JOURNAL OF CELLULAR PHYSIOLOGY 168:684–694 (1996)

Cell Kinetic Characterization of Cultured Human Keratinocytes From Normal and Psoriatic Individuals

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Psoriasis is a chronic skin disease characterized by epidermal hyperproliferation, disturbed differentiation, and inflammation. It is still a matter of debate whether the pathogenesis of psoriasis is based on immunological mechanisms, on defective growth control mechanisms, or possibly on a combination of both. Several in vivo cell biological differences between psoriatic lesional epidermis and normal epidermis have been reported. However, it is not clear whether these changes are causal or consequential. In case that keratinocytes from psoriatic patients have genetically determined deficiencies or polymorphisms with respect to autocrine growth regulation and the response to inflammatory cytokines, we hypothesize that these differences should be maintained in culture. Here we have started a systematic comparison of first passage keratinocytes cultured from normal skin and uninvolved psoriatic skin to address the question whether there are intrinsic differences in basic cell cycle parameters. In an established, defined culture system using keratinocyte growth medium (KGM) we have determined: (i) cell cycle parameters of exponentially growing keratinocytes, (ii) induction of quiescence by transforming growth factor β_1 (TGF-β_1), and (iii) restimulation from the G_0-phase of the cell cycle. Bivariate analysis of Iodo-deoxyuridine incorporation and relative DNA content was performed by flow cytometry. Within the limitations of this model no gross differences were found between normal and psoriatic keratinocytes with respect to S-phase duration (T_s), total cell cycle duration (T_c), responsiveness to TGF-β_1 and the kinetics for recruitment from G_0. In psoriatic keratinocytes we found a lower amount of cells in S-phase and a shorter duration of G_1, compared to normal keratinocytes. The methodology developed here provides us with a model for further studies on differences between normal and psoriatic keratinocytes in their response to immunological and inflammatory mediators.

Psoriasis is a polygenic, chronic skin disease of unknown etiology, affecting approximately 2–4% of the world population (van de Kerkhof, 1986). The disease is characterized by a marked increase in keratinocyte proliferation (Weinstein and van Scott, 1965), abnormal patterns of keratinocyte differentiation (Bernard et al., 1988), prominent alterations in dermal capillary vasculature (Braverman and Sibley, 1982), and the presence of dermal and epidermal T-cells, monocytes, macrophages, and polymorphonuclear leukocytes (PMNs; Hammar et al., 1984; Baker et al., 1984). Many in vivo cell biological differences between psoriatic lesional epidermis and normal epidermis have been reported. In psoriatic epidermis, a number of molecules that are absent in normal skin are strongly expressed (e.g., cytokeratins [CK] 6, 16, and 17 [de Jong et al., 1991; Weiss et al., 1984; De Mare et al., 1989], and the epidermal proteinase inhibitor SKALP which we have recently described [Alkemade et al., 1994; Molhuizen et al., 1993]). In addition, several molecules have a restricted expression pattern in normal skin, but are highly upregulated in psoriatic skin (e.g., psoriasis-associated fatty acid binding protein [PA-FABP; Madsen et al., 1992], psoriasin [Madsen et al., 1991], transforming growth factor α [TGF-α; Turbitt et al., 1990; Elder et al., 1989], amphiregulin [Cook et al., 1992], epidermal growth factor receptor [EGF-R; Nanney et al., 1992], interleukin 1ra [Hammerberg et al., 1992], interleukin 1β [Schmid et al., 1993], interleukin 6 and 8 [Nickoloff et al., 1991; Grossman et al., 1989], GRO α/β/γ [Tettelbach et al., 1993], and fibronectin [Bernard et al., 1988]), or show a sustained expression (e.g., the $\alpha_1\beta_5$ integrin [Hertle et al., 1992; Pellegrini et al., 1992]), or are prematurely expressed (e.g., involucrin [Bernard et al., 1988] and transglutaminase [Michel et

Received October 12, 1995; accepted April 22, 1996.

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al., 1992]). It is not clear whether these differences are involved in the pathogenetic process or are a mere consequence of the disease process.

Two main views exist on the etiology of psoriasis. Psoriasis could be based on immunological mechanisms (Gottlieb, 1990; Gottlieb et al., 1995; Grossman et al., 1989; Schmid et al., 1993) or, alternatively, psoriasis may be caused by defective growth control mechanisms (Gentleman et al., 1984; Nanney et al., 1986; Elder et al., 1989). Because of the presumed polygenic nature of the disease, these views cannot be strictly separated (Elder et al., 1994), and psoriasis could very well be caused by a combination of genetic polymorphisms affecting the immune response and an increased sensitivity of psoriatic keratinocytes to T-cell derived cytokines (Bata-Csorgo et al., 1995; Leung et al., 1993; Baadsgaard et al., 1990). We hypothesized that if keratinocytes from psoriatic patients harbor genetically determined deficiencies or polymorphisms with respect to autocrine growth regulation and their response to inflammatory cytokines, these differences are maintained in culture, and we should be able to quantify the abnormalities keratinocytes cultured from the clinically unaffected skin. In order to address these questions, we have started a systematic comparison of first passage cultured human keratinocytes derived from normal healthy epidermis and uninvolved psoriatic epidermis. Using bivariate flow cytometry, a method which allows accurate quantitative characterization of cell populations, we have determined cell cycle parameters of exponentially growing keratinocytes, induction of quiescence by addition of transforming growth factor β_1, and restimulation from the G_0-phase (quiescence) of the cell cycle.

MATERIALS AND METHODS

Biopsies

Biopsies (0.2-mm thickness) from healthy volunteers and volunteers with psoriasis were taken with a keratome as previously described (Schalkwijk et al., 1990) and used for primary keratinocyte cultures.

Keratinocyte primary culture

Human epidermal keratinocytes were initially cultured according to the Rheinwald-Green system (Rheinwald and Green, 1975). Primary cultures of keratinocytes were seeded on lethally irradiated (3,000 rad, 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 (3:1) (v/v; Flow Laboratories, Irvine, Scotland) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research Inc., Lexington, MA), isoproterenol (10^{-6} M; Sigma, St. Louis, MO), 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 6% fetal calf serum (FCS; Seralab, Nistelrode, The Netherlands), and 10 ng/ml mouse epidermal growth factor (EGF; Sigma). Cells were grown at 37°C, 95% relative humidity and 8% CO_2 in air. EDTA-treated, trypsinized and liquid nitrogen-stored keratinocytes from the primary culture were used in further experiments.

Keratinocyte secondary culture

For the experiments, the human keratinocytes were seeded at 10^5 cells in Keratinocyte Growth Medium (KGM) in 60-mm culture dishes. KGM was composed of Keratinocyte Basal Medium (KBM) (Clonetics, San Diego, CA; 0.15 mM calcium) supplemented with ethanolamine (0.1 mM; Sigma), phosphoethanolamine (0.1 mM; Sigma), bovine pituitary extract (BPE; 0.4% v/v; Clonetics), EGF (10 ng/ml; Sigma), insulin (5 μg/ml; Sigma), hydrocortisone (0.5 μg/ml; Collaborative Research Inc.), penicillin (100 U/ml; Gibco), and streptomycin (100 μg/ml; Gibco). Cultures were grown at 37°C, 95% relative humidity and 5% CO_2 in air.

Keratinocyte growth arrest

For experiments in which cells were required in the quiescent state, the medium was removed at 20–30% confluence (usually about 5 days), the culture washed briefly with phosphate-buffered saline (PBS) supplemented with calcium (120 μM; Merck, Darmstadt, Germany), and growth-arrest medium was added as we have described previously (van Ruissen et al., 1994). During the experiments we used KGM supplemented with TGF-β_1 (10 ng/ml; R&D systems Europe Ltd. Abingdon, UK) as growth-arrest medium. All cultures were grown at 37°C, 95% relative humidity and 5% CO_2 in air.

Keratinocyte growth stimulation

Growth arrested keratinocyte cultures were washed briefly with PBS supplemented with calcium (120 μM) to remove TGF-β_1, and new growth medium (KGM) was added.

IdUrd labeling

Cultured keratinocytes were either pulse-labeled (30 min) or continuously labeled with IdUrd (Sigma), in either case at a final concentration of 10 μM. After pulse labeling, the cultures were washed twice with PBS supplemented with calcium (120 μM) and refed with fresh medium. Experiments were terminated by washing the cultures twice with PBS followed by one wash with 0.25% trypsin (DIFCO Laboratories, Detroit, MI), 0.02% EDTA, and 0.1% glucose. After 15 min incubation with the same solution (to allow complete detachment of cells), the cells were collected in PBS containing 5% FCS. After centrifugation the keratinocytes were fixed by resuspending the pellet in 70% ethanol (−20°C), and the suspension was stored at −20°C until further use.

Immunocytochemical staining of IdUrd and DNA labeling

Staining was basically as previously described (van Erp et al., 1988). In brief, about 10^5 ethanol-fixed cells were washed once with phosphate-buffered saline supplemented with 1% heat-inactivated normal calf's serum (PBS/HINCS). The cells were hydrolyzed for 30 min at room temperature with 0.1 mg/ml pepsin in 2 N HCl. Hydrolysis was terminated with excess 1 M TRIZMA base (Sigma). The cells were pelleted and washed with PBS containing 0.1% Nonidet P40 (BDH Chemicals Ltd., Poole, England). After sedimentation, the cells were incubated with a 1:50 dilution of the monoclonal antibody DAKO-BrdUrd (Dakopatts, Copenhagen, Denmark) for 30 min. This monoclonal antibody specifically detects BrdUrd and IdUrd. After washing the cells with PBS/HINCS, a second incubation step (15 min, 4°C) using a 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (RAM-FITC; Dakopatts) containing 3% normal rabbit serum (NRS), was carried out to visual-

ize the IdUrd incorporation. Following a final wash with PBS/HINCS, the cells were resuspended in 300 µl PBS containing 40 µg/ml propidium iodide (PI; Calbiochem, San Diego, CA) and incubated for 15 min with 50 µl of 1% (w/v) RNAse A (Sigma).

Flow cytometry

Cells stained with PI and fluoresceine isothiocyanate (FITC) were analyzed on the Epics® Elite flow cytometer (Coulter Corporation, Hialeah, FL) equipped with a 40 mW air-cooled argon-ion laser set at 15 mW and tuned at a wavelength of 488 nm. FITC and PI signals were separated by a 550 nm dichroic mirror. The FITC signals (green fluorescence) were detected through a 525-nm band pass filter, and the PI signals (red fluorescence) were detected through a 630-nm long pass filter.

Usually 10^4 cells were measured at a flow rate of approximately 50 cells per second. The data were recorded in listmode and analyzed on the Epics® Elite workstation. The ratio area/peak of the red fluorescence is an excellent discriminator between artifacts due to doublets of diploid cells and real single tetraploid (or late S) cells when intact cells are used (Bauer and Boezeman, 1983). The data were further analyzed on the Epics® Elite workstation with the program Multicycle (Version 2.5; Phoenix Flow Systems, San Diego, CA) to determine the percentages of G_0/G_1, S and G_2/M.

Calculations of cell cycle time and duration of S-phase

Two-parameter flow cytometry of DNA versus IdUrd allows assessment of the number of cells in S-phase (N_s) and the duration of the S-phase (T_s) even from one single sample (Begg et al., 1985). The calculation of T_s is based on the assumption that there is a linear increase in mean relative DNA content of the IdUrd-labeled S-phase cells in time. At time zero after IdUrd pulse-labeling, the mean DNA content of the IdUrd-labeled S-phase cells is in the middle of the interval between the unlabeled diploid G_0/G_1 cell population and the unlabeled tetraploid G_2/M cell population. As the IdUrd-labeled cells move through the S-phase, the mean DNA content of the population will approach the DNA content of the G_2/M population. The IdUrd-labeled cells which have divided and appeared in the (labeled) diploid G_1 region were excluded from the calculation of mean DNA content. In Figure 1, two examples are given for the movement of IdUrd-positive cells through the cell cycle. Movement of IdUrd-labeled S-phase cells relative to the position of G_0/G_1 and G_2/M is expressed as relative movement (RM) and is calculated as follows:

$$RM = \frac{(F_{IdUrd} - F_{G0/G1})}{(F_{G2/M} - F_{G0/G1})} \quad (1)$$

where F_{IdUrd} (R4) is the mean DNA content of the IdUrd-labeled cell (IdUrd-labeled G_0/G_1 (R3) cells were excluded), $F_{G0/G1}$ (R2) is the mean DNA content of the unlabeled diploid G_0/G_1 population (will also contain the differentiated cell population, if present) and $F_{G2/M}$ (R5) is the mean DNA content of the G_2/M cells.

RM will increase in time from RM = 0.5 at time zero (IdUrd-labeled cells half-way between the G_0/G_1 cells and the G_2/M cells) to RM = 1 when all IdUrd-labeled cells have reached tetraploid DNA content. The time needed for labeled cells to reach tetraploid DNA content is equal to T_s. T_s is calculated from one single sample using the formula:

$$T_s = \frac{0.5}{(RM - 0.5)} \times \Delta t \quad (2)$$

where Δt is the time between pulse-labeling and sampling.

The absolute cell cycle time (T_c) can be calculated if the cells have a uniform distribution through the various phases of the cycle. It follows that:

$$T_c = T_s \times \frac{Ns}{Nc} \quad (3)$$

where N_s is the number of cells in S-phase, N_c is the number of cycling cells (growth fraction, GF), T_s is the duration of the S-phase and T_c is the cell cycle time.

Johansson et al. (1994) recently described a refinement of the method to improve the determination of T_s values by correcting for the nonlinearity of the Begg approach. We have compared the values of T_c calculated both according to Begg et al. (1985) and Johansson et al. (1994) with our data. The T_c is also measured directly as we monitored the movement of IdUrd-positive cells through the cell cycle after pulse labeling. To make use of all our measured data for each specimen, we postulate a function that could approximate the movement of IdUrd-positive cells through the middle of the S-phase. The pattern of IdUrd-positive cells passing through the window in the middle of the S-phase can be approximated with a damped oscillation:

$$y(t) \approx e^{-t/2} * \mathrm{Sin}\left(2\prod \frac{t}{T}\right) \quad (4)$$

where T is the period of the sine function (the cell cycle time). If necessary this formula can be adjusted to calculate the cell cycle time for the passing wave of IdUrd-positive cells.

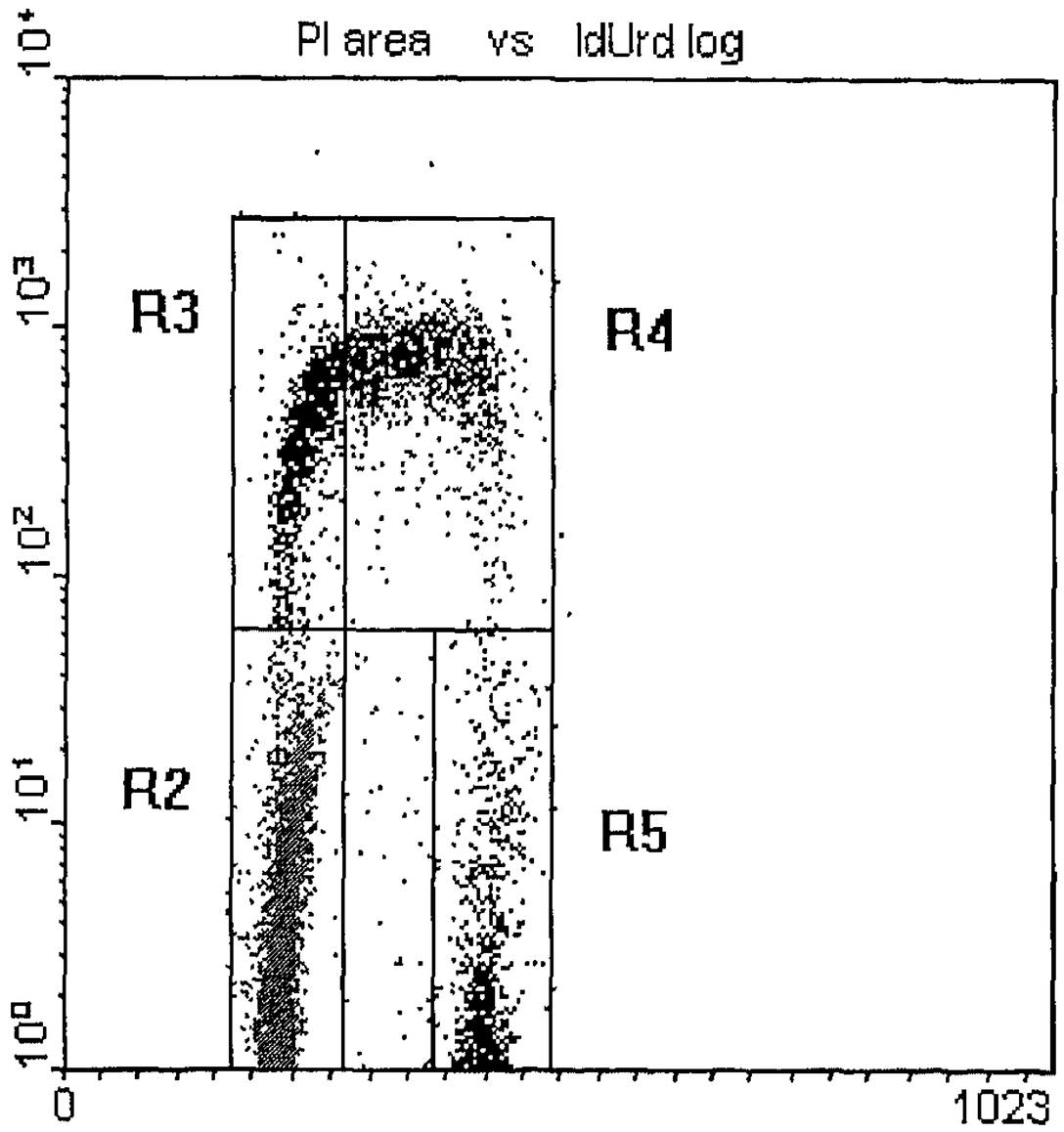
Statistical analysis

Statistical analysis was performed using the statistical analysis software from the SAS Institute BV, Hilversum, in the Netherlands. To estimate the cell cycle times, the measured IdUrd percentages were fitted to formula 4. For comparison of the different cell cycle times and growth restimulation between normal and psoriatic uninvolved keratinocytes, we used a two-way ANOVA with diagnosis (psoriasis and normal) and methods (Begg, Johansson and our model) as factors followed by a Duncan's multiple range test. To test significances between normal and psoriatic cell cycle parameters we used the Student t-test.

RESULTS

Cell cycle analysis

To determine cell cycle parameters, first-passage human keratinocytes were cultured in KGM. When cultures reached about 30–40% confluence they were either pulse-labeled or continuously labeled with IdUrd. Pulse-labeled cultures were harvested at intervals of 4 hours, during a period of 48 hours. Continuously labeled cultures were harvested after 48 hours. From the pulse-labeled cultures the percentage of cells in the



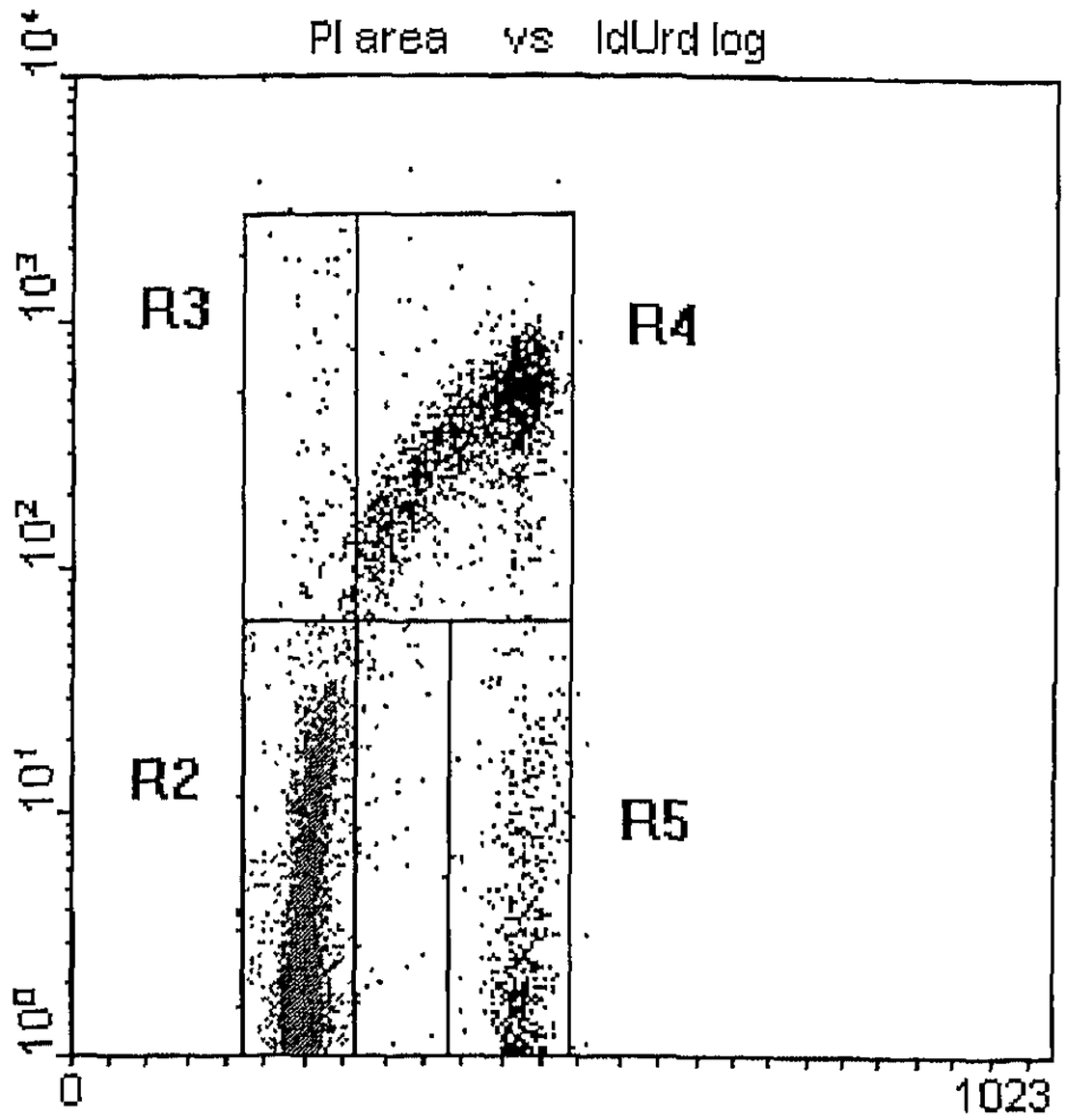


Fig. 1. Dot plot of IdUrd-positive cells after 0 hours and 4 hours of pulse labeling. The windows R2, R3, R4 and R5 represent the following population of cells: R2, the unlabeled diploid G_0/G_1 population (which will also contain the differentiated cell population, if present); R3, the IdUrd-labeled G_0/G_1; R4, the IdUrd-labeled cells from S and G2M; and R5, the unlabeled cells in G_2/M. These regions are necessary to calculate the relative movement. At t = 0 h the figure demonstrates that the mean of the IdUrd-labeled cells lies in the middle of G_0/G_1 and G_2/M, and at t = 4 h the figure shows the movement of cells from the G_0/G_1 position into the S-G_2/M position. Using formulas 1, 2 and 3, T_s and T_c are calculated.

middle of the S-phase was determined. In Figure 2 a typical experiment is shown for the movement of cells in time after pulse labeling with IdUrd. This pattern is identical for normal and psoriatic keratinocytes. Figure 3 represents an example of the fraction of IdUrd-labeled cells in the middle of the S-phase during normal growth in KGM of keratinocytes from normal healthy epidermis. The movement of IdUrd-positive cells through the cell cycle is similar for psoriatic keratinocytes. The fitted curve represents the approximation of the movement of IdUrd-positive cells through the middle of the S-phase by our proposed model (see Materials and Methods).

We have determined the percentages of actively cycling cells (N_c), also known as the growth fraction (GF), using a continuous labeling with IdUrd. As previously demonstrated (van Ruissen et al., 1994) a 48-hour continuous label of IdUrd is long enough to ensure that all cycling cells incorporate IdUrd into the DNA. Growth fractions were in a range of, respectively, 78.8–91.9% and 71.2–94.9% for normal human keratinocytes and psoriatic keratinocytes.

In Table 1 we have summarized the cell kinetic parameters calculated according to Begg et al. (1985). For the same cultures we calculated the cell cycle time according to formula 4. In this model we make use of all the data collected during a 48-hour time period, yielding a calculated cell cycle time based on 13 timepoints measured in duplo. In Table 2 we present our calculated cell cycle times for normal keratinocytes and psoriatic keratinocytes. Furthermore, we have summarized our data obtained from the DNA content analysis and the cell cycle parameters calculated from these data.

To compare our data with data derived from Begg et al. (1985) and Johansson et al. (1994), we performed a two-way ANOVA with methods (Begg, Johansson and formula 4) and diagnosis (normal or psoriasis) as factors followed by a Duncans' multiple range test. From this test we could conclude that the diagnosis was not significant, but that the methods were significantly different. We could not detect a significant difference between our calculated T_c and the T_c calculated according to Begg et al. (1985). Using the calculations proposed by Johansson et al. (1994; data not shown) we find significantly higher T_c values.

Statistical analysis of cell kinetic data from normal and uninvolved psoriatic keratinocytes did not reveal significant differences in cell cycle times and the duration of S-phase. This was found either using the method of Begg et al. (1985) or according to our model. We observed significant differences between the number of cells in the S-phase during normal growth and the duration of the G_1-phase (see Table 2).

Growth restimulation

Keratinocyte cultures were restimulated by removal of TGF-β_1 after 48 hours and addition of fresh growth medium. At successive timepoints 0, 8, 16, and 24 hours after restimulation, the cells were harvested for flow cytometric analysis. In the 8 hours prior to each sample point, cells were labeled with IdUrd. The number of labeled cells is indicative for the number of cells in the cohort and the appearance of labeled cells represents the kinetics as they move through the cell cycle (illustrated by Fig. 4).

We have to realize that the fraction of labeled cells is influenced by T_s, T_c and the labeling duration T_{pulse}

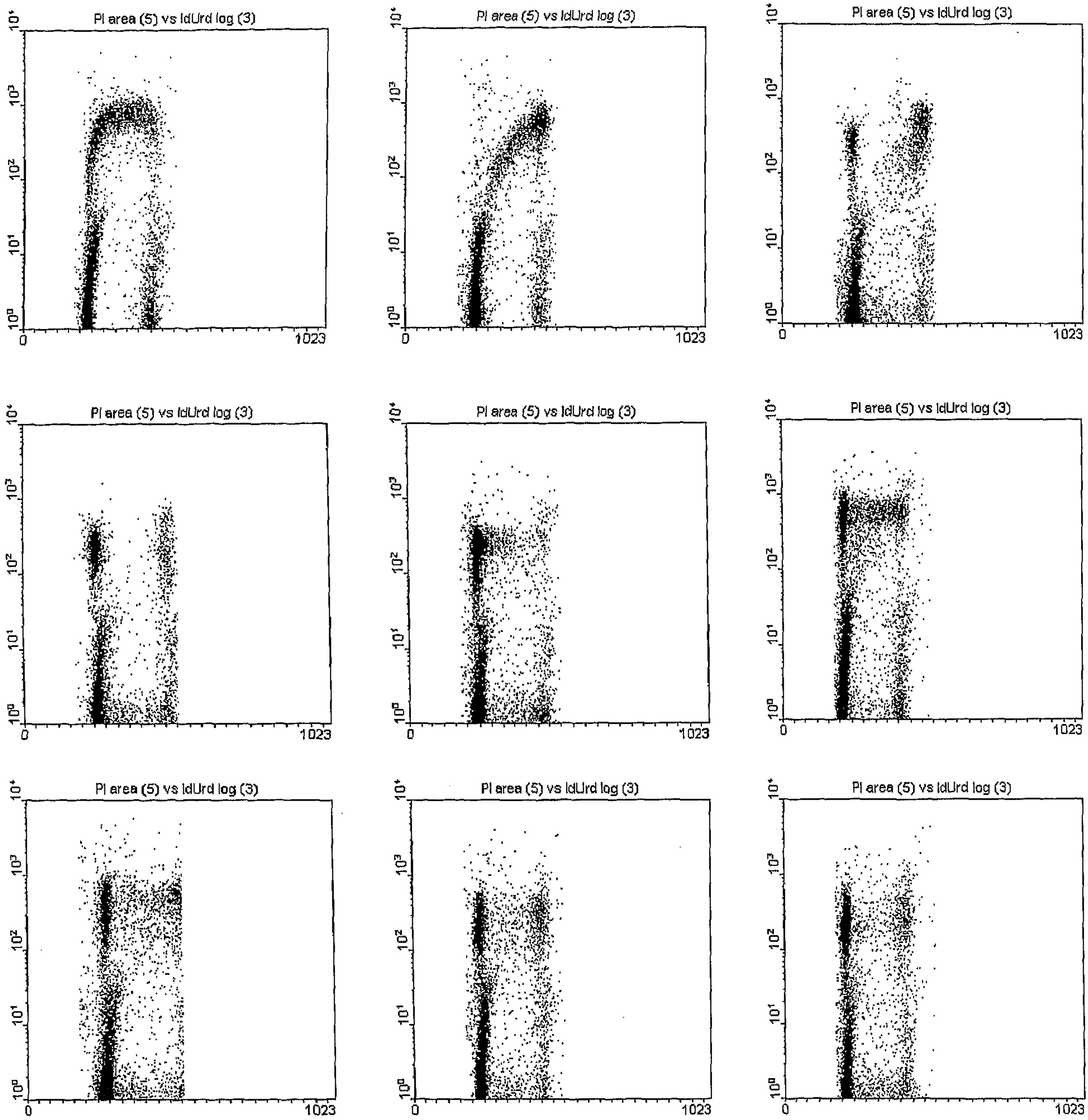


Fig. 2. Demonstration of the movement of IdUrd-positive cells through the cell cycle. From top to bottom and left to right we have arranged the plots of green fluorescence (IdUrd) versus red fluorescence (PI, DNA content) at t = 0, 4, 8, 12, 16, 20, 24, 28, and 32 hours after pulse labeling. With this figure it is demonstrated that the cells move through the cell cycle. At 0 hours after pulse labeling the IdUrd-positive cells are positioned between the G_0/G_1 phase and the G_2/M phase. At 4 hours the cells move in towards the G_2/M phase and at 8 hours a population of cells returned into the G_0/G_1 phase and a population resides in the G_2/M phase (the S-phase is almost empty). Twelve hours after labeling almost all IdUrd-positive cells have returned to the G_0/G_1 phase, and at 16 hours cells are entering the S-phase. This movement continues during the culture period but is damped at later time points, because the IdUrd-positive signal will be dispersed over the daughter cells and finally acquires a random distribution over the cell cycle.

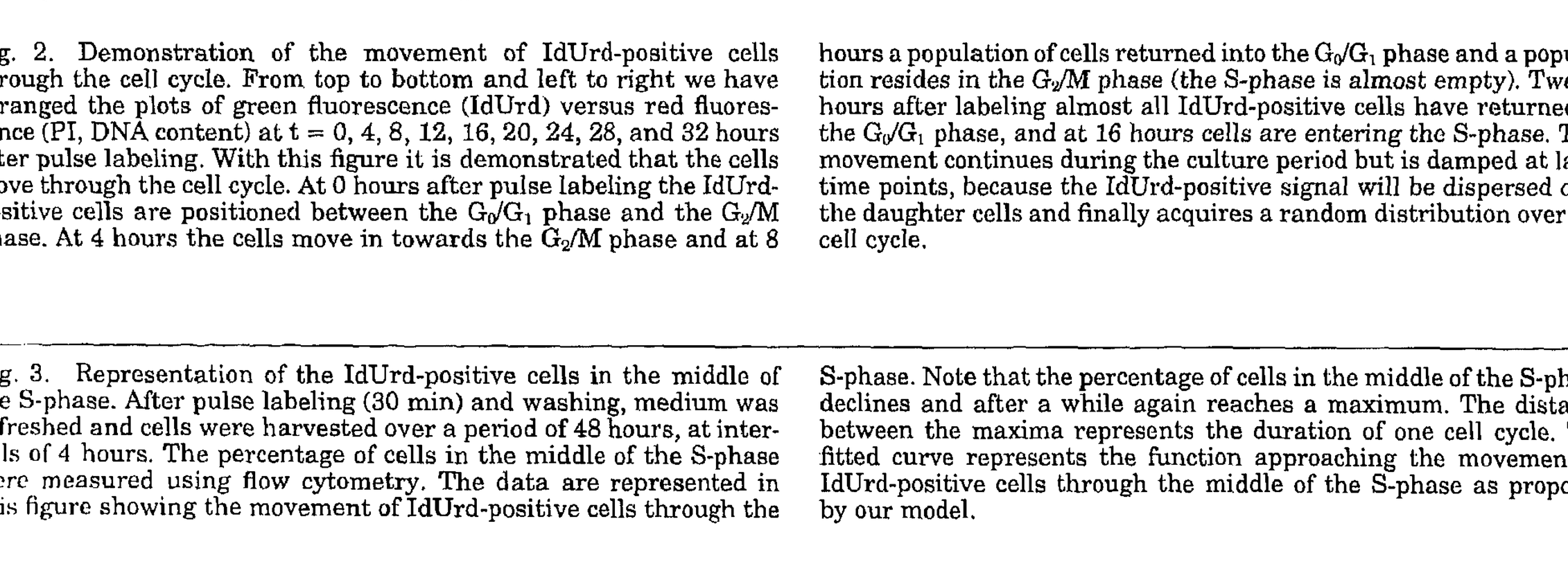


Fig. 3. Representation of the IdUrd-positive cells in the middle of the S-phase. After pulse labeling (30 min) and washing, medium was refreshed and cells were harvested over a period of 48 hours, at intervals of 4 hours. The percentage of cells in the middle of the S-phase were measured using flow cytometry. The data are represented in this figure showing the movement of IdUrd-positive cells through the S-phase. Note that the percentage of cells in the middle of the S-phase declines and after a while again reaches a maximum. The distance between the maxima represents the duration of one cell cycle. The fitted curve represents the function approaching the movement of IdUrd-positive cells through the middle of the S-phase as proposed by our model.

Sheet1 Chart 8

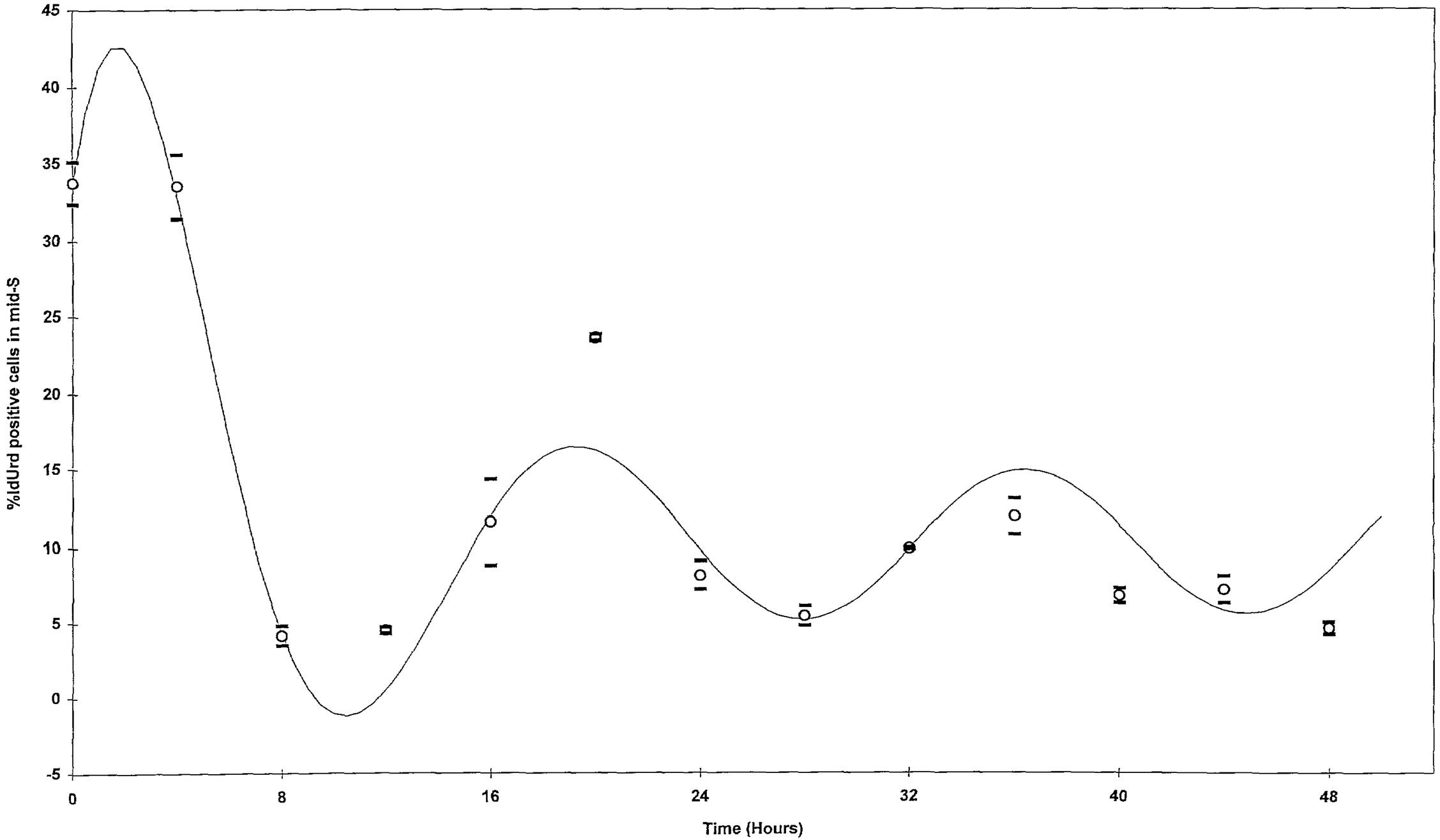


TABLE 1. Summary of cell kinetic data calculated according to Begg et al. (1985)

	Normal					Psoriatic (uninvolved)				
	LI	N_s	N_c (GF)	T_s	T_c	LI	N_s	N_c (GF)	T_s	T_c
	0.244	22.40	91.90	6.62	27.14	0.261	21.75	83.20	7.58	29.01
	0.379	34.05	89.75	5.75	15.16	0.259	21.05	81.35	7.08	27.36
	0.378	29.75	78.80	6.83	18.08	0.207	19.70	94.95	5.62	27.09
	0.329	30.55	92.75	5.34	16.22	0.233	16.60	71.20	5.29	22.71
	0.286	26.30	91.90	7.38	25.78					
Mean	0.32	28.61	89.02	6.38	20.48	0.24	19.78	82.68	6.39	26.54
S.D.	0.06	4.43	5.82	0.82	5.58	0.03	2.28	9.74	1.11	2.69
S.E.M.	0.03	1.98	2.60	0.37	2.50	0.01	1.02	4.35	0.50	1.20

TABLE 2. Comparison of cell kinetic data derived from keratinocytes cultured in serum-free medium

	Normal		Psoriasis (uninvolved)	
	Mean	S.E.M.	Mean	S.E.M.
T_c[2]	21.93	3.15	20.77	1.82
T_s[5]	7.17	1.42	4.92	0.20
Ng_0[3]	10.98	2.60	17.33	4.35
Ng_1[3]	50.83	2.46	51.66	3.52
Ng_2/m[3]	12.59	2.29	15.06	0.81
N_s[1,3]	25.63	1.86	16.04	1.05
Tg_1[1,4]	11.73	1.53	16.53	0.69
Tg_2/m[4]	3.01	0.82	4.87	0.41
T_s[4]	5.74	0.41	5.17	0.39

[1]Significant difference.
[2]Determined from direct measurement.
[3]Determined from the DNA histogram analysis with multicycle and formula [5].
[4]Determined with the multicycle parameters and the calculated T_c and GF.
[5]Determined from the measured T_c.

of 8 hours. The maximum fraction of labeled cells in an asynchronously growing culture can be expressed by

$$\frac{N_{labeled}}{N_c} = \frac{(T_s + T_{pulse})}{T_c} \quad (5)$$

As T_s and T_c differ for psoriatic and normal keratinocytes, the maxima are 51% and 60% respectively and the measured data are normalized in this respect.

Statistical analysis, using a two-way ANOVA (with diagnosis and IdUrd percentages as factors) followed by a Duncans' multiple range test (with different sample periods as factors), showed no significant difference in the kinetics of growth restimulation of psoriatic uninvolved and normal keratinocytes.

DISCUSSION

Early studies on keratinocyte proliferation have suggested that basal epidermal cells were equipotent with similar cell cycle times and probabilities of cell division. However, in later studies it became clear that the basal cell population is heterogeneous (reviewed in Dover, 1994). The main attempt in studies with respect to keratinocyte proliferation was to demonstrate the presence and localization of stem cells in the epidermis. With reference to the epidermis, stem cells are defined as keratinocytes with the capacity for unlimited self-renewal and whose daughters may either be stem cells or else be committed to undergo terminal differentiation and (ultimately) become a squame (Watt, 1988). At present there is no direct evidence for a stem cell population in the basal layer of the epidermis. Several publications describe the existence of epidermal stem cells in the hair follicle (reviewed in Dover, 1994), based on the idea that the epidermis may be reepithelialized after injury by migration of keratinocytes from the hair follicle. Other reports describe the existence of epidermal stem cells based on different surface expression of β_1-integrins, adhesion to collagen, and colony-forming efficiency (Watt and Jones, 1993; Jones and Watt, 1993). A recent report describes the existence of a β_1-integrin-positive, cytokeratin 1-, cytokeratin 10- and PCNA-negative epidermal subpopulation (containing stem cells), and a β_1-integrin, cytokeratin 1-, and cytokeratin 10-positive subpopulation, (transit amplifying cells; Bata-Csorgo et al., 1993, 1995). From these observations it is evident that the basal layer of normal epidermis consists of a heterogeneous population of keratinocytes containing progenitor cells that are responsible for continued local renewal of the epidermis. Some of these basal cells become committed to differentiation (expressing involucrin) and have the ability to undergo several rounds of amplification (transit amplifying cells; Wilke et al., 1988a; Bata-Csorgo et al., 1993).

Previously it was believed that the turnover of epidermis was regulated by variations in the cell cycle time of the stem cell population and that the GF was unity. In 1976, the first argument that germinative cells could reside in a noncycling state was presented by Gelfant (1976). He introduced the existence of noncycling (G_0) cells. This hypothesis was confirmed by several investigators (Gelfant, 1982; Eaglstein and Weinstein, 1975). We have previously shown that between 36 and 52 hours after tape-stripping, 23% of the viable epidermal cells moved as a cohort through the cell cycle (Boezeman et al., 1987). Such a well-defined cohort only results from a population of diploid cells simultaneously entering the cell cycle, meaning that these cells have the same starting point in the cell cycle. These results indicated that these recruited cells have a static position somewhere in the G_1 phase of the cell cycle, termed the G_0 phase. It is clear that the epidermal cell population comprises two distinct subpopulations termed "cycling" and "quiescent." The rate of cell production is determined by the ratio of these two populations, called the GF. In normal epidermis the growth fraction is around 10% (Bata-Csorgo et al., 1993; van Erp et al., 1991); following injury there is a transient increase, and in lesions of psoriatic epidermis the growth fraction remains permanently increased in the range of 50–100%. This concept is supported by a number of studies using bivariate flow cytometric analysis (IdUrd incorporation and DNA content) or the mono-

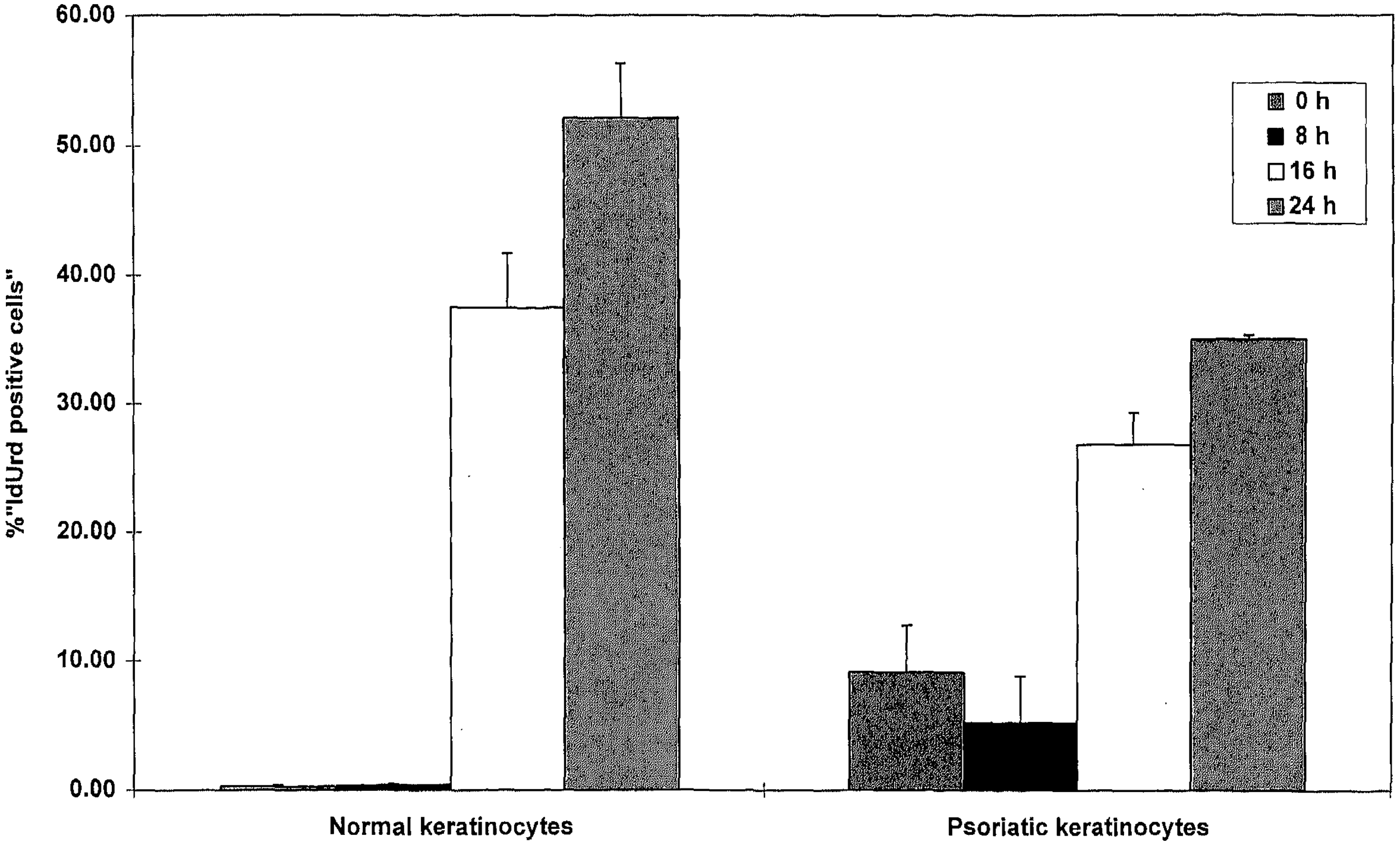


Fig. 4. Growth restimulation after induction of quiescence with TGF-β_1. Keratinocyte cultures of normal and uninvolved psoriatic keratinocytes were growth-arrested with TGF-β_1, cultures were washed and restimulated with fresh growth medium. Cultures were harvested after an 8-hour pulse label, at 0, 8, 16, and 24 hours following growth restimulation and analyzed using flow cytometry. The percentages of IdUrd-positive cells (y-axis) were normalized according to formula 6. The kinetics of growth restimulation of normal and psoriatic keratinocytes was not significantly different.

clonal antibody Ki-67 (Rijzewijk et al., 1989; van Erp et al., 1989).

During the past decade, many investigators have studied cell cycle parameters of human keratinocytes. Most of these papers give only ratiometric information (i.e., mitotic index and/or labeling index). The data given in the literature show a great deviation in the observed cell cycle times ranging from 12 to 316 h for normal keratinocytes (epidermis), 28 to 279 h for psoriatic involved keratinocytes (epidermis) and 28 to 149 h for psoriatic uninvolved keratinocytes (epidermis). The S-phase durations show less variation between the various observations, ranging from 6 to 16 h and 6 to 20 h for, respectively, normal and psoriatic (uninvolved) epidermis. On the one hand, the differences might be explained by clinical variations between patients, methodological errors, or statistical problems due to low numbers of labeled cells. On the other hand, there are discrepancies in the definition of the growth fraction. Some authors interpret their calculations on the assumption that the germinative pool is identical to the actively cycling pool. In other words, the growth fraction is unity. In psoriatic epidermis and in cultures this might be the case, but in normal epidermis it certainly is not. Furthermore, we have to keep in mind that passaged keratinocytes differ from the in vivo situation in their expression of cytoskeletal proteins, membrane composition, cytokine production and cell surface integrins (Bata-Csorgo et al., 1995).

They also differ in the composition of cells from the proliferative compartment, because cell cycle quiescent (G_0) cells are underrepresented and highly cycling clonogenic stem cells and transit amplifying cells are overrepresented (Bata-Csorgo et al., 1995). It has also been demonstrated that cultures enriched in cells isolated from the suprabasal stratum spinosum can be cultured in serum-free medium (Wilke et al., 1988a). The colony-forming efficiency of these cultures was at least comparable with cultures enriched with basal cells. It was even demonstrated that involucrin-containing cells (committed to differentiation) were able to proliferate in vitro. Studying cell cycle parameters in vitro means that we measure the parameters of a population containing basal cell and suprabasal cells, these include "stem cells" and "transit amplifying cells." These cells may differ in their growth characteristics, but the analysis finally leads to the expression of average cell cycle parameters.

The cell kinetic analysis that we made for normal and psoriatic keratinocytes uses flow cytometry combined with IdUrd labeling using time courses with many sampling points. This methodology allows a very accurate determination of cell cycle parameters for each cell line. Obviously this study also suffers from a number of limitations. Firstly, the cells that are obtained from the primary cultures reflect the clonogenic population in vitro and are not necessarily representative for the populations in normal or psoriatic skin in vivo. Secondly,

although the methodology used allows a very accurate determination of the cell cycle parameters for each cell line, we found that the interindividual variation of the cell cycle parameters found in the 5 normal cell lines and the 4 psoriatic cell lines is considerable (see Table 1). From our cell kinetic analysis we conclude that there are no gross differences between normal and uninvolved psoriatic keratinocytes with respect to the S-phase duration and total cell cycle duration. According to the statistical analysis we could not detect a significant difference between the data calculated according to Begg et al. (1985) and the data calculated according to our model. However, we prefer to use the data generated using our model since these are based on many time points, without making the assumptions as has been done by Begg et al. (1985). When we examine the other cell cycle parameters we can conclude that there are significant differences between normal and uninvolved psoriatic keratinocytes with respect to the percentage of cells performing DNA synthesis and the duration of the G_1-phase. Psoriatic keratinocytes display a longer duration of the G_1-phase of the cell cycle and have a smaller percentage of cells in S-phase. This apparent difference is only found when our model for calculation of cell cycle parameters is used, and not when the method of Begg et al. (1985) is applied. This apparent difference requires further examination using a larger series of cell lines. Our findings are in contrast with the data of Kragballe et al. (1985) who observed an increased DNA synthesis that is maintained in vitro, when using primary keratinocyte cultures cultivated on lethally irradiated 3T3 cells. Furthermore, they measured DNA synthesis by the incorporation of ^{3}[H]-thymidine after the addition of 10% normal human serum. Our cell cycle data of normal keratinocytes are in agreement with previous investigations using different methodology in the same serum-free culture medium (van Ruissen et al., 1994; Wille et al., 1984), whereas we provide new data on psoriatic keratinocytes.

It has been described that TGF-β_1 has potent antiproliferative effects on a variety of epithelial cell types in vitro, including keratinocytes (Moses et al., 1987; Matsumoto et al., 1990; Reiss and Sartorelli, 1987; Shipley and Pittelkow, 1987; Shipley et al., 1986). The inhibition of human keratinocyte proliferation in vitro by the addition of TGF-β_1 to the culture medium is reversible, and results in growth arrest predominantly in the G_0/G_1-phase of the cell cycle (Reiss and Sartorelli, 1987; Wilke et al., 1988). This growth arrest state is comparable to the situation in normal skin, where approximately 90% of the cells are in the G_0/G_1-phase of the cell cycle (van Ruissen et al., 1994). On the basis of these investigations it has been hypothesized that TGF-β_1 could function as a negative growth regulator in normal skin (Shipley et al., 1986; Wilke et al., 1988). It might be possible that keratinocytes derived from psoriatic epidermis could be deficient in either the production of TGF-β_1 or the responsiveness to TGF-β_1 resulting in the characteristic hyperproliferation in psoriasis. Investigation of the induction of quiescence showed that both normal and uninvolved psoriatic keratinocytes can be growth-arrested within 56 hours after the addition of TGF-β_1. We therefore conclude that epidermal hyperproliferation in psoriasis is not caused by a decreased responsiveness of the keratinocytes to TGF-β_1. These results are in agreement with the data from Elder et al. (1990). They have shown that TGF-β_1 mRNA expression was similar in normal epidermis and psoriatic lesional epidermis. They further report that keratinocytes derived from normal and psoriatic lesional epidermis do not differ in antiproliferative responsiveness to TGF-β_1 and that the number of TGF-β_1 receptors per cell and binding constants are comparable (Elder et al., 1990).

Quiescent keratinocyte cultures can be restimulated by removal of TGF-β_1 and addition of fresh growth medium. To investigate if there were differences in growth restimulation between normal and uninvolved psoriatic keratinocytes, we compared three cultures of each type. Analysis of the data suggested a difference in IdUrd-positive cells after 8 to 16 hours and 16 to 24 hours of growth restimulation between normal and uninvolved psoriatic keratinocytes and not between −8 to 0 hours and 0 to 8 hours. Statistical analysis did not show a significant difference in the kinetics of growth restimulation between normal and uninvolved psoriatic keratinocytes.

From this study we can conclude that the hyperproliferation seen in psoriasis is not due to intrinsic differences in cell cycle duration or growth fraction between normal and psoriatic keratinocytes. In addition, normal and psoriatic keratinocytes are equally responsive to TGF-β_1. If the hyperproliferative response found in vivo is based on mechanisms inherent to the keratinocyte, it is likely to be due to factors that affect responsiveness to external factors (growth factor receptors, signal transduction pathways) rather than the "setting" of cell cycle times. Recent studies by Bata-Csorgo et al. (1995) indeed suggest that epidermal β_1-integrin-positive subpopulations are hyperresponsive to T-cell-derived cytokines. The culture model and the methodology for analysis of proliferation, induction of quiescence, and recruitment from G_0 will be used for further studies on the involvement of T-cell-derived factors in the hyperproliferative process seen psoriasis.

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