Flow Cytometric Identification of Proliferative Subpopulations within Normal Human Epidermis and the Localization of the Primary Hyperproliferative Population in Psoriasis

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Summary

In this study we define the proliferative compartments of in vivo human epidermis, using specific antibodies related to cell differentiation ($\beta 1$ and $\beta 4$ integrins and K1/K10 differentiation keratins) and cell cycle (proliferating cell nuclear antigen [PCNA]) in combination with flow cytometric quantitation of the DNA content and optical characteristics of the cells. The $\beta 1$ integrin (CD29) marked both of the potentially proliferative subsets in normal epidermis. One subset of normal epidermis is CD29$^+$K1/K10$^-$, which was predominantly basal, and found to be comprised of slow cycling, small cells with primitive cytoplasmic organization. The vast majority (95.5%) of these cells were in a quiescent state (G0/early G1) as indicated by their lack of the cyclin, PCNA. The other proliferative subset of normal epidermis was CD29$^+$K1/K10$^+$, which was suprabasal and occasional basal, highly proliferative, larger in size, and which exhibited a more complex cytoplasmic structure. Because early differentiation (K1/K10 expression) has begun in the CD29$^+$K1/K10$^+$ subset, it is highly likely that they represent the proliferative population which is capable of transiently amplifying itself before terminal differentiation. Within lesional psoriatic epidermis, similar proliferative cell populations were present as in normal epidermis, and the hyperproliferative defect was localized to the $\beta 1$ and $\beta 4$ integrin$^+$, K1/K10$^-$ populations, which in normal epidermis is basally located and quiescent with regard to cell cycle. In psoriatic epidermis, a six- to sevenfold increase in the number of cells in the S/G2+M phase of cell cycle was found among CD29$^+$K1/K10$^-$ cells (p <0.05). Furthermore, all lesional K1/K10$^-$ cells showed high PCNA positivity, indicating that all these cells had been recently induced into cell cycle. By contrast, the proportion of cycling cells among lesional psoriatic CD29$^+$K1/K10$^+$ keratinocytes was similar to normals. Anti-HLA-DR, CD45, and vimentin antibodies were used to concomitantly track the proliferative states of Langerhans cell, melanocyte, and infiltrating leukocyte populations. In normal epidermis, the cycling fractions (cells in S/G2/M phase) of these cells were similar to the CD29$^+$K1/K10$^-$ keratinocytes, whereas in lesional epidermis their cycling pools were increased relative to normal, but not so much as the proliferative fractions of psoriatic CD29$^+$K1/K10$^-$ keratinocytes. These data demonstrate the use of simultaneous analysis of integrin expression, differentiation keratins, cyclin, cell cycle status, and optical characteristics of freshly isolated human epidermal cells. Such analysis allowed the physical identification and quantification of cycling populations in normal human skin, and has enabled the precise localization of the primary epidermal proliferative defect in psoriasis.

T he epidermis traditionally has been divided into three functionally different compartments: germinative, differentiated, and cornified. Increasing evidence suggests that the germinative compartment is heterogeneous (1, 2). In human epidermis, Lavker and Sun (3, 4) described two different proliferating cell types: one consisted of slow cycling, "nonserrated" cells with primitive cytoplasm, termed stem cells, and the other consisted of highly proliferative, "serrated" cells with more complex cytoplasmic organization, termed transiently amplifying cells. In vitro observations of human keratinocyte cultures have provided additional evidence of the existence of at least two functionally and morphologically different proliferating keratinocytes. Small cell size has been observed to correlate with the colony-forming ability of ker-

atinocytes in vitro (5). Analysis of exponentially growing cultures of human epidermis revealed two proliferating populations according to cellular RNA and DNA content (6, 7).

Dynamic assessment and physical identification of in vivo proliferative compartment subsets within human epidermis have been difficult because of the limitations of injecting radiolabeled nucleotides into subjects and the lack of a precise definition of the epidermal proliferative cell compartments. To identify pathological changes of epidermal proliferation in hyperproliferative skin conditions such as psoriasis, it is first critical to directly quantitate proliferation in the morphologically and functionally different cell compartments of in vivo epidermis (8). 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 (9). Although there is agreement that epidermal proliferation is elevated in psoriasis (10) 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 (11, 12).

We used four to 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 (13). DNA dyes circumvent the problem of cell metabolism of thymidine which can influence proliferative data acquired by radiolabeled thymidine incorporation (14, 15). Two groups of antigens are closely related to microanatomic location and stage of differentiation in the epidermis: adhesion molecules (16–25) and keratins (26–32). Using antibodies to the $\beta1$ and $\beta4$ integrins (the entire proliferative and strictly basal compartments, respectively), 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 proliferating cell nuclear antigen (PCNA)[1] expression then allowed quantitation of the proliferative status of each subset in normal and psoriatic skin. Melanocyte, Langerhans cell, and leukocyte contributions can be tracked by anti-CD45, anti-vimentin, and anti-HLA-DR. With this approach, we 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 before terminal differentiation (3, 4). Furthermore, we localize the upregulation in psoriasis to occur only in the normally slow cycling, less differentiated subset.

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 mo before the time when specimens were taken. In addition, in the psoriatic group, external treatment was not allowed 2 wk before 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 μm) in OCT (Tissue Tec II; Miles Laboratories, Elkhart, IN) were fixed in 70% cold ethanol for 10 min at $-20°C$. Indirect immunofluorescence staining was performed with anti-CD29, anti-K1/K10 antibodies, and isotype controls, followed by FITC or rhodamine-conjugated second antibodies, as previously described (33).

Epidermal Cell Suspension. After overnight treatment of keratome biopsies with Dispase (Collaborative Biomedical Products, Bedford, MA) at 4°C, epidermis was removed from dermis. The epidermal sheet was placed into 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) and incubated for 20 min at 37°C. A single cell suspension was then prepared in the presence of 0.01% DNase (Sigma Chemical Co.) and 10% FBS (Hyclone Laboratories, Logan, UT) by gentle teasing. The suspension was filtered through a 112 μm nylon mesh and washed. After the final wash, cells were resuspended in 1 ml HBSS without phenol red, Ca^{2+} and Mg^{2+} (Irvine Scientific, Santa Ana, CA) and slowly pipetted into 30 ml 70% cold ethanol. Samples were kept at $-20°C$ in ethanol until staining and flow cytometric analysis.

Staining Procedure. The ethanol-permeabilized cells were centrifuged (10 min, 3,000 rpm) and resuspended in Hank's solution. The suspension was syringed through a 30 G needle before staining. mAbs used were: anti-CD29 (4B4; Coulter Immunology, Hialeah, FL), 1:20 dilution; anti-K1/K10 (AE2; ICN, Costa Mesa, CA), 1:50 dilution; anti-CD45 (Becton Dickinson & Co., Mountain View, CA), 1:5 dilution; anti-HLA-DR-PE (Becton Dickinson & Co.), 1:5 dilution; anti-vimentin (ICN), 1:800 dilution; anti-$\beta4$ (Chemicon International, Inc., Temecula, CA), 1:5,000 dilution; anti-PCNA, 1:100 dilution (Boehringer Mannheim, Indianapolis, IN), and isotype controls, which included purified mouse IgG1 and ascites mouse IgG1 (both from Sigma Chemical Co.), and purified mouse IgG2a (Pharmingen, San Diego, CA). 10% normal goat serum (Cedarlane Laboratories, Hornby, Ontario, Canada) was used to block nonspecific binding. Goat anti-mouse IgG1-FITC and IgG2a-PE (Boehringer Mannheim) were used as secondary antibodies at 1:100 and 1:80 dilutions, respectively. Double staining with anti-CD29 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 & Scientific Corp., Westbury, NY) for 30 min on ice and stained in the fourth step with PE-conjugated anti-CD29. The cells were then resuspended either in 50 μg/ml propidium iodide (PI) with 100 U/ml RNase A (Sigma Chemical Co.) or 7 amino-actinomycin D [7AAD] (25 μg/ml, Calbiochem Novabiochem Corp., San Diego, CA). Samples were analyzed within 24 h by flow cytometry. HLA-DR-PE and CD29-FITC double staining were performed before ethanol permeabilization, because ethanol pretreatment reduced HLA-DR visualization.

Flow Cytometry. Flow cytometry was performed using an Epics Elite Flow Cytometer (Coulter Cytometry, Hialeah, FL). Light scatter, forward and 90°, was used for gating out debris and lym-
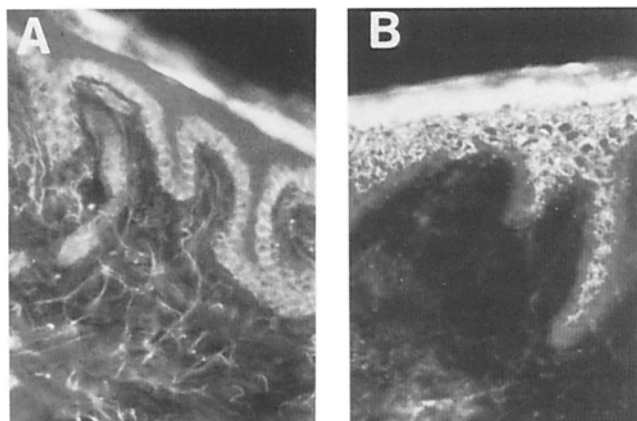
phocytes in the lesional psoriatic samples (a distinct, small, low granularity cell population of CD29+CD45+ cells). Cell aggregates were eliminated from the DNA analysis based on the ratio of integrated to peak fluorescence of PI or 7AAD. Listmode data were analyzed using Elite Software (Coulter Corp.), and for cell cycle analysis, multicycle software from Phoenix Flow Systems (San Diego, CA) was used.
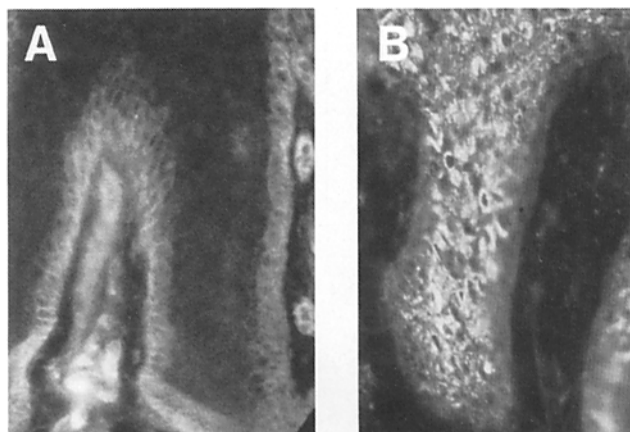
Results

Anti-CD29 and Anti-K1/K10 Antibodies Distinguish Basal from Suprabasal Epidermal Cells in Tissue Sections. To use flow cytometry to quantitate proliferative compartments in the epidermis, antibodies that preferentially react with the proliferative or nonproliferative 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 mAb to the β1 subunit of the CD49–CD29 integrin complex stained the basal layer and occasional second layer cells in the epidermis (Fig. 1 *A*). 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. 1 *B*).

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 CD29+ layers (Fig. 2 *B*). However, psoriatic skin differed from normal skin in CD29 expression, in that CD29 was expressed on the first two to three rows of lower cells in psoriatic epidermis (Fig. 2 *A*).

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 CD29 and K1/K10 keratins or isotype controls and analyzed by flow cytometry. Consonant
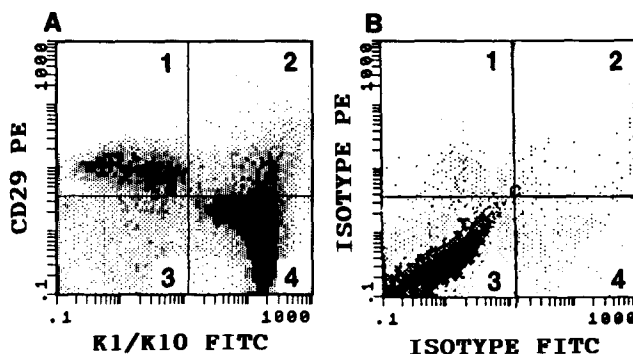

Figure 2. Expression of β1 integrin (CD29) and K1/K10 keratins in psoriatic skin. Sections of lesional psoriatic skin stained with anti-CD29 (*A*) and anti-K1/K10 (*B*) antibodies. Anti-CD29 positivity is apparent on the first two to three rows of basal cells, whereas anti-K1/K10 staining occurs above the CD29-positive cell layers.

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 CD29+K1/K10− and consisted of 20.7 ± 1.6% of the stained epidermal cells (Fig. 3 *A*, quadrant 1). 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 CD29. The majority of the epidermal cells were found in the CD29−K1/K10+ population (Fig. 3 *A*, quadrant 4). Another population of epidermal cells, which coexpressed CD29 and K1/K10 (CD29+K1/K10+ population) comprised

Figure 1. Expression of β1 integrin (CD29) and K1/K10 keratins in normal human skin. Sections of normal human skin were stained with anti-CD29 (*A*) and anti-K1/K10 (*B*) mAbs. Whereas anti-CD29 stains the basal layer and occasional second layer cells, anti-K1/K10 staining is limited to upper layer cells, sparing the basal layer.


Figure 3. Identification of normal human epidermal cell populations by flow cytometry. Two-parameter scatter plot diagrams of a double-stained (anti-CD29 PE and anti-K1/K10 FITC) epidermal cell suspension from normal human skin. Cells with PE and FITC binding levels that fall within quadrant 3 (*A*) exhibit negative staining that is not different than background fluorescence that occurs with double-isotype staining (*B*). Double-positive cells exhibiting higher than background fluorescence intensity for both fluorochromes are in quadrant 2. Cells positive for only a single marker fall into quadrant 1 (CD29+K1/K10−) and quadrant 4 (CD29−K1/K10+ FITC).
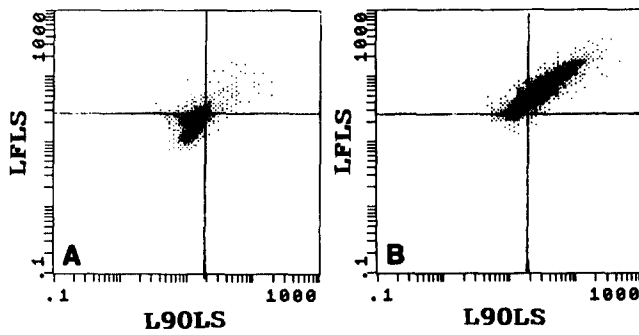
Figure 4. Light scatter analysis reveals two distinct CD29+ cell populations. Larger cell size and cytoplasmic complexity of CD29+K1/K10+ cells versus CD29+K1/K10- cells. Anti-CD29 PE and anti-K1/K10 FITC double-stained normal epidermal cells were separated according to their PE and FITC fluorescence. CD29+K1/K10- (A) and CD29+K1/K10+ (B) cells appear as distinct groups on the dual-parameter forward light scatter (y axis, expressed as log) versus 90° angle light scatter (x axis, expressed as log).

13.8 ± 3.2% of stained epidermal cells (Fig. 3 A, quadrant 2). The CD29+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 (34) and reports of occasional CD29+ cells in the second layer of the epidermis (21).

Light Scatter Analysis Separates the CD29+ Compartment into Two Populations Based upon their Expression of K1/K10. Light scatter analysis of the CD29+ cell population in normal epidermis revealed that the CD29+K1/K10- basal cells and the CD29+K1/K10+, more differentiated, cells represent different populations by size and cytoplasmic complexity (35). In comparison with the K1/K10- population (Fig. 4 A, lower left quadrant), K1/K10+ cells are larger and exhibit the more complex cytoplasmic structures (Fig. 4 B, upper left and upper right quadrants) expected of differentiating keratinocytes.

CD29 Expression Distinguishes between Proliferative and Non-proliferative Compartments in Normal Epidermis. To define the epidermal proliferative compartment, epidermal cells were simultaneously stained with anti-CD29, anti-K1/K10, or iso-

type control antibody in combination with DNA dyes such as PI and 7AAD. DNA staining in combination with specific antibody permitted determination of cycling (S/G2/M phase cells) and noncycling (G0/G1) states of specific epidermal compartments (Fig. 5). With this approach, it is clear that the entire pool of proliferating cells (S/G2/M) are CD29+ (Fig. 5 A). Using anti-K1/K10, the proliferating CD29+ cell population can be subdivided into two main subsets (Fig. 5 B). One lacked K1/K10 and the other coexpressed K1/K10.

In studies of keratinocytes cultured long-term in vitro, two populations of proliferating keratinocytes have been observed (7). Small cells were found to be slowly cycling whereas 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 that 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 CD29+ and large CD29+ cells were analyzed separately. Among the small CD29+ cells, 5.16 ± 1.8% cells were found in S/G2/M phase, whereas 16.6 ± 4.54% of the large CD29+ cells were in cycle (n = 5; data not shown).

Separation of Proliferative Compartment of Normal Epidermis into Slow-Cycling CD29+K1/K10- Cells and Rapidly Cycling CD29+K1K10+ Cells. Because there is a continuity in size and granularity among the CD29+ 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 CD29+ cells into subpopulations, and triple-color analysis utilized to simultaneously quantitate DNA content with another DNA dye, 7AAD. Indeed, the DNA analysis (Fig. 6) revealed a great difference in mitotic activity between CD29+K1/K10- (4.53 ± 1.1%, Fig. 6 A) and CD29+K1/K10+ cells (48.8 ± 18.92%, Fig. 6 B) (n = 3). This disparity in cycling pool was greater than that seen between the CD29+ small and CD29+ large group, confirming our suspicion that some slow cycling K1/K10- cells can exhibit a log forward light scatter (LFLS) indistinguishable from K1/K10+ cells.
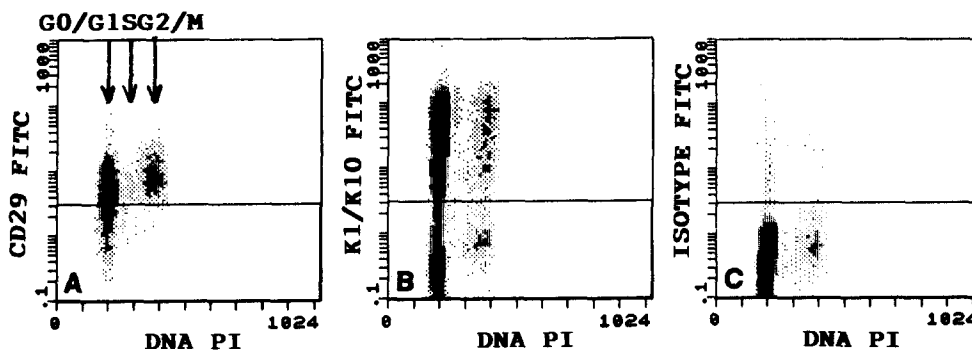
Figure 5. Proliferative state of epidermal cell subsets in normal skin determined by DNA content. Two-parameter scatter plot of normal human epidermal cells stained separately with anti-CD29 (A, y axis), anti-K1/K10 mAbs (B, y axis), and isotype control (C, y axis) in combination with propidium iodide (PI, x axis). Proliferating cells (cells in S/G2/M phase) all express CD29 (A, upper right) relative to isotype control (C, lower right). Proliferating cells can be either K1/K10+ (B, upper right) or K1/K10- (B, lower right).
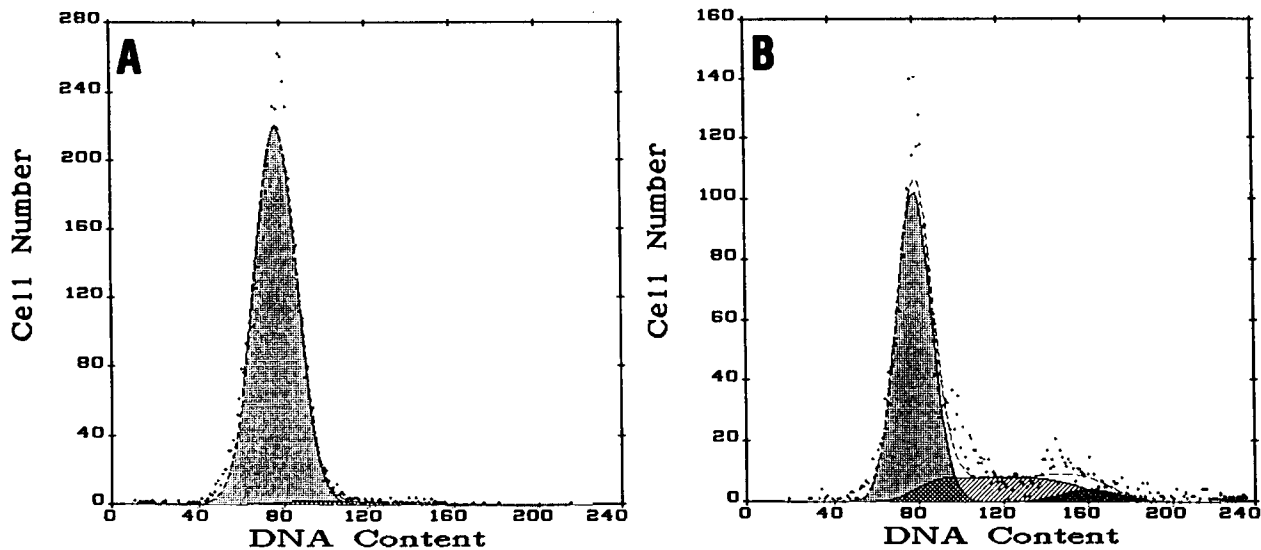
Figure 6. Cell cycle analysis of epidermal cell subsets by triple-color flow cytometry. Normal epidermal cells simultaneously costained with anti-CD29 PE, anti-K1/K10 FITC, and 7AAD. Cells were separated according to their PE and FITC fluorescence intensity into CD29$^+$K1/K10$^-$ (*A*) and CD29$^+$K1/K10$^+$ (*B*) groups. The number of cells with a given DNA content is expressed as number of cells versus 7AAD fluorescence (*x* axis). (*Dashed line*) Histogram of the experimental data (*dots*). (*Shaded areas*) The best curve fit calculated by the multicycle software package (Phoenix Flow Systems) to separately resolve the distribution of cells in G0/G1 (*gray shading*), S (*diagonal lines*), and G2/M (*dense shading*) phase of the cell cycle.

These data provide, for the first time, a direct demonstration that the proliferative compartment of in vivo human epidermis is characterized by CD29 expression and consists of at least two morphologically and functionally different compartments. One compartment mainly contains slowly cycling CD29$^+$K1/K10$^-$ small cells with a relatively simple internal structure. The other proliferative compartment contains rapidly proliferating CD29$^+$K1/K10$^+$ larger cells with more complex cytoplasmic organization.

Contribution of Nonkeratinocyte CD29$^+$ Cells to the Proliferative Compartment of CD29$^+$K1/K10$^-$ Epidermal Cells. Although expression of K1/K10 is specific for keratinocytes, anti-CD29 recognizes the β1 integrin chain and may be expressed on other cells in the epidermis, such as melanocytes, Langerhans cells, or T cells. To verify whether our DNA analysis truly represents the proliferative state of the keratinocyte CD29$^+$K1/K10$^-$ population, we utilized monoclonal anti-vimentin, anti-HLA-DR, and anti-CD45 antibodies to identify melanocytes, Langerhans cells, and lymphocytes in our epidermal cell suspension. In agreement with previous results (21, 36–38), in normal epidermis, all the DR- and vimentin-positive cells showed double staining with anti-CD29 (data not shown), indicating that the β1 integrin is indeed present on Langerhans cells and melanocytes. Although the number of Langerhans cells and melanocytes in the normal epidermis is relatively low compared with the CD29$^+$K1/K10$^-$ epidermal cells (Table 1), DNA staining (PI) of CD45 and vimentin-stained normal epidermal cells was performed to determine the contribution that melanocyte and Langerhans cell proliferation could have on the DNA analysis of the CD29$^+$K1/K10$^-$ population. Vimentin- and CD45-positive cells exhibited a cycling pool (percentage of that cell type that is in S/G2/M

phase of the cell cycle) similar to CD29$^+$K1/K10$^-$ cells in normal epidermis (5, 6, and 4%, respectively, Table 1), and they comprised one fourth of the cycling population in normal epidermis (0.25% vimentin-positive cycling cells within 1.05% total CD29$^+$ cycling cells) (Table 1).

CD29$^+$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-CD29 and anti-K1/K10 as were found in normal: CD29$^+$K1/K10$^-$ (Fig. 7 *A*, quadrant *1*), CD29$^+$K1/K10$^+$ (Fig. 7 *A*, quadrant *2*) and CD29$^-$K1/K10$^+$ (Fig. 7 *A*, 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. 7 *A*). Although the cells in quadrant 1 must be considered to be K1/K10$^-$ based on the isotype staining (Fig. 7 *b*), the cells in the leftmost portion of quadrant 2 (Fig. 7 *A*) can be considered to be K1/K10^{dim}, indicating a transitional state of early K1/K10 expression.

As in normal epidermis, the proliferative compartment (S/G2/M) of the psoriatic epidermis was entirely comprised of CD29$^+$ cells (Fig. 8 *A*), cells above the horizontal cursor for CD29$^+$ cells defined by the isotype control (Fig. 8 *C*). One difference, however, was the presence of a CD29$^-$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. 8, cells with >2 n DNA below the horizontal cursor). This population is never present in normal samples.

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Table 1. *The Elevated Proliferative Rate of CD29$^+$ Cells in Lesional Psoriasis Is Due To an Enlarged Cycling Pool of CD29$^+$K1/K10$^-$ Keratinocytes and Not to CD29$^+$ Lymphocytes, Langerhans Cells, Macrophages, or Melanocytes*

		Cycling subset cells as a proportion of each cell subset	Cell population size (% total EC)	Cycling subset cells as a proportion of total EC (% total EC)
Normal				
Vimentin$^+$	(n = 2)	5%*	4.5 ± 0.5‡	0.25 ± 0.05§
CD45$^+$	(n = 4)	6%	2.08 ± 0.08	0.125 ± 0.0025
CD29$^+$K1/K10$^-$	(n = 3)	4%	23.2 ± 1.6	1.05 ± 0.27
Lesional psoriatic				
Vimentin$^+$	(n = 2)	11%	6.75 ± 3.3	0.75 ± 0.15
CD45$^+$	(n = 4)	17%	5.25 ± 1.6	0.9 ± 0.3
CD29$^+$K1/K10$^-$	(n = 3)	25%	30.76 ± 8.1	7.8 ± 0.9

* The percentage of cells within each cell subset that is in S/G2/M [cycling subset cells as a proportion of each subset = 100(cell population size/cycling subset cells as a proportion of total EC)].
‡ The percentage of cells expressing this marker as a percentage of total epidermal cells (EC).
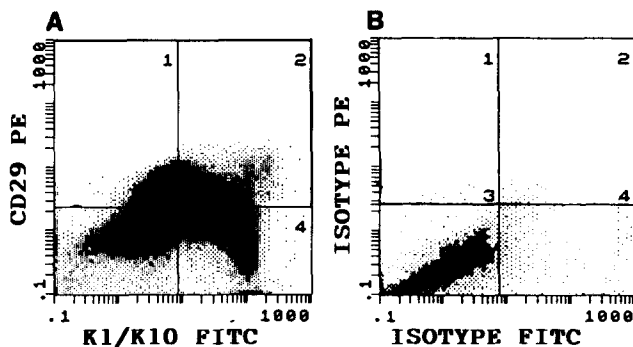§ Proportion of each subset that is in S/G2/M as a percentage of total EC.

In psoriasis, the vast majority of the proliferating cells are clearly K1/K10$^-$ (Fig. 8 *B*, S/G2/M cells below the horizontal cursor), based on the isotype staining (Fig. 8 *C*). Of interest is the slightly increased presence of a cluster of G2/M cells just above the horizontal cursor in psoriasis (Fig. 8 *B*) relative to normals (Fig. 5 *B*), 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 2), suggesting a parallel expansion of these two populations in lesional psoriatic skin. 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. 9). On the one hand, a major difference in the number of
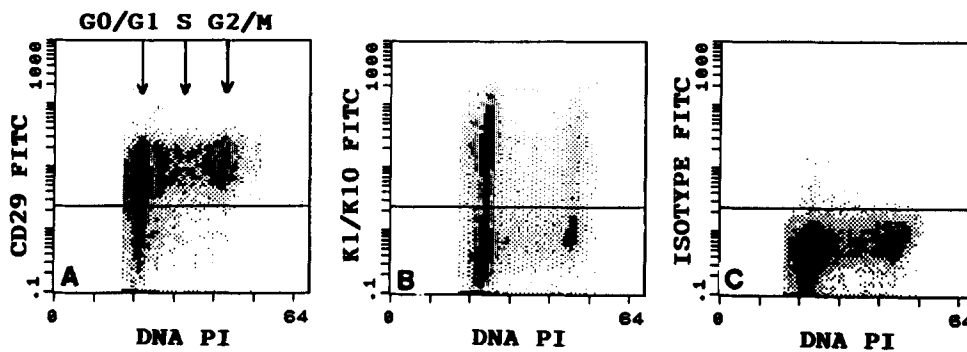

Figure 7. Identification of psoriatic epidermal cell subsets by flow cytometry. Two-parameter scatter plot of lesional psoriatic epidermal cells co-stained with anti-CD29 PE and anti-K1/K10 FITC. Background staining and cursor placement is determined by double isotype staining (*B*). Cells expressing both antigens fall within quadrant 2 (*A*). Quadrant *1* contains CD29$^+$K1/K10$^-$ cells and CD29$^-$K1/K10$^+$ cells are in quadrant *4* (*A*).

S/G2/M phase cells among the CD29$^+$K1/K10$^-$ cells was detected in lesional psoriatic epidermis upon comparison with 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/G2/M phase cells) within the CD29$^+$K1/K10$^+$ differentiating transiently amplifying cells was almost the same in normals and psoriatics.

The finding that the same mitotic activity is present in the normal and lesional psoriatic CD29$^+$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 CD29$^+$K1/K10$^-$ cells that differentiate to express K1/K10 is the same for both normal and psoriatic CD29$^+$K1/K10$^-$ cells.

Hyperproliferation in the Psoriatic Epidermis Is Due to Activation of the Normally Quiescent Cells in the CD29$^+$K1/K10$^-$ Basal Cell Compartment. Although stem cells must be contained within the CD29$^+$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 G1 phase of the cell cycle, is most abundant during S phase, and declines during G2/M phase (39). In normal epidermis the vast majority (95.5%) of the K1/K10$^-$ cells were PCNA negative, indicating quiescent state (Fig. 10 *A*), consistent with a large component of slow cycling cells. By contrast, in psoriatic epidermis, all of the K1/K10$^-$ cells showed PCNA positivity (Fig. 10 *B*, upward shift of dark dot plot relative to isotype-stained cells represented by counter plot). These data suggest that the entire CD29$^+$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).
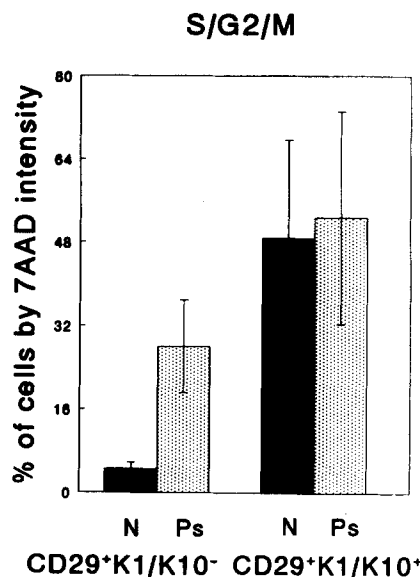
Figure 8. Proliferative state of epidermal cell subsets in lesional psoriatic skin determined by DNA content. Two-parameter scatter plot of lesional psoriatic epidermal cells stained with anti-CD29 FITC (*A*) or anti K1/K10 FITC (*B*). Positivity is determined by isotype FITC staining (*C*). Cells in S/G2/M phase are CD29+ (*A*) and either K1/K10− or K1/K10+ (*B*). A few CD29− cells can be observed in early S phase.

Identification, Quantification, and Removal of the Leukocytic and Melanocytic Proliferative Compartment. In addition to Langerhans cells and melanocytes, activated T lymphocytes and macrophages are present in the CD29+ compartment of lesional psoriatic epidermis (40–45). Among the total epidermal cell population, the percentage of CD45+ cells is consistently higher in the lesional psoriatic samples (5.25 ± 1.6%) than in normals (2.08 ± 0.06%) (Table 1) (*n* = 4). However, the proportion of cells in S/G2/M phase within each subset among the vimentin- and CD45-positive cells is less than among the entire CD29+K1/K10− cell population, (vimentin+, 11%; CD45+, 17%; and CD29+K1/K10−, 25%; Table 1), suggesting that the 25% figure is a minimal estimate of the lesional psoriatic keratinocyte CD29+K1/K10− subset cycling pool. Vimentin+ leukocytes, Langerhans cells, and melanocytes comprised less than one tenth of the cycling population (0.75% vimentin+ versus 7.8% CD29+K1/K10− epidermal cells are in cycle) (Table 1). The above findings cause DNA analysis of the lesional psoriatic CD29+K1/K10− population to somewhat underestimate the proliferation of the keratinocytes (because of the inclusion of less proliferative melanocytes, Langerhans cells, and lymphocytes in the analysis). Thus the difference in the number of S/G2/M cells, among CD29+K1/K10− keratinocytes between normal versus lesional psoriatic epidermis, is even higher than the sevenfold increase demonstrated in Table 1, column 3.

Hyperproliferation among Strictly Basal β4 Integrin+ Keratinocytes. To further verify that stimulation of the normally slow

cycling strictly basal cell population is responsible for hyperproliferation in psoriatic lesions, and not a CD29+ population which has differentiated but delayed K1/K10 expression, we used β4 integrin to further subdivide the CD29+K1/K10− cell population. β4 integrin is strongly localized to hemidesmosomes, suggesting that it plays a role in epidermal cell adhesion to the basement membrane (25). Contrary to β1 integrin in psoriatic lesional tissue, β4 integrin staining in psoriasis is identical to normals, in that it is restricted to the

S/G2/M

Figure 9. Cell cycle analysis localizes the hyperproliferative defect in lesional psoriasis to be within the 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-CD29 PE, anti-K1/K10 FITC, and the DNA dye, 7AAD. Based upon PE and FITC fluorescence, cells were electronically separated into CD29+K1/K10− (*left bars*) and CD29+K1/K10+ (*right bars*) populations and the DNA distribution based on 7AAD fluorescence was analyzed by the Multicycle software package (Phoenix Flow Systems). The percentage of cells in each epidermal cell subset that has entered cell cycle (S/G2/M) is expressed as the average ± SE from three normals and three psoriatics. The difference in the percentage of S/G2/M cells among the CD29+K1/K10− cells between normal and psoriatic epidermal cells is significant (*p* <0.05) as assessed by two-tailed Student's *t* test. By contrast, the more differentiated K1/K10+ subset exhibits identical proliferation in normal and psoriatic skin.

Table 2. *The Relative Proportions of the CD29+ Proliferative Cell Compartments Are the Same in Normal and Psoriatic Epidermis*

	CD29+K1/K10−	CD29+K1/K10+
Normal	65.4 ± 4.8	34.6 ± 4.8
Psoriatic	65.4 ± 9.3	34.6 ± 9.3

Percentages were calculated in five independent anti-CD29 PE and anti-K1/K10 FITC double-staining experiments for both the normal and psoriatic groups. Values are expressed as averages ± SE in each group.
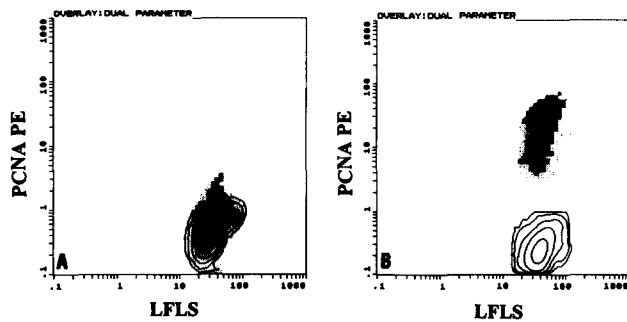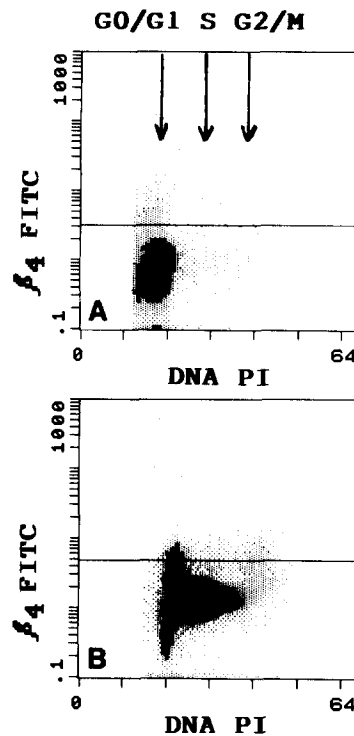
Figure 10. PCNA and K1/K10 double staining of normal and psoriatic involved epidermis. K1/K10⁻ cells are electronically gated according to FITC (K1/K10) fluorescence in normal (*A*) and in psoriatic involved (*B*) epidermis. Two-parameter diagrams show PCNA PE staining (*dot plots, y axis*) and isotype control staining (*counter plots, y axis*) versus forward angle light scatter (*x axes*). In normal epidermis, only 4.5% of the K1/K10⁻ cells show PCNA positivity (*dots* above the outermost line of the counter plot diagram, *A*) whereas in psoriatic epidermis all the K1/K10⁻ cells exhibit high PCNA PE fluorescence (*dot plots B*).

Figure 11. β4 integrin–positive basal cells in psoriatic epidermis are in hyperproliferative state. DNA content of normal (*A, x axis*) and psoriatic involved (*B, x axis*) epidermal cells. β4 integrin–positive cells in the psoriatic sample (*top, B*) contain a higher number of cells in S/G2/M phase (*dot plots*) than β4 integrin–positive cells in the normal sample (*top, A*). Cursors for positive FITC fluorescence (*y axes*) are set according to isotype background staining (data not shown).

basal pole of the very first layer of cells above the basement membrane (46). In normal epidermis β4 integrin–positive cells (β4 FITC, Fig. 11 *A*) were identified as a minor population exhibiting FITC fluorescence above the cursor for positivity defined by the isotype control (data not shown). Qualitatively, it can be appreciated that many β4 integrin–positive cells in lesional psoriatic epidermis express DNA content (DNA PI) >2 n (Fig. 11 *B*). Quantitation of the cell cycle status of β4 integrin–positive lesional psoriatic keratinocytes revealed a marked hyperproliferation (31% of β4⁺ cells in S/G2/M phase) in comparison with normal cells (3.9% of β4⁺ cells in S/G2/M phase).

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 (17, 34, 47–49). 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 (50, 51).

The β1 integrin chain is expressed predominantly on basal cells, but also on some suprabasal cells upon staining of vertical sections of epidermis. In this report, we show by flow cytometric analysis that CD29 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 (CD29⁻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 (29, 31). 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/G2/M) CD29⁺ 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 (34). Thus, the proliferative compartment of the human in vivo epidermis contains two subpopulations characterized by CD29 and K1/K10 expression. Undifferentiated CD29⁺K1/K10⁻ cells in the basal layer express K1/K10 upon commitment to differentiation and disattachment from the basement membrane. Initially, they retain CD29, 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 CD29 expression. At present, there are no data available of the precise three-dimensional organization of the human epidermis. Comparison of data acquired by flow cytometry with the staining of tissue sections can only be approximative.

Because CD29 staining can separate the heterogeneous proliferative compartment from the nonproliferative compartment and K1/K10 expression further subdivides the prolifer-

ative subpopulations, we were able to quantify the proliferative state of each CD29$^+$ subpopulation separately. DNA analysis revealed that the different subpopulations of CD29$^+$ keratinocytes in normal human epidermis differ greatly in the number of proliferating cells. The CD29$^+$ cells that have initiated their differentiation program, as indicated by the expression of K1/K10 keratins, are highly proliferative in comparison to the nondifferentiating cells (CD29$^+$K1/K10$^-$). The undifferentiated basal CD29$^+$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). Besides keratinocytes, Langerhans cells and melanocytes are included in the CD29$^+$K1/K10$^-$ population. Using mAbs that bind Langerhans cells and melanocytes, we were able to further subdivide the CD29$^+$K1/K10$^-$ populations, and found that Langerhans cells and melanocytes exhibit a cycling pool similar to the CD29$^+$K1/K10$^-$ slow cycling keratinocytes in normal epidermis. They comprise about one fourth of the proliferative pool because of their low frequency in the epidermis, relative to CD29$^+$K1/K10$^-$ keratinocytes.

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 (14). In addition, we found that psoriatic epidermis, relative to normal epidermis, demonstrates markedly increased thymidine phosphorylase activity (15), 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 (52). The existence of such a stem cell population in the human epidermis was strongly supported by the observations of Lavker and Sun (3). In human palmar epidermis they observed two distinct basal cell types. One type, resembling stem cells, was non-serrated, slowly cycling, and primitive in cytoplasmic structure, whereas the other type, considered to be transiently amplifying 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 CD29$^+$K1/K10$^-$ and β4 integrin$^+$ basal cells are slow cycling, small in cell size (low LFLS), and primitive in cytoplasmic structure (low complexity or L90LS). By contrast the CD29$^+$K1/K10$^+$ cells are highly proliferative, larger in size, and exhibit a more complex cytoplasm (higher L90LS). These data suggest that the CD29$^+$ keratinocyte subpopulations indeed contain stem and transiently amplifying subpopulations comprising the epidermal proliferative compartment. Although we cannot further specify the CD29$^+$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 two to three rows of proliferating cells are CD29$^+$, whereas K1/K10 occurs above the proliferative layers. Flow analysis of the anti-CD29 and anti-K1/K10 double-stained samples revealed the presence of similar proliferating keratinocyte subpopulations (CD29$^+$K1/K10$^-$ and CD29$^+$K1/K10$^+$) and nonproliferative compartment cells (CD29$^-$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/G2/M phase of the cell cycle among the CD29$^+$K1/K10$^-$ as well as β4 integrin$^+$ basal cells. By comparison, the CD29$^+$K1/K10$^+$ more differentiated cells exhibited almost the same high frequency of cycling cells in psoriatic as in normal epidermal cells. The striking difference apparent 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. In contrast to this data, in a previous study using flow cytometry to analyze basal (low cellular RNA) and amplifying (high cellular RNA) keratinocytes, Staiano-Coico et al. (9) found an increase in the number of the amplifying, high cellular RNA cells in psoriatic epidermis relative to normal, and they concluded that the induction of the psoriatic plaque involves the induction of an unbalanced growth state within the more rapidly dividing keratinocyte population. The main reason for the contradicting results lies in the markers used for defining and separating the epidermal cell compartments. Cellular RNA content similar to cell size and granularity is continuous, resulting in somewhat arbitrary separation between cell populations. Furthermore, cellular RNA, cell size, and granularity can change with proliferative activation, therefore proliferating basal cells could show up as transient cells in psoriatic epidermis based on these markers, regardless of their true differentiation status.

Our flow cytometry-based data confirm and precisely localize data suggesting that the growth fraction (proliferating cells at any given time) is considerably increased in the psoriatic tissue (53). However, according to our data, the increased number of cycling keratinocytes in the psoriatic CD29$^+$K1/K10$^-$ compartment did not result in an increase in the size of this cell compartment in psoriatic epidermis relative to the CD29$^+$K1/K10$^+$ cells with transient amplifying cell features. This suggests that the proportion of CD29$^+$K1/K10$^-$ cells that self-renew (to CD29$^+$K1/K10$^-$) is the same in psoriatic epidermis as it is in normal epidermis, and that excess cells are committed toward differentiation (CD29$^+$K1/K10^{dim}). The ratio of CD29$^+$K1/K10$^-$ to CD29$^+$K1/K10$^+$ cells could only be kept constant (Table 2) if there was accelerated maturation among the newly generated transient amplifying cells, and concomitant loss of CD29. Such an event is consistent with the accelerated differentiation pattern that psoriatic keratinocytes are known to undergo (54, 55).

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. This finding will facilitate the elucidation of the signal(s) responsible for the marked- over stimulation of this subset. Because these presented data provide a better understanding of the proliferative function of normal skin, the application of this technique in the investigation of other hyperproliferative skin disorders will most likely help to precisely identify proliferative changes in different dermatoses.

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