control media cultures to 2.3±0.5% in lesional T cell-stimulated cultures. Thus, T cell lymphokines increase cycling among the culture-initiating keratinocytes of stem cell phenotype (55). Furthermore, in keeping with the cell number data, and in contrast to psoriasis, epidermal cell cultures from normals did not exhibit an increase in the S/G_2M phase β1 integrin^{bright} population in response to T cell stimulation (p=0.66).

These data provided the first evidence that elements of the immune system, in particular, lesional psoriatic T cells, can directly upregulate the growth of keratinocyte stem cells. Of additional importance is that psoriatic keratinocyte stem cells appear to be in an altered state relative to normals, as they are hyperresponsive to the growth promoting effect of T cell lymphokines.

T cell induction of increased cells with bright β1 integrin expression is preceded by the appearance of β1 integrin^{+} cells expressing the early G_1 marker, PCNA.

Increased β1 integrin^{bright} proliferating cells could be due to enhanced β1 integrin expression without increased cycling, to accelerated cycling of the small percentage of stem cells already in cycle in vivo (4.5%) (63) or to a recruitment of previously quiescent (G_0) stem cells into cell cycle. Because DNA content can not detect G_1 entry (the cells are still diploid), we utilized another marker of cell cycle entry into G_1. The proliferating cell nuclear antigen (PCNA) is an auxiliary DNA polymerase molecule which appears early in G_1. It becomes somewhat more abundant during S and then declines during G_2/M (62,65). Psoriatic uninvolved cultured epidermal cells were costained with anti-β1 integrin and anti-PCNA antibodies 48 and 72 hours after initiation of the cultures (Fig. 12. and 13.).

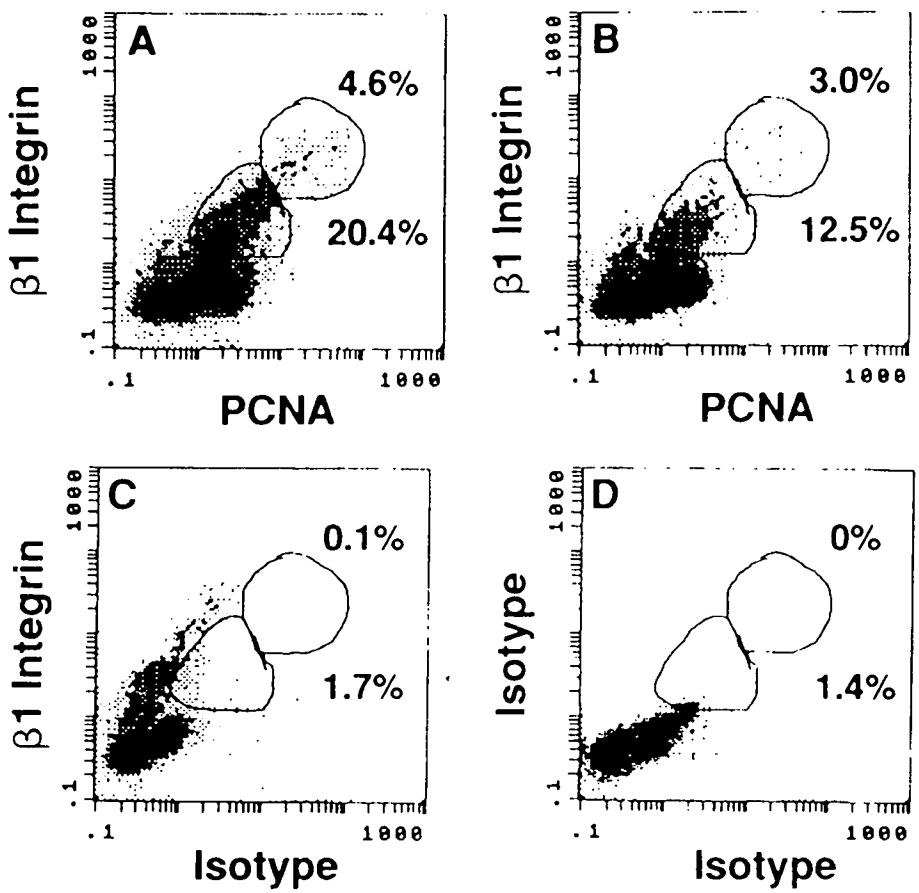
Figure 12. Cell cycle entry (PCNA expression) precedes acquisition of β1 integrin^{bright} phenotype, growth stimulatory T cells accelerate the appearance of PCNA^{+} β1 integrin^{bright} cells in non-lesional psoriatic epidermal cell cultures.



Cells were grown in media either supplemented with a growth-stimulatory T cell supernatant (4F4) or AIM V control media. After 48 hours of culture, none of the cultures contained β1 integrin^{bright} cells, as evidenced by the single, homogenous population of β1 integrin^{+} cells (circles, Fig. 12D vs. A-C). Despite the lack of β1 integrin^{bright} cells, we were able to detect an increase in PCNA^{+} cells in the T cell-treated culture at 48 hours. In cultures containing the T cell supernatant, 2.0% of total epidermal cells were PCNA^{+} (Fig. 12A, encircled cells minus Fig. 12C encircled cells) as opposed to 0% PCNA^{+} β1 integrin^{+} keratinocytes in culture containing control media (Fig. 12B). The lack of PCNA^{+} cells over background flourescence in control media-containing cultures indicates that initial keratinocyte colony formation in vitro depends entirely on quiescent, PCNA^{-} cell recruitment into cell cycle. Even though bright β1 integrin expression was not yet detectable, all PCNA^{+} cells in the T cell-treated culture were within the

β1 integrin$^+$ population. The PCNA$^+$ cells tended to arise within the cells expressing somewhat higher amounts of β1 integrin, however (Fig. 12A), consistent with the concept that these cells arise from the in vivo stem cell population, which exhibits slightly higher β1 integrin than neighboring transient amplifying cells (66,67).

Figure 13. Accelerated recruitment and proliferation of two subsets of β1 integrin ^{bright} PCNA$^+$ cells by T cell lymphokines in psoriatic non-lesional epidermal cell cultures.



After 72 hours, two populations of β1 integrin$^+$ cells could be detected, one bright and the other dim in intensity (Fig. 13A and B upper and lower encircled areas; vs. Fig. 13D). Comparison of cells stained with anti-β1 integrin plus isotype (Fig. 13C) with 72 hours cells stained with both anti-β1 integrin and anti- PCNA (Fig. 6A,B) revealed that virtually all β1 integrin$^+$ cells were PCNA$^+$, and the high PCNA expressing cells were all now β1 integrin^{bright} (Fig. 13A and B upper circles).

Increased cell numbers of the PCNA$^+$ β1 integrin$^+$ and PCNA^{bright} β1 integrin^{bright} populations were detectable in T cell supernatant-treated (Fig. 13A) relative to control media-containing cultures (Fig. 13B). The total PCNA$^+$ population comprised 23.2% vs. 13.7% of 72 hour cultured epidermal cells, respectively, and the β1 integrin^{bright} and PCNA^{bright} subset comprised 4.5% of the T cell stimulated culture as compared to 2.9% of the control media-treated culture (Fig. 13A vs. B minus C).

The reason that β1 integrin^{bright} cells in the 72 hour harvest have a β1 fluorescence intensity in the third logarithmic decade, and the single population of β1 integrin^{dim} cells in the 48 hour culture also have β1 fluorescence intensity in the 3rd decade, is that the data were acquired on two sequential days, and there was nonidentical voltage amplification during acquisition. We have measured PCNA$^+$ cells parallel with β1 integrin^{bright} cells in two other experiments in 72-hour cultures and detected similar differences in the numbers of PCNA$^+$ and β1 integrin^{bright} cells between T cell supernatants treated cultures and control cultures (data not shown).

These data suggests that the growth promoting effect of the lesional T cell supernatant is due to accelerated recruitment of β1 integrin$^+$ stem cells into G_1 and that cell cycle entry (PCNA expression) precedes the marked upregulation of β1 integrin which occurs among the clonogenic stem cells as they establish the culture. The very bright PCNA expression among the β1 integrin^{bright} cells likely indicates a build-up of PCNA protein during repeated, relatively rapid rounds of cell cycle (56), whereas the β1$^+$ PCNA$^+$ population's expansion may represent further stem cell recruitment.

Heterogeneity of cloned lesional psoriatic T cells in regard to keratinocyte clonal growth correlates with CD4 and CD8 expression of the T cells as well as differential lymphokine production.

Our finding that certain cloned lesional psoriatic T cells induced enhanced keratinocyte clonal growth whereas others inhibited growth provided an opportunity to examine whether distinct "growth stimulatory" lymphokine profiles could be identified. To answer this question we have measured, by ELISA, the IL-3, IL-4, GM-CSF, TNF-α and gamma-interferon levels in the activated lesional psoriatic T cell supernatants. High GM-CSF (10-11 ng/ml) and γ-IFN (38-51 ng/ml) levels were characteristic of the growth stimulatory (upper group, Table III), and some of the growth inhibitory (lower group, Table III) T cell supernatants.

Table III. Lymphokine profile of lesional psoriatic T cell subsets: correlation with keratinocyte growth.

	T cell phenotype[‡]				Lymphokine content in pg/ml[¶]				
Cloned T cells*	CD4	CD8	CD3	KC clonal growth[§]	GM-CSF	IL-3	γ-IFN	IL-4	TNF-α
1C11	+	−	+	+	10,040	<100	45,420	<100	1,438
4F1	+	−	+	+	10,360	2,900	39,700	15,364	77
4F4	+	−	+	+	10,920	2,640	38,780	8,181	1,551
3D11	+	−	+	±	10,540	<100	32,380	252	36
1H8	+	−	+	±	10,300	<100	36,040	<100	5,974
1D3	+	−	+	±	10,260	9,220	38,560	9,352	2,714
1C4	NT	NT	NT	−	9,100	33,940	51,140	6,002	NT
1B3	−	+	+ (α,β)	−	<100	34,060	39,940	6,648	2,629
2G10	−	+	+ (α,β)	−	9,340	32,780	NT	8,000	6,853

* T cells expanded in low dose IL-2 from untreated expanding psoriatic papule were cloned by limiting dilution and screened for keratinocyte clonal growth regulatory activity. ‡ T cells were phenotyped by dual color flow cytometry. § Keratinocyte clonal growth in KBM+1% FBS/1% Aim V media was assessed after addition of 48 h supernatants derived from anti-CD3+ fibronectin-stimulated T-cells. Relative to Aim V media control, growth promotion was scored as + if repeated experiments demonstrated an increase in colony density and size. Growth inhibition was scored as − if reduced colony density and size was observed. ¶ Lymphokine levels in the 48-h stimulated T cell supernatants were determined by ELISA. NT, not tested.

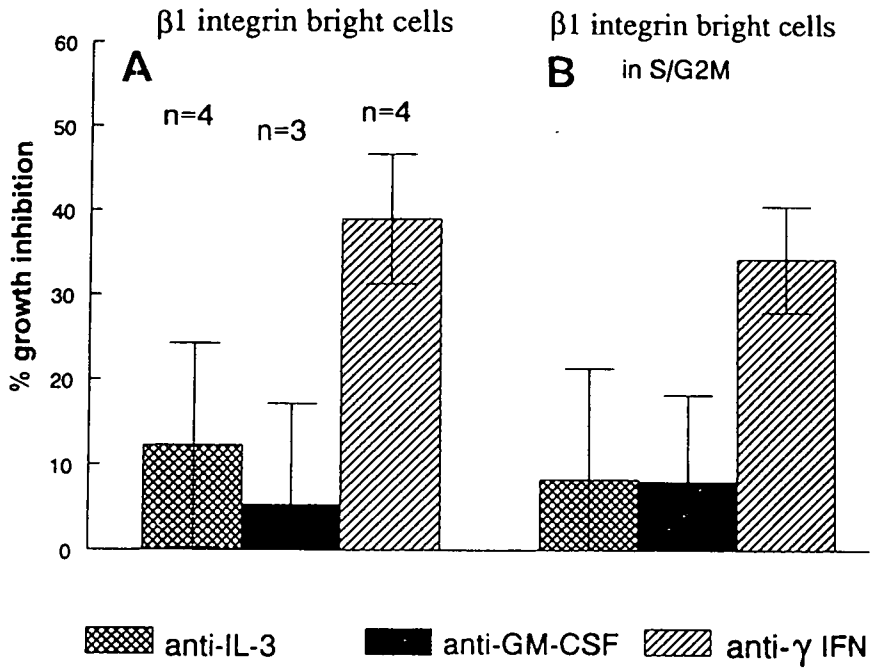
Growth inhibition correlated with high IL-3 (32-34 ng/ml) and moderate TNF-α (2.5-6.8 ng/ml) levels, whereas growth stimulatory supernatants contained <3 ng/ml IL-3 and <1.5 ng/ml TNF-α. No obvious connection was found between cell growth and IL-4 levels; with most of the T cells producing substantial levels of both γ-IFN and IL-4, only three exhibiting a type I lymphokine profile (high γ-IFN, absent or very low IL-4), and none exhibiting a type II profile (low γ-IFN, high IL-4) (Table III). Thus, enhanced keratinocyte growth can not be obviously associated with high levels of any of the tested lymphokines. However, phenotypic analysis of the T cells revealed that growth stimulatory lymphokine production was exclusively mediated by $CD4^+$ T cells, whereas T cells producing the inhibitory supernatants which were stained were composed of $CD4^-CD3^+$ T cells which contained $CD8^+$ cells and $CD8^-$ cells, but which all expressed α,β TCR and not γ,δ TCR (Table III).

Lesional T cell stimulation of psoriatic keratinocyte stem cell hyperproliferation is multifactorial: γ-IFN appears to be a critical component for growth stimulation, but requires the presence of other growth factors.

To determine which lymphokines present in the activated lesional T cell supernatants are critical for the observed enhancement of keratinocyte stem cell growth by the activated lesional T cell supernatants, we used neutralizing antibodies against IL-3, GM-CSF and γ-IFN. Each antibody, or its isotype control, was added to the T cell supernatant to block the effect of these

individual lymphokines in the 72-hour short term psoriatic uninvolved epidermal cell culture assay (Fig. 14).

Figure 14. Identification of specific lymphokines responsible for psoriatic uninvolved keratinocyte stem cell growth activation.



Neutralizing antibodies and appropriate isotype controls were added separately to psoriatic uninvolved epidermal cell cultures before addition of the growth promoting T cell supernatant (4F4). After 72 hours in culture, cells were harvested, stained and analysed by flow cytometry. Epidermal stem cell growth was measured either by the number of β1 integrin^{bright} cells (A) or the number of β1 integrin^{bright} cells in S/G_2/M phase (B). Control cultures containing species and isotype matched immunoglobulins were considered to represent 100% growth.

The neutralizing antibody concentrations were calculated to achieve a 100 fold excess of neutralizing activity based upon the known amounts of lymphokines in the supernatants and the neutralizing activity of each antibody. In each of 4 experiments anti-γ-IFN neutralization resulted in inhibition of keratinocyte growth, with a 39±7.7% inhibition of β1 integrin^{bright} cell numbers and a 34±6.3% inhibition in the numbers of S/G_2/M phase β1 integrin^{bright} cells, relative to isotype control cultures. No consistent growth inhibition was observed upon addition of either anti-GM-CSF or anti-IL-3 (Fig. 14). Addition of the neutralizing anti-GM-CSF and anti-IL-3 to the epidermal cell cultures without the growth promoting supernatant 4F4 also did not influence keratinocyte stem cell growth, suggesting that autocrine production of these lymphokines were not responsible for growth regulation. Addition of neutralizing antibody against TNF-α also did not affect keratinocyte stem cell growth.

To test the possibility that γ-IFN alone could be responsible for the growth stimulatory effect on psoriatic keratinocyte stem cells, we cultured psoriatic uninvolved epidermal cells in

KBM+1%FBS supplemented with human recombinant γ-IFN at 10 and 100 U/ml. (These concentrations were chosen based on the spesific activity/mg protein of the this recombinant γ-IFN. The ELISA-determined γ-IFN concentrations in the T cell supernatants fall within this 10-100 U/ml range.) We found that, in each instance, cell growth in the cultures was suppressed (39% and 44±19% at 10 and 100 U/ml respetively). Taken together with the above data that γ-IFN is critical for growth promotion, the growth inhibitory effect of the isolated γ-IFN indicates that γ-IFN requires the presence of additional factors (i.e. GM-CSF, IL-3 or others) simultaneously produced by activated lesional T cells in order to stimulate psoriatic keratinocyte stem cell growth.

Discussion

An accurate comparison of keratinocyte proliferation between normal and pathological proliferative conditions requires the development of methods to simultaneously define and quantitate proliferative compartment subsets within the epidermis. Several specific markers are known to bind to the basal cells of normal epidermis, but none of them have proven to be exclusive markers of the proliferative populations (10,60,68-70). It is widely accepted now that cell markers specifically expressed on basal layer cells do not define the total proliferative compartment in normal epidermis; mitotic cells as well as thymidine incorporation occur among suprabasal cells (71,72).

The β1 integrin chain is expressed predominantly on basal cells, but also on some suprabasal cells upon staining of vertical sections of epidermis. In our work, we showed by flow cytometric analysis that β1 integrin identifies all of the proliferating cells in the epidermis. Cytoplasmic expression of the differentiation keratin pair, K1/K10, was used to more precisely relate in vitro flow cytometric results to in vivo tissue location and to separate more highly differentiated cells (β1 integrin$^-$K1/K10$^+$) from the proliferative compartment cells. The appearance of K1/K10 keratin in keratinocytes is considered to be one of the first signs of differentiation (22,24). Surprisingly, K1/K10 which by light microscopy stains the epidermis from the suprabasal layer up to the stratum granulosum, was expressed on a large number of cycling ($S/G_2/M$) β1 integrin$^+$ cells in normal epidermis. By immunoelectron microscopy it has been shown that K1/K10$^+$ proliferating cells are present in the basal layer but that they are slightly unattached from the basal membrane (60). Thus the proliferative compartment of the human in vivo epidermis contains two subpopulations characterized by β1 integrin and K1/K10 expression.

Undifferentiated β1 integrin$^+$ K1/K10$^-$ cells in the basal layer express K1/K10 upon commitment to differentiation and disattachment from the basament membrane. Initially, they retain β1 integrin, but begin migrating up into suprabasal layers. Cells in this latter state of differentiation proliferate briskly, but proliferative capacity ceases in association with loss of β1 integrin expression. At present there are no data available of the precisious three dimensional organization of the human epidermis. Comparison of data aquired by flow cytometry with the staining of tissue sections can only be approximative.

Because β1 integrin staining can separate the heterogeneous proliferative compartment from the non-proliferative compartment and K1/K10 expression further subdivides the proliferative subpopulations, we were able to quantify the proliferative state of each β1 integrin$^+$ subpopulation separately. DNA analysis revealed that the different subpopulations of β1 integrin$^+$ keratinocytes in normal human epidermis differ greatly in the number of proliferating cells. The β1 integrin$^+$ cells that have initiated their differentiation program, as indicated by the expression of K1/K10 keratins, are highly proliferative in comparison to the non-differentiating cells (β1 integrin$^+$ K1/K10$^-$). The undifferentiated basal β1 integrin$^+$ K1/K10$^-$ cell subset has few cells in cycle (4.5% PCNA$^+$ cells in one particular sample), the vast majority of the cells are in quiescent state (PCNA$^-$ cells). Determining the proliferative state of the keratinocytes by DNA content using DNA dyes circumvents the problem of differential thymidine metabolism in normal and diseased keratinocytes. It has been shown that thymidine salvage and catabolism can be substantially different in proliferating versus differentiating cells in vitro (7). In addition, psoriatic epidermis, relative to normal epidermis, demonstrates markedly increased thymidine phosphorylase activity (8) which could result in decreased incorporation of exogenous thymidine in psoriatic tissue.

It is now considered that the major difference between stem cells and differentiated cells in self renewing tissues lies in their "unlimited" versus "limited" proliferative potential. In other words, differentiated cells can be highly reproductive through a limited number of cell cycles, whereas stem cells have the potential for unlimited numbers of cell cycles, but are not necessarily highly reproductive (73). The existence of such a stem cell population in the human epidermis was strongly supported by the observations of Lavker and Sun (26). In human palmar epidermis they observed two distinct basal cell types: one type, resembling stem cells, was nonserrated, slowly cycling, and primitive in cytoplasmic structure, whereas the other type, considered to be transiently amplifying (TA) cells, exhibited more complex cytoplasmic structure and was highly

proliferative. Our flow cytometric analysis of the two different proliferative subpopulations in normal epidermis reveals similar subset features; the β1 integrin$^+$ K1/K10$^-$ basal cells are slow cycling, small in cell size (low LFLS), and primitive in cytoplasmic structure (low complexity or L90LS). By contrast the β1 integrin$^+$ K1/K10$^+$ cells are highly proliferative, larger in size, and exhibit a more complex cytoplasm (higher L90LS). These data suggest that the β1 integrin$^+$ keratinocyte subpopulations indeed contain stem and transiently amplifying subpopulations comprising the epidermal proliferative compartment. Altough we can not further specify the β1 integrin$^+$ K1/K10$^-$ stem cell containing population, it is clear that the putative stem cells must be present within this population.

In tissue sections of lesional psoriatic epidermis the first 2-3 rows of proliferating cells are β1 integrin$^+$ while K1/K10 occurs above the proliferative layers. Flow analysis of the anti-β1 integrin and anti-K1/K10 double-stained samples revealed the presence of similar proliferating keratinocyte subpopulations (β1 integrin$^+$ K1/K10$^-$ and β1 integrin$^+$ K1/K10$^+$) and non-proliferative compartment cells (β1 integrin$^-$K1/K10$^+$) in lesional psoriatic epidermis as in normal epidermis.

Characterization of the germinative compartment within the proliferative compartments permitted identification of the major proliferative difference between normal and lesional psoriatic tissue. Epidermal expansion appears due to the considerably increased percentage of cells in S/G_2/M phase of the cell cycle among the β1 integrin$^+$ K1/K10$^-$ basal cells. By comparison, the β1 integrin$^+$K1/K10$^+$ more differentiated cells exhibited almost the same high frequency of cycling cells in psoriatic as in normal epidermal cells. The sriking difference appearent with PCNA staining among K1/K10$^-$ cells in the psoriatic tissue indicates that all the cells in this compartment of the psoriatic tissue left quiescent state.

Techniques involving simultaneous analysis of multiple parameters of epidermal cells have allowed us to precisely localize the epidermal proliferative defect in psoriasis to be within the normally slow cycling basal keratinocyte cell population which includes the stem cells. To determine whether the behavior of human keratinocyte stem cells could be studied ex vivo in a state as close as possible to their in vivo state (prior to multiple in vitro passage modulation), we analyzed primary ex vivo epidermal cell cultures and tracked the numbers and cell cycle status of cells of stem cell phenotype by multiparameter flow cytometry. In a time course study, we demonstrated that in this culture system (KBM+1%FBS media), keratinocytes responsible for initial colony formation are of the β1 integrin$^+$ K1/K10$^-$ PCNA$^-$ stem cell phenotype. Thus, at

the time when small colonies, (5-6 cells of monolayered keratinocytes) were seen in the culture, all the cells were K1/K10$^-$. Parallel with accelerated proliferation, the β1 integrin expression of these clonogenic cells became very bright in vitro. Later, as the colonies enlarged, differentiation occurred (K1/K10 expression appeared). In confluent cultures the majority, but not all, of the cells were K1/K10$^+$. β1 integrin expression remained, however the intensity diminished somewhat as the proliferation of the cells slowed down.

The β1 integrin^{bright} K1/K10$^-$ clonogenic cell population was detectable as early as 72 hours after initiation of epidermal cell cultures. It has been shown recently that in multiply passaged neonatal foreskin keratinocyte cultures, cells with bright β1 integrin expression were the most efficient cells for colony formation (55). Because such cells have survived multiple proliferative activations (2-12 passages), yet could still regain proliferative capacity without showing signs of differentiation, they most likely represent the progeny of the in vivo stem cell population (55).

In vivo, under steady state conditions, epidermal stem cells are believed to divide infrequently and to have a long cell cycle time (3). However, when placed into culture, label retaining cells thought to be epidermal stem cells are known to be actively cycling and clonogenic (56,74). Congruent with these prior findings, the majority of epidermal stem cells are in a quiescent cell cycle state in in vivo normal epidermis (diploid DNA content,PCNA$^-$) (63) and some are recruited spontaneously from quiescent state into cell cycle in the culture between 48 hours and 72 hours (PCNA$^+$). Thus, clonal growth in the early primary culture depends on the rate of stem cells leaving cell quiescence (G_0-G_1 transition).

After the initial death of existing differentiated cells (K1/K10$^+$), the stem cell-containing cell population (β1 integrin$^+$ K1/K10$^-$) expanded through proliferation, but a substantial fraction simultaneously and asymmetrically generated K1/K10$^+$ cells. The β1 integrin$^+$ K1/K10$^+$ phenotype is characteristic of the population in in vivo epidermis which has committed to differentiation but undergoes transient amplification before terminal differentiation. Thus, in confluent primary culture, keratinocytes are composed of two basic proliferative populations: β1 integrin$^+$ K1/K10$^-$ (stem cells) and β1 integrin$^+$ K1/K10$^+$ (transient amplifying) cells.

Using the above monoclonal antibody markers in combination with cell cycle analysis, we have found that the primary hyperproliferative cell subset in lesional psoriatic epidermis was the normally slow-cycling β1 integrin$^+$ K1/K10$^-$ subset. The ability to model events leading to stem cell hyperplasia in psoriasis is critically important in understanding stem cell homeostasis and is

relevant not only to psoriasis but to epithelial cancer and immune regulation of tissue proliferation as well. By tracking the behavior of the β1 integrin$^+$ K1/K10$^-$ stem cells after exposure to T cell products, we provide definitive evidence for a direct link, via secreted lymphokines, between lesional T cells and the relevant target cell in psoriasis, the epidermal proliferative compartment keratinocytic stem cell (β1 integrin$^+$ K1/K10$^-$). That the growth activation was actually occurring in the keratinocyte stem cell population was confirmed by vimentin staining which identified Langerhans cells and melanocytes in the cultures (data not shown).

We demonstrated, using the supernatants of anti-CD3 plus fibronectin-activated T cells cloned from an expanding psoriatic lesion, differential growth regulation of clonal keratinocyte growth in vitro. Both normal and psoriatic uninvolved keratinocyte clonal-growth stimulation could be linked to a group of CD4$^+$ T cells whose supernatants contained relatively high levels of GM-CSF and γ-IFN, and moderate to low levels of IL-3. Growth stimulatory T cell clones could not be exclusively assigned to a Type I lymphokine profile (48), because at least 2 clones produced substantial IL-4 as well as γ-IFN (Table II.). Interestingly, we observed growth inhibition by cloned T cells containing CD8$^+$CD3$^+$CD4$^-$TCR α/β$^+$ cells which demonstrated much higher IL-3 production. We do not yet know whether high IL-3 can inhibit growth directly or whether it can enhance differentiation with resultant reduced clonal growth. The reason for the normal keratinocyte response to growth stimulatory T cell in these long term clonal growth assays, but not the short term stem cell assay is not entirely clear, but may relate to the more complex composition of proliferative cells in the more mature colonies of the long term assay. Measurement of growth in the latter assay includes the growth of both stem and transiently amplifying keratinocytes, as well as melanocytes. Thus, because it is not possible to identify the actual target cell of action, it is difficult to interpret our long term clonal growth assay and other proliferative assays involving cultured keratinocytes treated with T cell supernatants (50) or recombinant lymphokines (75-77).

Although both IL-3 and GM-CSF seem to be growth promoting factors for keratinocytes in vivo (78-80) as well as in vitro on passaged cells (75) our neutralization experiments in the short term primary culture model suggest that γ-IFN, and not GM-CSF or IL-3, is a potent stimulatory cofactor in psoriatic uninvolved keratinocyte stem cell proliferation. γ-IFN has been reported to inhibit normal, and to a lesser degree, psoriatic, epidermal cell growth in vitro in primary cultures (76,77), despite its ability to induce TGF-α (81). We have observed a

similar result in our stem cell growth assay. However, in vivo-administered γ-IFN to psoriatic uninvolved skin resulted in hyperproliferation of the epidermis (82). It is possible that in vivo growth factors (such as GM-CSF,IL-3 or other factors) are sufficiently present in the epidermis to provide the necessary costimulatory signals for γ-IFN to become growth stimulatory. Similar to our observation on psoriatic uninvolved keratinocyte stem cells, γ-IFN also exhibits a growth promoting effect on haemopoietic progenitor cells in the presence of GM-CSF and IL-3 (83).

The observed hyperresponsiveness of psoriatic uninvolved β1 integrin$^+$ K1/K10$^-$ keratinocytes to the growth promoting effect of T cell lymphokines relative to normal keratinocytes is of particular interest. The hyperresponsive status of uninvolved psoriatic stem cells can either be the result of genetic susceptibility or the result of a chronic low grade stimulatory priming of the keratinocytes in vivo. These in vitro data correspond to the clinical observations that, although epidermal hyperplasia occurs in a variety of skin conditions containing activated T cells (84-86), the most significant hyperproliferation occurs in psoriasis. The finding is also consistent with the Koebner-phenomenon, in which inflammation or wounding of psoriatic uninvolved skin can result in development of a psoriatic lesion in the presence of an epidermal infiltrate dominated by CD4$^+$ T cells (87), whereas similar perturbation of normal skin does not result in such changes.

These data characterize a sensitive in vitro model of T cell and keratinocyte stem cell growth-regulatory interactions which appears highly relevant to the human skin disease, psoriasis. According to this model, lesional CD4$^+$ T cells, through the release of γ-IFN in the context of other cytokines, act on resting β1 integrin$^+$ K1/K10$^-$ keratinocyte stem cells to enter cell cycle (PCNA$^+$) and subsequently progress through S/G_2/M, resulting in expansion of the proliferative compartment population. This model may be useful in understanding regulation of epidermopoiesis and in determining specific markers of genetic susceptibility to psoriasis.

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