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Human keratinocyte cell culture for studying skin irritation in man?

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Summary

Four basic compounds, i.e., imipramine, norephedrine, nicotine and 8-aminoquinoline, were evaluated for their toxic effects on cultured epidermal keratinocytes and the results compared to in vivo irritancy testing. Morphological changes and cell proliferation inhibition on confluent cultures were examined at concentrations ranging from 0.1 to 10% (w/v). Exposure to four bases resulted in dose-dependent changes in cell morphology and cell growth inhibition. The rank order of decreasing damage was as follows: imipramine > 8-aminoquinoline > norephedrine > nicotine. The relevance of in vitro toxicity data was assessed in terms of human skin irritation response obtained after applying four bases on the back of 16 healthy women volunteers for 24 h. Except for 8-aminoquinoline, in vitro results appeared to correlate with in vivo skin irritation data.

Introduction

Various drugs, when applied topically, elicit primary skin irritation (Fisher et al., 1984; McBurney et al., 1989; Hogan et al., 1990). This irritation may vary with the ability of the agent to cross the stratum corneum barrier and subsequently interact with the viable cells of the epidermis and dermis. Skin irritation of investigational compounds is currently evaluated in experimental animals or in human trials (Draize et al., 1944; Marks et al., 1985; Patrick et al., 1985). Development of an in vitro cell culture model would be valuable for humane reasons, for convenience, and to study the mechanisms of irritation and inflammation. While it should be borne in mind that epidermal keratinocytes may not be the principle cells involved in inflammation, i.e., the cells surrounding the dermal vasculature may be most appropriate, there is current interest in commercial keratinocyte cell culture systems.

This communication presents the results of a probe study designed to test the correlation of in vitro morphological changes and the number of keratinocytes in epidermal keratinocyte cell cul-

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ture with an in vivo skin irritation trial in man using four basic compounds.

Materials and Methods

The four selected basic compounds were 8-aminoquinoline, nicotine, norephedrine, and imipramine. Details for these compounds, their sources, and their purification, including the preparation of imipramine free base from the hydrochloride salt, may be found elsewhere (Berner et al., 1990).

Human epidermal keratinocytes from a specimen of adult female breast skin obtained at surgery performed for reasons unrelated to dermatological problems were cultured on disks using standard methods (Tsao et al., 1982; Boyce et al., 1983, 1985). The culture medium was keratinocyte growth medium, a modified MCDB 153 supplemented with growth factors (Clonetics Corp., San Diego, CA). All experiments were performed on second or third passage cells.

Upon achieving 70–80% of confluence, the cultures were inoculated with the test compounds so that the final concentrations were 5, 50, and 500 μg/ml for each compound. To achieve these final concentrations, each culture well received 10 μl of freshly prepared aqueous solution (norephedrine and nicotine) or ethanolic solution (imipramine and 8-aminoquinoline). The final concentration of ethanol was 0.5% in both the treated and control cultures. As controls, replicate culture disks were examined that received either 10 μl water, ethanol alone or water adjusted to pH 12, the most extreme pH of the test compound solutions (imipramine; Berner et al., 1990). The medium contained 1.2 mg/ml phenol red, a pH indicator, and no changes in color were observed in any of the cultures. Gross cellular morphological alterations were graded as absent or present at 1 +, 2 +, or 3 + levels. The parameters were: 1, granulation; 2, vacuolation; 3, blebbing or pseudopod formation; 4, cell rounding; 5, frank cell loss; and 6, cell enlargement. Following 24 h exposure to the test compounds, the cell

TABLE 1

Morphological changes observed after treatment of cultured keratinocytes with various basic compounds at different concentrations after 24 h

Treatment	Granulation	Vacuolation	Blebbing[a]	Cell rounding	Frank cell loss	Cell enlargement	Cell shrinkage
Alcohol	–	–	–	–	–	–	–
pH 12 solution	+	–	–	–	–	–	–
Imipramine							
(0.1%)	+	–	+	–	+	–	–
(1.0%)	+ +	+ + +	+ +	+ + +	+ + +	–	+
(10.0%)	+ + +	+ + +	+ + +	+	+ +	–	+ + +
Norephedrine							
(0.1%)	–	–	–	+	–	–	–
(1.0%)	+	–	–	+	+	+	–
(10.0%)	+ + +	+	+	+ +	+ +	+ + +	–
Nicotine							
(0.1%)	–	–	–	–	–	–	–
(1.0%)	+	–	–	–	–	–	–
(10.0%)	+	–	+	+	+ +	+	–
8-AQ							
(0.1%)	–	+	–	–	–	+	–
(1.0%)	–	+	+ +	+	+	+	–
(10.0%)	+ + +	+ +	–	+ + +	+ + +	+ +	–

[a] Blebbing includes pseudopod formation.

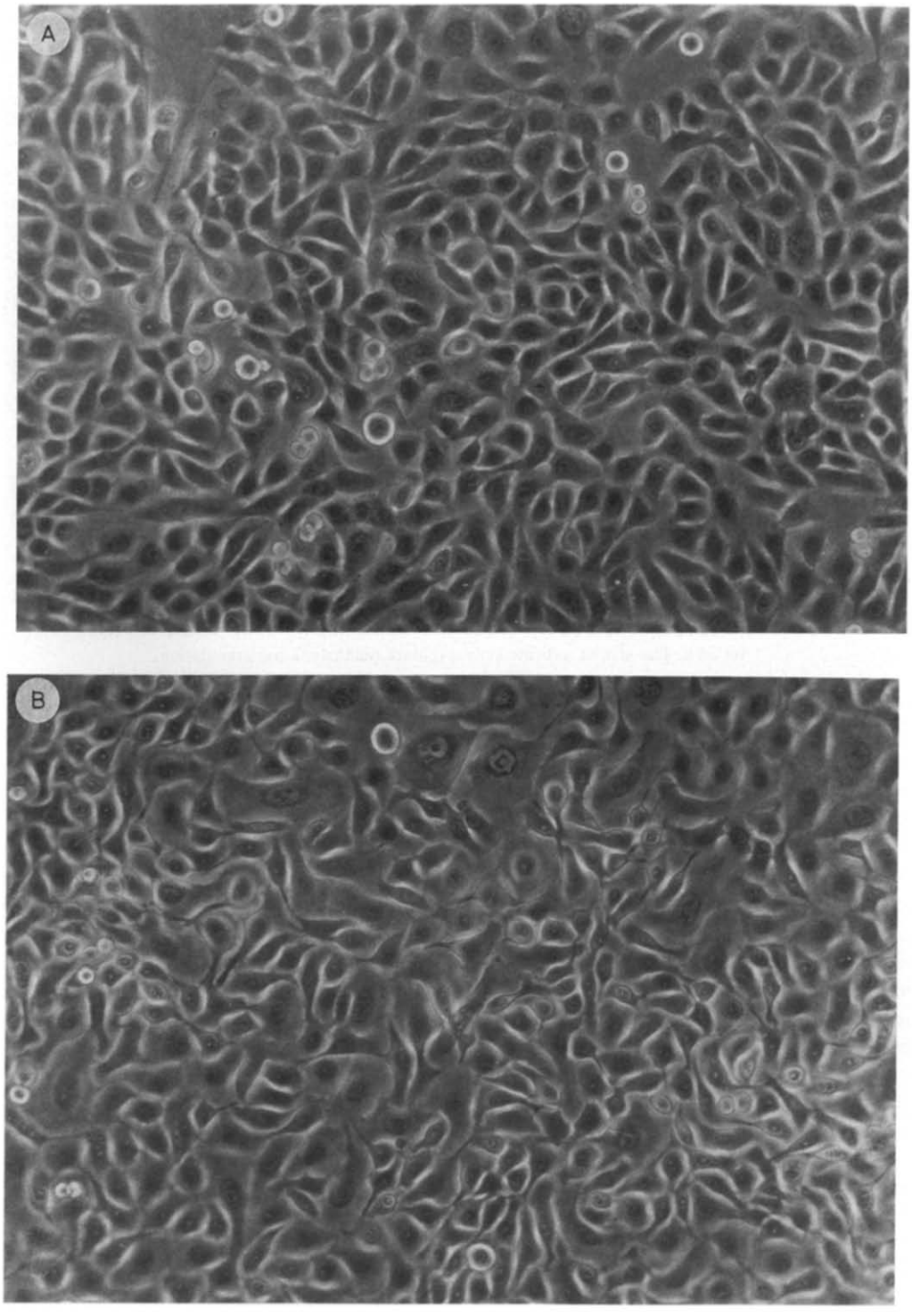

Fig. 1(A, B).

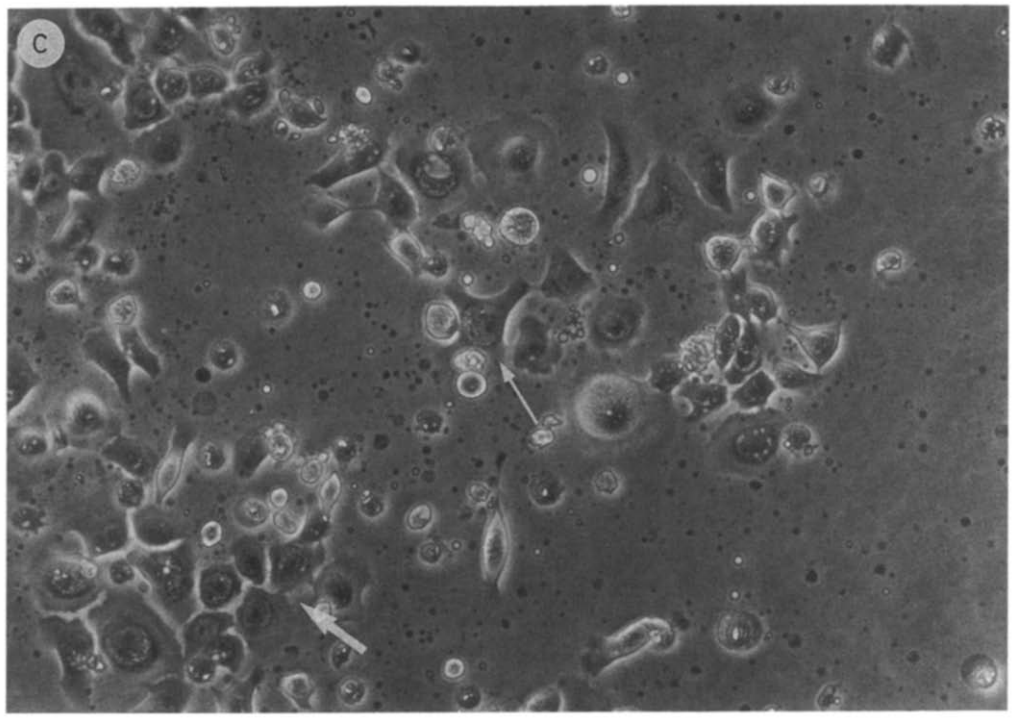

Fig. 1. Phase-contrast photomicrographs of keratinocyte cell culture treated with (A) 5, (B) 50, and (C) 500 μg/ml norephedrine for 24 h. The arrows indicate cells with dark nucleation and granulation.

monolayers were separated from the disk with trypsin solution and the released cells were counted with a Coulter counter.

The skin irritation of these four compounds was assessed in 16 healthy women volunteers between 35 and 46 years of age (41.2 $\pm$ 3.4, S.D.) after having given their written informed consent. Each compound was studied at three concentrations of 2.5, 5, and 10% (w/v). Either 0.2 ml aqueous solution (nicotine and norephedrine) or ethanolic solution (imipramine and 4-aminoquinoline) was applied for 24 h in occlusive polypropylene chambers (Hill Top, Cincinnati, OH) according to a double-blind protocol using premarked test sites on the scapular region of the upper back avoiding the midline. Details of the testing and grading procedures may be found elsewhere (Berner et al., 1990).

Results and Discussion

The addition of these four bases to nearly confluent cell cultures resulted in dose-dependent changes in cell morphology (Table 1). In 500 μg/ml imipramine-treated cultures the most profound morphological changes occurred within 15–20 min. Most cells remained in contact with neighboring cells, but perhaps due to cell shrinkage, this was through narrow pseudopods. The cells also showed strong evidence of granulation and vacuolation. While exposure to 5 and 50 μg/ml norephedrine (Fig. 1A and B) produced subtle morphological changes, in particular some enlarged flattened, rounded cells, the highest concentration of norephedrine, 500 μg/ml (Fig. 1C), resulted in reduced confluency and cells with dark nuclei and granulation. Based on these morphological changes, the rank order of decreasing damage was as follows: imipramine > 8-aminoquinoline > norephedrine > nicotine.

The number of cells observed is shown in Fig. 2. Both the 50 and 500 μg/ml imipramine treated cultures were statistically different from the controls. This more quantitative test exhibits the same dose dependent trends and same rank order of toxicity of compounds as the more subjec-

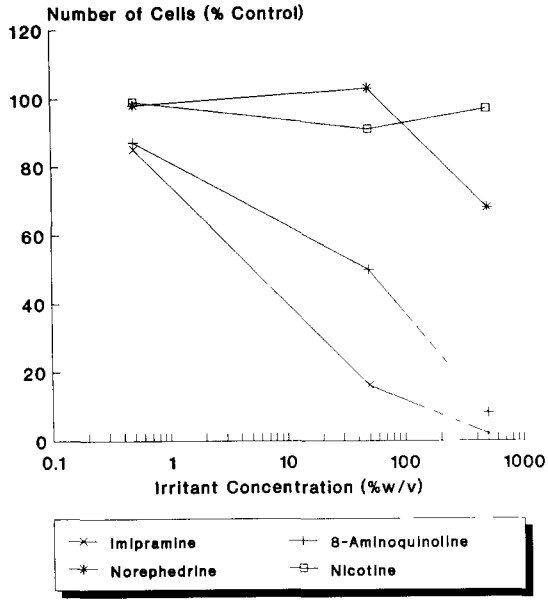

Fig. 2. The number of keratinocytes in confluent cultures after exposure to increasing concentrations of imipramine, 8-aminoquinoline, norephedrine, and nicotine solutions for 24 h. The measurements were performed in duplicate and differences between the values were less than 10%. The results are expressed as percentage of control.

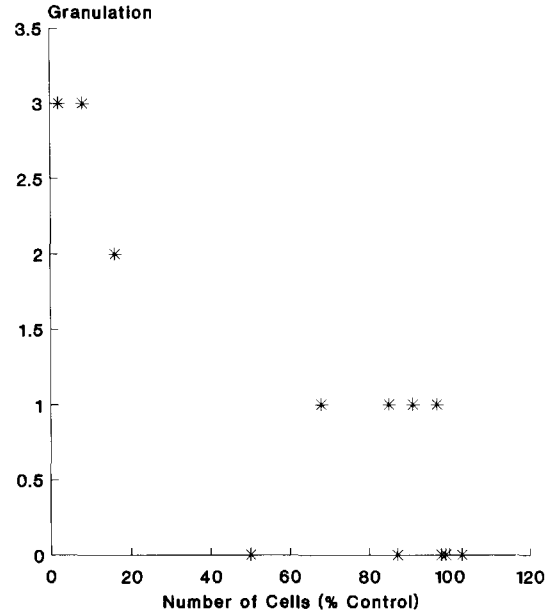

Fig. 3. The relationship of granulation with cell proliferation for the four treatments.

TABLE 2

Erythema score observed after topical application of basic compounds at three concentration levels

Compound	Erythema score		
	2.5% (w/v)	5% (w/v)	10% (w/v)
8-Aminoquinoline	0.28 ± 0.40	0.69 ± 0.91	0.31 ± 0.36
Nicotine	0.38 ± 0.59	0.34 ± 0.35	0.66 ± 0.54
Norephedrine	0.59 ± 0.58	0.56 ± 0.44	1.03 ± 0.43
Imipramine	1.70 ± 1.10		2.60 ± 1.50

tive morphological changes. The number of cells was highly linearly correlated with granulation or blebbing ($p < 0.01$, Fig. 3) and the number of cells appears to be a more sensitive measure.

The results of the skin irritation study in man are summarized in Table 2 and as in the previous study (Berner et al., 1990), the rank order of erythema correlates well with pK_a, i.e., in descending order of irritation and pK_a, the order was: imipramine (pK_a 9.5) > norephedrine (9.0) > nicotine (8.0) > 8-aminoquinoline (3.8).

While cellular toxicity should correlate with erythema, there was a major reversal in the rank of 8-aminoquinoline. For one of four compounds to show such a large reversal suggests a deficiency in this potential cell culture model for irritation. This is particularly true given the more direct correlation of erythema and edema with pK_a (Berner et al., 1989, 1990). Given the reversal in the rank order of the number of keratinocytes and erythema, investigation of other dermal cell lines and further biochemical studies of keratinocytes would be intriguing.

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