Involucrin-Positive Keratinocytes Demonstrate Decreased Migration Speed but Sustained Directional Migration in a DC Electric Field

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When skin is wounded, keratinocytes from the cut edges of the epidermis migrate over the wounded area to re-epithelialize the wound. It is not clear which cells of the epidermis have the capacity to migrate and contribute to this re-epithelialization: the less differentiated cells of the basal layer, or the more differentiated, involucrin-positive suprabasilar cells. Here we demonstrate that both involucrin-negative and involucrin-positive cells are able to respond to a directional cue for migration with sustained directional migration. When cultured keratinocytes are exposed to a physiologic DC electric field of 100 mV per mm as a cue to guide migration (galvanotaxis) they migrate toward the cathode with equivalent directionality. The involucrin-positive cells, however, display mean migration speeds approximately one half (23.6 μm per h) of the mean rate achieved by involucrin-negative cells (46.5 μm per h). Despite their decreased migration rates, involucrin-positive cells appear to possess an intact mechanism for sensing a directional signal, transducing that signal, and responding with sustained directional migration. Because electric fields are endogenous in skin wounds, it is likely that both the basal, involucrin-negative cells and the involucrin-positive suprabasilar cells respond to this cue with directional migration. The new observation that involucrin-positive cells can indeed migrate suggests that these cells may also contribute to wound re-epithelialization *in vivo*. *Key words: cell motility/galvanotaxis/wound current/wound healing. Journal of Investigative Dermatology 113:851–855, 1999*

Re-epithelialization of wounded skin is essential for re-establishment of normal function. The re-epithelialization process requires not only the induction of locomotion in epithelial cells, but also their migration with sustained directionality, so that they effectively move to cover the wound defect. Directional stimuli likely to be present in the wound include gradients in components of the extracellular matrix, gradients in soluble chemotaxic agents (Anand-Apte and Zetter, 1997), and a gradient in the lateral electric field that is generated by the wounded epithelium (Jaffe and Vanable, 1984).

The importance of these endogenous skin wound fields in wound healing is becoming increasingly evident. In mammals, lateral fields of 10–100 mV per mm have been measured at the borders of skin wounds, with the positive pole directed toward the center of the wounded area directly beneath the stratum corneum of the epidermis (Barker, 1981; Barker *et al*, 1982; Chiang *et al*, 1992; Olivotto *et al*, 1996), and it has been suggested that lateral fields of the opposite polarity, with the negative pole pointing to the center of the wound, are present at the lower layers of the epidermis (Vanable, 1989). Recently we have shown that normal human keratinocytes (NHK) migrate to the cathode with a rate of about 1 μm per min in fields of physiologic strength (100 mV per mm) (Nishimura *et al*, 1996; Fang *et al*, 1999). In the absence of the field, NHK move at a similar rate but without directionality, indicating that electric fields affect only the directionality of cell motility. Whereas this cathodal galvanotaxis response would be in the opposite direction for wound closure at the uppermost layers of the epidermis, at the basal layer, where migration is first noted to occur, it is oriented appropriately: the center of the wound is negative relative to the wound edge. For these reasons, lateral electric fields have been proposed to provide one of the earliest cues for guiding the migration response of keratinocytes resurfacing skin wounds (Jaffe and Vanable, 1984; Nishimura *et al*, 1996; Sheridan *et al*, 1996; Fang *et al*, 1999)

Skin wound re-epithelialization has been studied *in vivo* (Ortonne *et al*, 1981; Mansbridge and Knapp, 1987) and in an *in vitro* skin wound model (Garlick and Taichman, 1994). These studies use histologic or immunohistochemical techniques to examine a resurfacing wound and characterize the chronology of the re-epithelialization process. Studies such as these have suggested that cells expressing the differentiation markers keratin 1/10, or involucrin, normally present only in the suprabasilar layers of uninjured epidermis, are the first cells to migrate to form the advancing epithelial tongue of the healing wound margin. This is in marked contradistinction to the reported inability of differentiated keratinocytes to migrate in an *in vitro* system (Nickoloff *et al*, 1988). These two contradictory findings leave the question of the ability of differentiated keratinocytes to migrate unresolved.

In this study, we use galvanotaxis as a tool to study the very early migration response of keratinocytes to a directional signal.

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Abbreviations: DC, direct current; EGFR, epidermal growth factor receptor.

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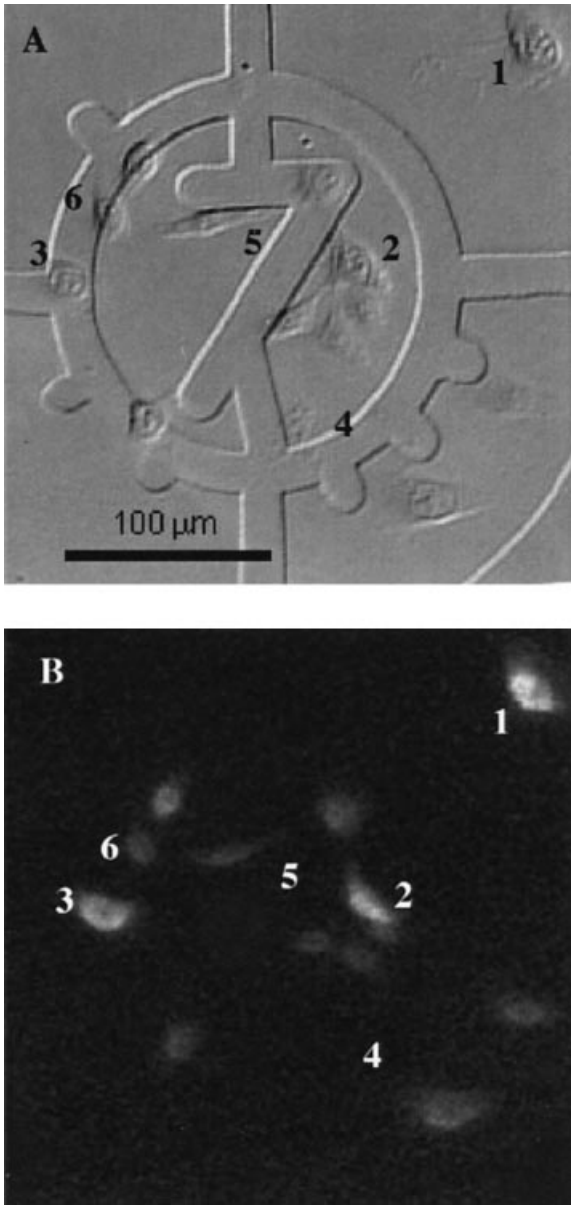


Figure 1. The migration pattern of involucrin-positive and involucrin-negative keratinocytes can be mapped. Keratinocytes plated on coverslips were subjected to DC electric fields of 100 mV per mm for 1 h, during which time their positions were captured using time lapse videomicroscopy, and each cell's migration characteristics calculated. At the end of the experiment, the coverslips were stained to immunohistochemically detect involucrin. Part (*A*) shows the positions of the cells on a gridded coverslip using phase contrast optics. Part (*B*) shows the same microscope field after immunostaining for involucrin. Cells marked 1, 2, and 3 were scored as involucrin-positive, using the criteria delineated in the *Materials and Methods* section. All other cells, including those visible under phase optics and only faintly visible with fluorescence optics (e.g., cells marked 4, 5, and 6), were scored as involucrin-negative. *Scale bar*: 100 μm.

This study provides information regarding the migration rates of individual keratinocytes based on direct, real-time observations. Because the system utilized in this report allows for the tracking of individual cells, we are able to determine the migration characteristics of individual, differentiated (involucrin-positive) or nondifferentiated (involucrin-negative) cells. We find that both differentiated and nondifferentiated cells can respond to an imposed electric field with directional migration. The rate of their response, however, differs significantly, with differentiated cells moving at approximately half the speed of nondifferentiated keratinocytes. The data suggest that both differentiated and nondifferentiated cells, by virtue of their ability to respond to an electric field of the same magnitude as that generated endogenously by a skin wound, may contribute to its re-epithelialization. The more rapid migration rate of the nondifferentiated keratinocyte, however, suggests that it would be the more effective cell for the task of resurfacing the denuded area.

MATERIALS AND METHODS

Cell culture Human epidermal keratinocytes are derived from neonatal foreskins discarded at the time of elective circumcision as we have reported previously (Sheridan *et al*, 1996). Briefly, the epidermis is separated from dermis by overnight trypsinization at 4°C, and after dispersal into a single cell suspension, keratinocytes are plated on a bed of mitomycin C-treated 3T3 feeder cells, as originally described by Rheinwald and Green (1975). After establishment of secondary cultures, cells are routinely maintained in a low calcium (0.2 mM) serum-free keratinocyte growth medium (KGM, Cascade Biologics, Portland, OR), supplemented with 100 IU each of penicillin and streptomycin and 0.25 5 g amphotericin. Cultures are maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells between passage 3–4, from strains derived from three different donors, are used for all experiments.

Electric field application and analysis of migration Keratinocytes are plated on 2% collagen I-coated (Vitrogen, Collagen Biomaterials, Palo Alto, CA.), alphanumerically gridded plastic coverslips (CELLocate, Eppendorf Scientific, Hamburg, Germany) at low densities (5.2×10^3 cells per cm^2) to allow attachment of single cells without the formation of groups. After cell attachment and spreading for 2 h, the coverslip is mounted in a plexiglass chamber constructed so that there is a reservoir containing medium on either side of the coverslip with flow from one side to another, as previously described by Erickson and Nuccitelli (1984). The chambers are sealed with tape and silicone high-vacuum grease to prevent evaporative loss an pH change in the medium. The experimental chamber contains serum-free, supplemented KGM with a final calcium concentration of 1.8 mM, and 10 mM HEPES, pH 7.4. Medium is first added to one well of the chamber and only after a clear path for medium flow over the cells is established, is medium added to the second well. A constant DC voltage of 100 mV per mm is applied across the chamber, applied through Ag-AgCl electrodes in each well. Agar-filled glass bridges separate the electrodes supplying the current from the wells of the chamber to prevent diffusion of electrode products into the medium. The cells are subjected to an electric field of 100 mV per mm for 1 h, with continuous monitoring of current. Other coverslips are simultaneously subjected to 0 mV per mm on identical set-ups to serve as controls.

During application of the electric field, coverslips are observed using phase contrast optics on an inverted microscope. Images are captured directly from a video camera with a frame grabber on a Macintosh power PC, for which we have written a subroutine program that interfaces with the program NIH Image, version 1.60. Cells in the field are identified at the beginning of the observation period and images automatically captured and recorded every 10 min from the duplicate experimental and control set-ups. After each cell is tracked, the data are automatically exported to FileMaker Pro 3.0 where the data are analyzed and stored. Cell migration is quantitatively analyzed for both speed and directionality of migration. Cosine ø describes the direction of migration, where ø is the angle between the electric field axis and the vector drawn between the initial and final cell position. The mean cosine $ø = \Sigma_i \cos ø_i/N$, where Σ_i is the summation of individual cells from 12 separate experiments. Angle zero ($ø = 0°$) is assigned to the negative pole and increasing angles assigned in a counterclockwise manner, with $ø = 180°$ aligned with the positive pole. Therefore, taking the cosine of ø yields an index of directional migration: cosine $0° = 1$ and cosine $180° = -1$ and all other angles $1 > \cos ø_n > -1$. For example, if a cell were to move directly to the negative pole, $ø = 0°$ and cosine $ø = 1$. Likewise, if a cell were to move toward the positive pole, $ø = 180°$ and cosine $ø = -1$. "Translocation distance" refers to the average distance the cell travels in a period of time. It is important to note that the data are analyzed to describe two distinct characteristics of cell motility: the directedness of the migration response is described by cosine ϕ, whereas locomotory activity is described by the translocation distance, or translocation rate (mean translocation distance/time). Because only 10–15 cells are visualized in the field during the 1 h period of electric field application, data were collected from 12 separate experiments and pooled to achieve statistical significance. Analysis of statistical significance was performed using the unpaired Student's t test, with significance assigned to p values less than 0.05.

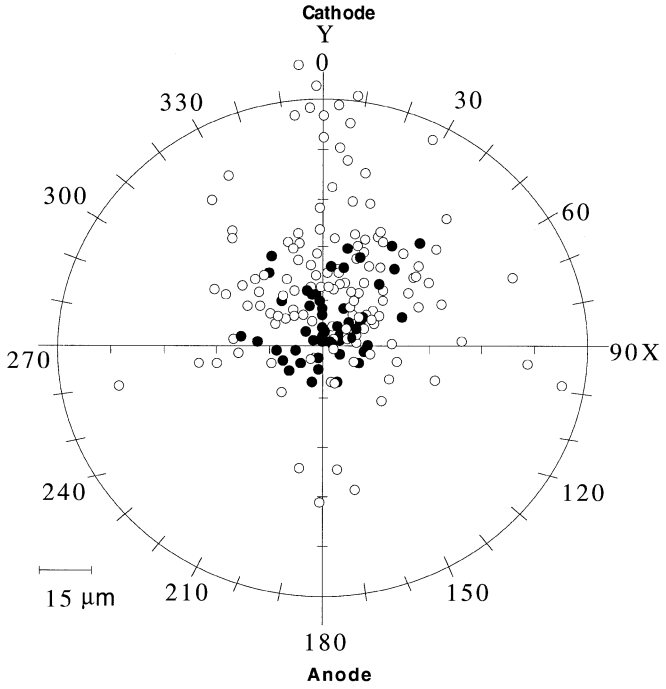


Figure 2. Involucrin-positive cells migrate directionally, but at a slower rate than involucrin-negative cells. Keratinocytes on coverslips were subjected to an electric field of 100 mV per mm for 1 h, and processed for immunostaining for involucrin, as described in the *Materials and Methods*. The directed migration in the electric field was quantified by calculating the mean cosine ϕ of the angles of migration in relation to the anodal-cathodal orientation. The starting position of each cell is represented by the center of the circle, and the final position was plotted using a filled circle (●) for an involucrin-positive cell and a clear circle (○) for an involucrin-negative cell. The 0° position represents the negative pole (cathode), therefore the predominance of cells at the upper half of the graph indicates their preferential directional migration toward the cathode. The grid marks on the X and Y axes represent 15 μm distance traversed by migrating cells. Cells closer to the graph's origin migrated shorter distances than did cells whose final position is further from the origin.

Analysis of keratinocyte differentiation Expression of involucrin, as detected by immunofluorescent staining, is used to identify differentiated keratinocytes. After the 1 h application of electric field, the coverslips are washed two times with phosphate-buffered saline (PBS) for 5 min each, then fixed with 4% paraformaldehyde in PBS for 7 min. After permeabilization with 0.5% Triton X-100 in PBS for 3 min, nonspecific sites are blocked by 1% bovine serum albumin in PBS for 15 min. The coverslips are then incubated in 1:50 dilution of anti-involucrin monoclonal antibody (Sigma Immuno Chemical, St. Louis, MO) for 1 h at room temperature, rinsed with PBS, and incubated with cy3 Goat Anti-Mouse IgG at 1:200 dilution for 45 min (Biomedical Technologies, Stoughton, MA). Coverslips are incubated with equilibrating buffer (Slowfade Component C, Molecular Probes, Eugene, OR) for 10 min, then mounted, sealed, and observed under a Nikon microscope. Negative controls (not shown) for all experiments included substituting blocking buffer for the first antibody and immunostaining dermal fibroblasts (which do not contain involucrin) with the anti-involucrin antibody. Images were captured by a CCD camera to a computer and stored on optical disks. The correlation between the differentiation state of the cells and their migration patterns is made by comparing the immunofluorescent images with the phase microscopic images captured during field application. A cell was scored as involucrin-positive if its mean fluorescence intensity was 6×, or above, the background fluorescence value. The average intensity of fluorescence of each cell was calculated using the program NIH Image, tracing the outline of each cell and determining the average pixel intensity of the enclosed area.

RESULTS

The migration pattern of involucrin-positive and involucrin-negative keratinocytes can be mapped At the end of the 1 h period of field application, images of identical microscope fields, viewed either with fluorescence or phase optics, were superimposed and the final positions of the involucrin-positive or -negative cells were determined. By analyzing the migration characteristics of each individual cell in the field, the translocation distance and cosine ϕ, the measure of directedness of migration, could be determined for each cell (**Fig 1**). Occasionally, cells appearing in the phase time lapse images were absent from the fluorescent image. It is likely that these cells were dislodged from the coverslip during the immunofluorescence fixation protocol. These cells were excluded from the motility analysis.

Table I. Involucrin-positive cells migrate directionally, but at a slower rate than involucrin-negative cells[a]

	Involucrin positive[b] cells (n = 50)	Involucrin negative cells (n = 139)	p value
Mean translocation distance	23.60 μm ±1.16	46.49 μm ±2.49	<0.0001
Mean cosine ø	0.42 ±0.042	0.56 ±0.095	>0.05

[a]The data are derived from the individual cell analysis, graphically depicted in **Fig 2**.
[b]Keratinocytes plated on coverslips were subjected to DC electric fields of 100 mV per mm for 1 h, during which time their positions were captured using time lapse videomicroscopy, and each cell's migration characteristics calculated. At the end of the experiment, the coverslips were stained to immunohistochemically detect involucrin. The migration characteristics of the involucrin-positive and involucrin-negative cells were determined. Values are means ± SEM.

Table II. Imposition of an electric field does not alter the percentage of involucrin-expressing keratinocytes[a]

	No field Field (100 mV per mm)	(0 mV per mm) (n = 189)	p value (n = 200)
Per cent of cells positive for involucrin	15.5 (±1.5)	26.4 (±3.9)	>0.05*

[a]Keratinocytes plated on coverslips were subjected to DC electric fields of 100 mV per mm for 1 h, then fixed and immunostained for involucrin expression. The average per cent of positive cells in 11 experiments from three different donors is given, ± the SEM. *Actual p value = 0.067.

Involucrin-positive cells migrate directionally, but at a slower rate than involucrin-negative cells Both involucrin-positive and -negative keratinocytes respond to the applied electric field with directional migration toward the cathode, with mean cosine ϕ of 0.56 and 0.42, respectively (**Table I**, and **Fig 2**); however, the translocation rate for involucrin-positive keratinocytes is much slower than for involucrin-negative keratinocytes during the 1 h electric field application. The differentiated keratinocytes traveled an average of 23.6 μm in 1 h in an electric field, whereas the undifferentiated keratinocytes averaged 46.5 μm over the same time period.

Imposition of an electric field does not alter the percentage of involucrin expressing keratinocytes To determine whether the imposition of an electric field upon keratinocytes alters their differentiation, we compared the percentage of involucrin-positive keratinocytes in a population of cells subjected to a constant DC field of 100 mV per mm to a control population subjected to 0 mV per mm. **Table II** shows that 15.5% of control cells (0 mV per mm) were positive for involucrin whereas 26.4% of those cells subjected to 100 mV per mm were positive for involucrin. Although this suggests that the imposition of the electric field may slightly increase involucrin expression, the differences between these two groups does not reach statistical significance (p = 0.067).

DISCUSSION

Skin wound healing requires the directional migration of keratinocytes from the wound edges into the wounded area (Odland and Ross, 1968; Croft and Tarin, 1970; Ortonne *et al*, 1981).

Although previous investigations have demonstrated the importance of soluble cytokines and basement membrane matrix proteins in stimulating motility in general (reviewed in Nickoloff *et al*, 1988; Sarret *et al*, 1992; Clark, 1993; Falanga, 1993; Woodley *et al*, 1993), the question of the directional stimulus that guides the migrating keratinocyte to the denuded wound surface is not yet answered. There is a body of evidence to support the notion that endogenously generated electric fields may serve as early guidance cues for this directional migration. Work by Borgens *et al* suggested that in the newt, the transepithelial potential can drive a current out of a cutaneous wound, and that this current may play a role in directing epithelial cell migration (Borgens *et al*, 1977). Subsequent findings of electrical fields in the immediate vicinity of mammalian wounds (Barker *et al*, 1982), and the directional migration response of human keratinocytes in electric fields of physiologic magnitude (Nishimura *et al*, 1996), further advance the hypothesis that endogenous wound electric fields function as an early stimulus for directed keratinocyte migration.

Keratinocytes found within the intact epidermis are heterogeneous: with the proliferative, nondifferentiated cells in the basal layer, and cells expressing a more differentiated phenotype in suprabasilar layers. Mansbridge and Knapp's (1987) work demonstrates that involucrin-positive cells can be found in newly re-epithelialized skin wounds. Because theirs is a histologic study of fixed tissue, the finding of involucrin-positive cells within the wound bed does not discriminate between two possibilities: (i) that involucrin-positive (differentiated) cells can migrate into the wound, or (ii) that nondifferentiated, involucrin-negative cells change their phenotype once exposed to a wound environment and encounter a "wound" matrix, i.e. the provisional matrix of fibrinogen/fibrin. This study asks the question of which subpopulation of keratinocytes, differentiated or nondifferentiated, are capable of migration in response to a directional signal. Here we use an *in vitro* system to examine the ability of keratinocytes to migrate as single cells on a collagen I matrix. Although this system differs from the *in vivo* wound environment, most notably by limiting the extracellular matrix components to collagen I, it allows the tracking of the migratory paths of individual cells. Using this system we demonstrate, for the first time, that differentiated keratinocytes expressing involucrin are indeed capable of migration. We find that differentiated keratinocytes can respond to a directional stimulus although their migration rate is much slower than that of nondifferentiated, involucrin-negative cells.

The application of an electric field during the course of the experimental observation period does not significantly change the percentage of involucrin-positive cells; however, it is only a 1 h exposure and we cannot say at this time whether longer exposures to DC fields of this magnitude alter differentiation characteristics. Because in the *in vivo* wound, keratinocytes at the wound margin are exposed to wound-generated electric fields for days or weeks [essentially until the wound is re-epithelialized and the current flow is blocked (Illingworth and Barker, 1980)], it is possible that longer term DC electric field exposures may alter either the differentiation or the migration characteristics of exposed cells. At present, the experimental constraints of our system, where cells can only retain viability and optimal motility for 1–2 h, precludes longer term observation. We are currently working on devising a longer term experimental apparatus.

Perhaps the notion that differentiated cells migrate with slower speeds than nondifferentiated cells is intuitive. As keratinocytes differentiate, the number of receptors for the motility-enhancing growth factor EGF decreases (Nanney *et al*, 1984; Green and Couchman, 1985; Kusumi *et al*, 1993). EGF not only induces keratinocyte migration (Barrandon and Green, 1987; Chen *et al*, 1993; Ando and Jensen, 1993) but also increases migration speed in fibroblasts (Ware *et al*, 1998). Thus, it is logical to suppose that differentiated keratinocytes, with decreased numbers of EGFR, exhibit decreased migration speed. Nevertheless, until this report, the decreased migration speed of differentiated keratinocytes has not been documented. Of interest, and perhaps counterintuitive, is the finding that the differentiated keratinocyte maintains the ability to respond to a directional cue with sustained directional migration. We have suggested that the two distinct properties of cell locomotion – cell motility and migration directionality – are subject to different regulatory mechanisms (Fang *et al*, 1999). At least one component of the signaling cascade for directional migration in response to a DC electrical field is the relocalization of the EGFR to the leading edge of the cell and activation of its receptor kinase function (Fang *et al*, 1999). Despite the reduction in total number of EGFR on the surface of differentiated keratinocytes, however, it is possible that the relocalization of the decreased numbers of receptors to the cell's leading edge and subsequent activation of the receptor kinase is sufficient to establish asymmetric signaling and a directional migration response. Our continued investigations are aimed at elucidating the signaling mechanisms involved in directional migration.

This study set out to determine whether the differentiation state of keratinocytes affects their ability to migrate. Our results demonstrate for the first time that differentiated keratinocytes can indeed migrate, albeit at a reduced rate when compared with nondifferentiated cells. Involucrin-positive cells appear to have an intact mechanism for sensing the DC electric field signal, transducing the signal and responding with sustained directional migration. Because outwardly directed ionic currents leave skin wounds *in vivo* generating an electric field with a negative pole (cathode) at the center of the wound at the lower layer of the epidermis, and both involucrin-positive and involucrin-negative cells can migrate cathodally in electric fields of physiologic strength, it is tempting to speculate that this field provides the directional cue for migration for both involucrin-negative cells in the basal layer, as well as the involucrin-positive keratinocytes normally present within the immediate suprabasilar layer of the skin. Both cell types have the capacity to respond to this endogenous signal for directional migration and thus may contribute to wound re-epithelialization.

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