U.S. ARMY MEDICAL RESEARCH INSTITUTE OF CHEMICAL DEFENSE

USAMRICD-TR-00-05

Fluorometric Assay for Intracellular pH in Human Epidermal Keratinocytes

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January 2000

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (*Leave blank*)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2000	July 1997 to September 1999

4. TITLE AND SUBTITLE
Fluorometric Assay for Intracellular pH in Human Epidermal Keratinocytes

5. FUNDING NUMBERS
30162384ATC2
62384A

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
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10. SPONSORING/MONITORING AGENCY REPORT NUMBER

USAMRICD-TR-00-05

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (*Maximum 200 words*)

Sulfur mustard (HD) is a powerful alkylating agent with cytotoxic, mutagenic, and vesicant properties. Pathology in humans results from ocular, respiratory and cutaneous exposure. Basal cells of the epidermis of the skin are damaged by exposure to HD, causing large, slow healing blisters. To protect individuals from the effects of HD, the mechanism by which it induces pathology must be determined. In this report, a fluorometric assay of intracellular pH was developed and used to examine the role of intracellular pH in cutaneous cellular toxicity.

HD undergoes a hydrolysis reaction in aqueous environments, generating thiodiglycol, protons, and chloride ions. One hypothesis of HD injury postulates the formation of hydrochloric acid (HCl), which could acidify surrounding cell and tissue milieu. All cellular metabolic pathways are dependent on carefully buffered systems that maintain the intracellular pH within strict boundaries. Any disruption in pH regulation has the potential to result in cell or tissue injury. The effect of HD on intracellular pH had not been previously examined.

The value of fluorescent assays is in their sensitivity and specificity. Using fluorescent probes capable of crossing the cell membrane and becoming trapped within the cytoplasm, we were able to directly determine proton concentrations within the cytoplasm and could determine intracellular pH levels before and after exposure to HD in vitro. While small changes in pH were noted, they were not determined to be of biological significance. We suggest, therefore, that vesicating agents do not produce their toxicity through alteration of intracellular pH.

14. SUBJECT TERMS
Sulfur mustard, HD, in vitro toxicity, intracellular pH

15. NUMBER OF PAGES
17

16. PRICE CODE

S17. ECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UNLIMITED

Objectives and Location of Data

Objective: The objective of these experiments was to establish a sensitive and precise method for measuring intracellular pH, both before and after sulfur mustard exposure.

This research was conducted at the United States Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, Maryland). The research was conducted under protocol number 1-01-89-000-A-555.

Location of Data: These data are located in notebooks 038-95, 062-97, and 011-99.

Acknowledgements

I would like to thank Robyn Lee for her assistance with the statistical evaluation of the data. I would also like to thank Mr. Clark Gross for his assistance in various areas of this report.

Introduction

Sulfur mustard (HD) is a powerful alkylating agent with cytotoxic, mutagenic, and vesicant properties (Papirmeister et al., 1991). Pathology in humans results from ocular, respiratory, and cutaneous exposure. Basal cells of the epidermis of the skin are damaged by exposure to HD, causing large, slowly healing blisters (Papirmeister et al., 1991). To protect individuals from the effects of HD, the mechanism by which it induces pathology must be determined. This report describes a fluorometric pH assay developed and used to examine the role of intracellular pH in cutaneous cellular toxicity.

In the early 1920's, the prevailing theory of HD toxicity was the acid theory (Marshall, 1919; Lynch et al., 1919; and Peters & Walker, 1923). Researchers were aware of the rapid hydrolysis reaction of HD that occurs in an aqueous environment, generating thiodiglycol and hydrochloric acid, which could in turn acidify the surrounding environment. In the absence of other research this model became the accepted theory of vesication. However, as more and more research was performed, this argument for toxicity was questioned and other models came to the forefront.

The acid theory reemerged in the 1940's as an intracellular theory. Researchers knew that HD would easily move into the cell because of its high lipid solubility. Once inside, it could hydrolyze, and HCl would form and be trapped in the cell causing damage. This theory was once again questioned: (1) various HD analogs did not show any correlation between rate of acid liberated and the degree of vesication; (2) the buffering system could not be overwhelmed with such a small amount of acid; and (3) other chemical agents that release HCl after hydrolysis did not have vesicant properties (Philips, 1950). However, the question was still left somewhat open in that researchers believed that acid liberation might be a contributing factor to the disturbance of homeostasis within the cells (Banks et al., 1946). All cellular metabolic pathways are dependent on a carefully buffered system that maintains the intracellular pH within strict boundaries. Those boundaries vary among different cell types, depending on their function and location in the body. Regardless of location, any disruption in pH has the potential to be lethal if the internal buffering system is not functioning or is overwhelmed. In two recent publications the vesicant properties of HD were cited as a direct consequence of hydrochloric acid formation in the cytosol (Barranco, 1991; Thomsen et al., 1998).

Considering the innate structure of HD, it is feasible that a decrease in intracellular pH could be created when cells were treated with the agent. Chloroethyl ethyl sulfide (CEES), a monofunctional sulfur mustard, and mechlorethamine (HN_2), a bifunctional nitrogen mustard, are compounds similar in structure and function to HD. It is reasonable to believe that both of these compounds would generate a change in pH if it were found that HD did. It is hypothesized that CEES would produce a smaller decrease in pH than does HD or HN_2 because it is a monofunctional agent and produces only one proton per molecule hydrolyzed. HD can produce two protons per molecule hydrolyzed.

The present study was initiated to investigate the effects of HD, CEES, and HN_2 on intracellular pH. Intracellular pH changes in cultured human epidermal keratinocytes

(HEK) were determined fluorometrically using the dye BCECF-AM. This research will provide data to support or disprove the acid theory.

Furthermore, this research will generate values for the standard intracellular pH of keratinocytes and outline a method that could be used in a variety of cell types.

Materials and Methods

I. Cell Suspension Protocol

Cell Culture. Human epidermal keratinocytes (HEK) were purchased from Clonetics Corporation (San Diego, CA) and cultured with Keratinocyte Growth Medium (KGM) in T-150 flasks. The flasks were kept in a 37°C / 5% CO_2 incubator. HEK in passage 2 were harvested using trypsin/EDTA and subcultured into T-75 flasks. At 80% confluency, the passage 3 HEK were harvested using trypsin/EDTA and a cell scraper. Cells were counted with a hemacytometer and adjusted to approximately 1.0 x 10^6 cells per mL in KGM.

Solutions and Sample Preparation. A stock solution (4 mM) of nigericin (Sigma Chemical, St. Louis, MO) in ethanol was made. A stock solution (5 mM) of 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probes, Inc., Eugene, OR) was prepared in dimethylsulfoxide (DMSO) (Sigma Chemical, St. Louis, MO) and stored at 4°C. Ten one-milliliter aliquots of HEK (10^6 cells) were placed in capped 12x75 mm tubes and washed with assay buffer: HEPES buffered Tyrodes (136 mM NaCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 0.36 mM NaH_2PO_4, 5 mM HEPES, 2.6 mM KCl, 1mM $MgCl_2 \cdot 6H_2O$). The cells were resuspended in 1 mL Tyrodes with 250 µl BCECF-AM (loading conc=1 mM). The tubes were covered in aluminum foil and placed in a 37°C water bath for 25 minutes. After the elapsed time, the tubes were centrifuged at 514 x g for 5 minutes. The excess dye solution was decanted, and the cells were resuspended in assay buffer. The cells were mixed, centrifuged, decanted, and finally resuspended in 1 mL of assay buffer.

Titration Experiment. This procedure to calculate intracellular pH by titration was modified from James-Kracke (1992). One capped tube (10^6 cells) of dye-loaded cells in assay buffer was placed in a quartz cuvette containing 1 mL assay buffer. A stirring bar was added to the cuvette. The scan was started and a baseline for the HEK was established. Nigericin was added to the cuvette (final conc=40 uM, 1% EtOH) to equilibrate intracellular pH with extracellular pH. Five minutes elapsed before any further additions were made to allow the anti-porter effects of nigericin to be established. After waiting five minutes a stepwise acidification, followed by neutralization, was performed. Twelve 2-µl additions of 3 M acetic acid (pH 3) were made, followed by sixteen 10-µl additions of 2.5 M Tris base (pH 11.3) and one 8-µl addition of Tris base (pH 11.3).

The parameters on the Spex Fluoromax (Edison, NJ) were set for a time-base scan with multigroup wavelengths: 530 nm emission wavelength and 490 nm and 439 nm excitation wavelengths. Bandpass was set at 5 nm. The scan ran for twenty minutes.

Control Experiment. This procedure was performed to generate a standard curve for pH in HEK. From one capped tube, dye-loaded HEK were transferred to a quartz cuvette and an additional 1 mL of assay buffer was added, along with a stir bar. The cuvette was placed into the Fluoromax with constant stirring and scanned using the same wavelengths as mentioned above for 60 seconds.

Eight capped tubes containing HEK were centrifuged and the original media decanted. The cells were resuspended in 2.4 mL potassium phosphate buffer containing nigericin (40 uM final conc). Potassium buffers were made, varying only in their pH: 6.4, 6.6, 6.5, 6.8, 7.0, 7.2, 7.4, 7.5. The variance in pH was generated by using different volume combinations of the two original solutions (solution A: 130 mM KH_2PO_4, 20 mM NaCl; solution B: 110 mM K_2HPO_4, 20 mM NaCl). Each of the seven remaining samples was resuspended in a different buffer and allowed to equilibrate for five minutes. Each of the samples was transferred to a cuvette, placed in the Fluoromax, and scanned for 60 seconds.

Agent Experiment. BCECF-AM loaded HEK in assay buffer (2.2 mL) were placed in a cuvette with a stirring bar. The number of cells in the cuvette was approximately 1.0 x 10^6. A scan was begun, using the same parameters as the titration experiment with a total time of 30 minutes. After five minutes HD (98% pure, obtained from Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD), CEES (Aldrich Chemical, Milwaukee, WI), or HN_2 (Merck & Co., West Point, PA) was added to the cuvette to yield final concentrations of 300 uM, 1 mM, and 1 mM, respectively. Different molarities of agent were used to have equally toxic doses.

II. Coverslip Grown HEK Protocol

Cell Culture. Cryopreserved HEK were seeded in T-150 flasks with KGM and incubated as mentioned above. HEK in passage 2 were harvested using Trypsin/EDTA and subcultured onto Thermanox coverslips (Fischer Scientific, Pittsburgh, PA) in a 6-well plate. When the HEK reached 90% confluency on the coverslips they were used in this experiment.

Solutions and Sample Preparation. The growth medium was removed from the wells of the plate as the coverslip remained in the bottom of the well. The cells were washed twice with 2 mL assay buffer. The second wash solution was removed and replaced with 140 μl of BCECF-AM in assay buffer (1 mM loading conc). The entire plate was wrapped in aluminum foil and incubated in the dark at room temperature for 25 minutes. After the elapsed time, additional assay buffer was added to the wells and the entire volume of solution was removed and repeated. The coverslips were left submerged in assay buffer until ready to begin data collection.

Titration Experiment. A dye-loaded coverslip was inserted into a glass coverslip holder and placed inside a quartz cuvette, and 2.4 mL of assay buffer was added. The scan was started with the same parameters as in the cell suspension protocol. A baseline was established, then nigericin was added (final conc 40 uM). After waiting five minutes for the nigericin to take effect, acid and base were added in the same quantities and concentrations as noted in the cell suspension protocol.

Control Experiment. A dye-loaded coverslip was inserted into a glass coverslip holder and placed inside a quartz cuvette, and 2.4 mL of Tyrodes buffer was added. A scan was performed as described above, and used as control data. After the first scan, the buffer was removed, replaced with 2.4 mL of a potassium phosphate buffer solution with nigericin (final conc 40 uM) and rescanned. That solution was removed and replaced with a different potassium buffer solution containing nigericin (40 uM) and scanned again. The same procedure was followed until all buffers of different pH had been scanned.

Agent Experiment. BCECF-AM loaded HEK on a coverslip were placed in a cuvette and 2.2 mL of Tyrodes buffer were added. A scan was performed, using the same parameters as the titration experiment, for 30 minutes running time. Five minutes after the scan began and a baseline was established, HD, CEES, or HN_2 was added to the cuvette to yield final concentrations of 300 μM, 1 mM, or 1 mM, respectively, as described earlier.

III. Data Processing

For both experimental protocols the ratio of the 490/439 wavelength group was calculated within the Datamax 32 software and then copied to MS Excel.

Titration Experiment. It is possible to calculate the pH of the HEK for each data point along the scan using a Henderson-Hasselbach derived equation (James-Kracke, 1992):

$$pH_I = pKa - \log[(((Rmax-R)/(R\_Rmin)) \times (Fbase439/Facid439))]$$

pKa is equal to 6.97 for BCECF-AM. Rmax represents the largest value of the 490/439 ratio in the base range. Rmin represents the smallest value of the ratio in the acid range. R corresponds to any arbitrary ratio point along the graph. Fbase is the value of the

maximum fluoresence of the 439 nm wavelength in the basic range. Facid is the value of the minimum fluoresence of the 439 nm wavelength in the acid range.

Control Experiment. The mean of all the data points of the collected ratio was calculated in MS Excel. These ratio values were transferred to Sigma Plot for that particular buffer or control (y-values). The data points were plotted against the value of the buffer (x-values) to generate a standard curve. A linear regression was performed in SigmaPlot on the standard curve, allowing the calculation of pH of the control cells from the slope of the standard curve line.

Agent Experiment. The ratio of the scan was calculated, and the data was transferred to MS Excel. The mean of the ratio data points over the first five minutes was compared with the mean of the data points over the last five minutes. The percent change was calculated and paired t-tests were performed on the pre- and postexposure values to determine whether they were significantly different ($p<0.001$).

Results

Standard curves were generated from both types of control experiments, suspended cells and coverslip cells. The r^2 values of the linear regression of the data were closer to one for the coverslip data, meaning that close to 100% of the data points were represented by that regression line. The suspended cells did not generate as precise a standard curve as the coverslip cells did. Through this observation we began concentrating our efforts on the coverslip model.

Standard curves were generated from the experiments with buffers of varying pH (Fig. 1). The different symbols represent sets of data points for different trials. Initially the data sets were looked at individually, and a regression line was calculated with r^2 values in the range 0.96-0.99. The regression line in Figure 1 is representative of all the data points shown on the graph ($r^2= 0.706$). This line has been generated so that the internal pH of control HEK could be interpolated using the equation of the regression line and inverse prediction, as outlined in Draper and Smith (1998). The regression line was tested for lack of fit (Draper and Smith, 1998) but was found to be an adequate model.

The titration experiments were used to more accurately calculate internal pH of control cells, using the Henderson-Hasselbach derived equation (Fig. 2). The titration experiments provide the values necessary to perform a direction calculation of pH: Rmin, Rmax, Fbase, and Facid. This type of calculation could not be performed on the buffer experiment data or on the agent experiment data because of the need for max and min values. Through this procedure it was possible to calculate the pH of HEK at every time point during the scan and most importantly, for this experiment, the initial control pH.

Experiments with vesicating agents (HD, HN_2, and CEES) revealed small decreases in the 490/439 nm ratio. Paired t-tests of the mean ratio values revealed that the changes in

the ratio were statistically significant, $p<0.001$. However, the mean difference between pre- and postexposure ratio values were 0.053 (CEES), 0.063 (HN_2), and 0.051 (HD), which, biologically, is quite small. This small difference most likely was statistically different due to a large sample size of approximately 200.

The pre- and postexposure ratio values were converted to a pH value by interpolation with the equation of the regression line. The difference between pre- and postexposure pH was small (Table 1). The percent change reaffirms that the change is small.

The statistical difference of the ratio values pre- and postexposure does not necessarily correlate with biologically significant difference of the pH values for pre- and postexposure.

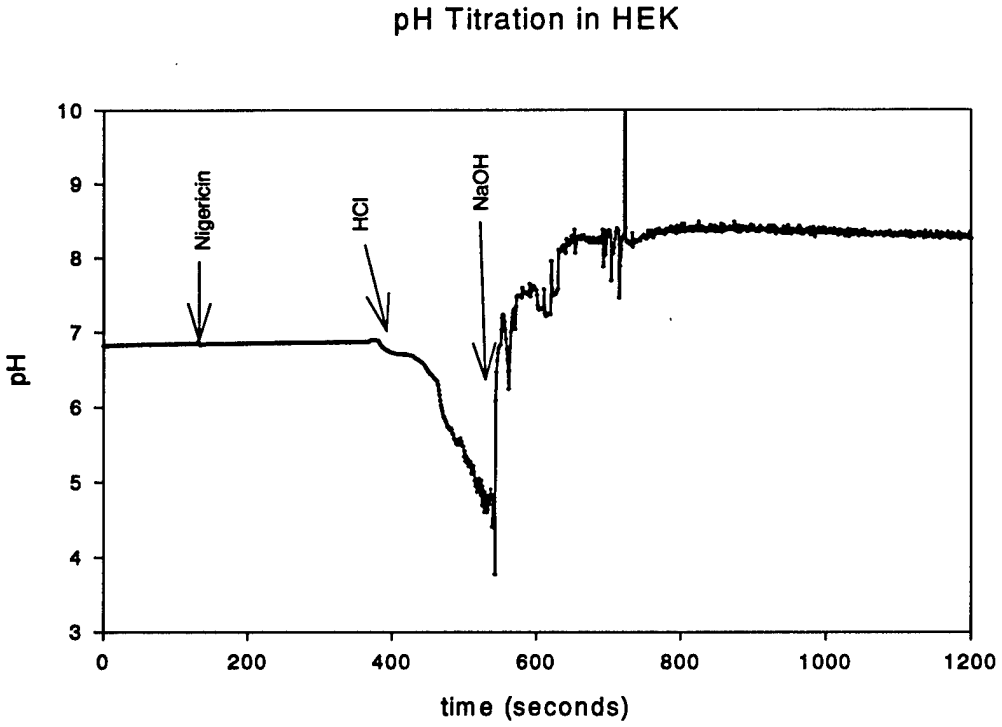

Figure 1. A titration experiment for internal pH in HEK treated with BCECF-AM. Titration was performed with HCl and NaOH. Each point on the graph was converted to a pH value by using the Henderson-Hasselbach-derived equation. The pH of the control cells was calculated to be 6.97. Nigericin was added to equilibrate the external pH environment with the internal pH environment. Upon addition of acid followed by base the pH changes from 6.97 to 3.4 and back up to 11.0.

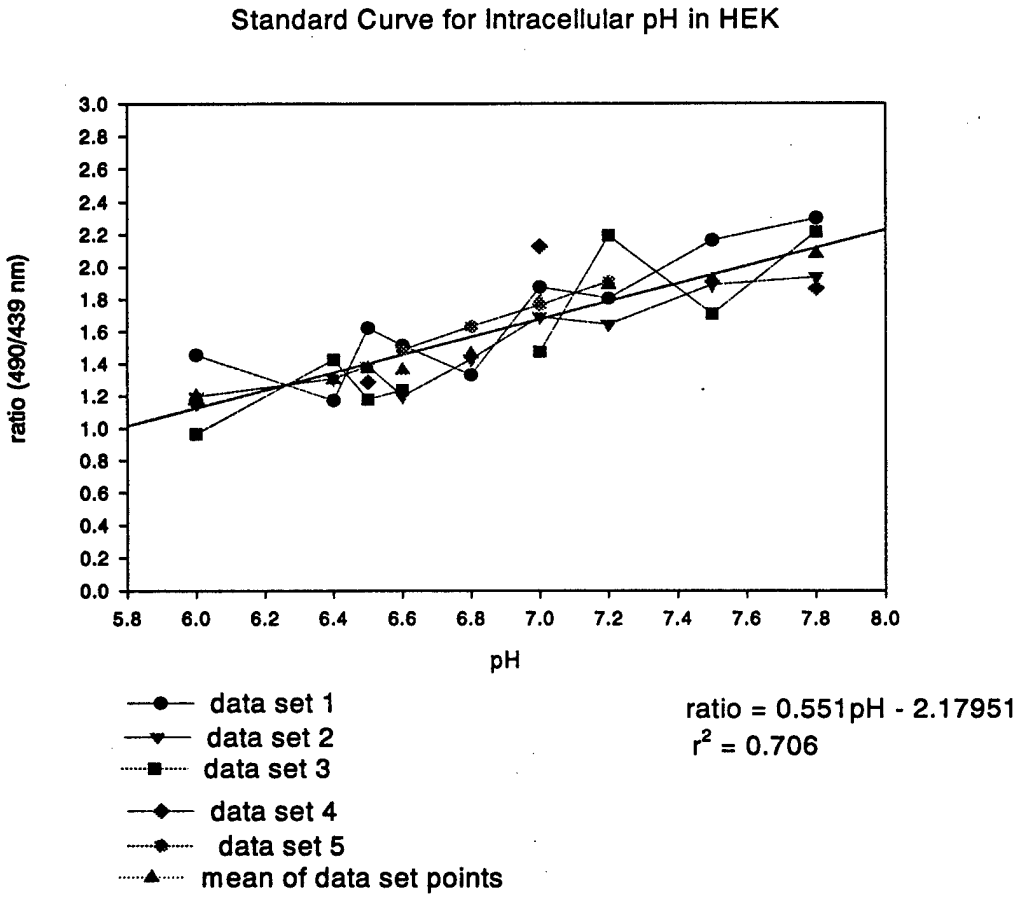

Figure 2. A standard curve for intracellular pH in BCECF-AM loaded HEK generated from five data sets with buffers of varying pH. Linear regression was performed to generate the line shown ($r^2 = 0.702$). The ratio value for control cells could be interpolated using the equation of the regression line.

A summary of data on changes in internal pH after treatment with vesicating agents: HD (300 μM) , HN_2 (1 mM) , and CEES (1 mM).

Agent	pH pre	pH post	change	%decrease
HD	7.13	7.0356	-0.0944	1.32%
CEES	7.828	7.7182	-0.1098	1.403%
HN2	7.637	7.51	-0.127	1.663%

Table 1. This table shows the percent decrease in pH after exposing HEK to HD, CEES, and HN_2. The pH before agent and after agent pH values were calculated using the equation of the regression line in Figure 2.

Discussion

In two recent publications, hydrochloric acid has been cited as the sole cause of vesication (Barranco, 1991; and Thomsen et al., 1998) based on citings of past research (between 40 and 80 years old). The citings were based on an argument for vesication built with the acid theory. The acid theory makes a valid argument for vesication, based on the interaction of HD with an aqueous environment, but it was never tested within the cell. The acid theory could not be completely set aside until intracellular studies were performed. Using fluorescent probes, we were able to determine whether or not a change in intracellular pH was occurring inside the cell. We were able to look directly at proton concentrations within the cell and determine the pH both before and after exposure to HD. In this report we document small decreases in intracellular pH after exposure to vesicating agents. We believe, based on knowledge of cell response and buffered systems, that the change documented is not biologically significant.

As further support of this belief we look to the experiments with HN_2. HN_2, in an aqueous environment, releases two Cl^- ions to the intracellular milieu. In these experiments a higher concentration of HN_2 was used to keep all the doses equally toxic. That higher concentration would have produced an even greater decrease in intracellular pH, if the acid theory were correct. We believe that the HEK's inherent buffering system would quickly act to buffer any change in internal pH and maintain the cell in a physiologically neutral range (pH 6-8).

Through the course of this assay determining standard intracellular pH of a keratinocyte proved difficult. The titration procedure was the best way to directly calculate intracellular pH. The buffer procedure required that we use the equation of the regression to calculate standard HEK pH. Both procedures provided pH values that fell into the pH 6.0-8.0 range. The mean pH with data from both the titration and buffer experiments, over many trials, was 7.4. This value seems reasonable, but this pH value was observed experimentally only about 35% of the time.

As with any in vitro fluorescent assay there are certain considerations to take into account. First, the extracellular environment cannot mimic the body; certain proteins, hormones, and organic compounds will be absent and can lead to variation in results. Secondly, it is possible for dye to be compartmentalized, and when calculations are carried out the values could represent the gradient across an organelle membrane. Also, the value obtained in these assays is an average that represents approximately 500,000 cells. It is improbable for all of them to have the dye loaded only into the cytoplasm and not anywhere else in the cell.

The data acquired has been reproduced and found to be relatively standard, based on comparisons of regression line slopes in the buffer experiments and percent change comparisons in the agent experiments. The statistics completed on the standard curves suggest that our data has a good fit, with 70% of the data from five trials represented (r^2 = 0.706). A lack of fit test revealed that this was an adequate model. In the agent

experiments the data show a slight decrease in pH after treatment. Paired t-tests revealed that the decrease was statistically significant. Does statistical significance correlate with biological significance? In this experiment it is more valuable to compare pH values before agent and after agent to determine biological significance. Based on the small decrease observed, we suggest that the vesicating agent does not significantly affect the intracellular pH of keratinocytes. We also feel that the buffering capacity of the keratinocyte is powerful enough that none of the agents we examined would disrupt the intracellular pH. We do not support the acid theory with our findings, and do not believe that a change in intracellular pH plays a role in vesication. There is definitely the chance that it may have a role somewhere in the toxicity pathway, but not immediately after challenge with agent.

Troubleshooting

During the course of this assay various changes were implemented to improve the procedure and output.

Problem. All cell types respond differently to reagents and conditions. The assay had to be modified to suit keratinocytes.

Resolution. One of the first changes made was to use cells grown on coverslips as opposed to cells in suspension. The natural state of keratinocytes is to be attached, not suspended, so this change was valuable across all assays. The suspension protocol produced good data, but it is hypothesized that the coverslip cells are in a more natural state and therefore may behave more like those in the human body.

Problem. The standard curve experiment did not produce increasing linear results.

Resolution. Through a dose-response experiment the optimal concentration of nigericin was determined to be 40 uM. It was under this concentration that a good standard curve was generated using the buffers. It was found to be very important that the buffers were high in potassium, since nigericin is a hydrogen ion/potassium ion antiporter. Changing the buffers to high potassium ones improved the function of the nigericin.

Problem. Varying results with different cell confluencies.

Resolution. Cell confluency is a confounding issue. When the cells are 90% confluent or above they begin to stack up on one another, normal cellular processes are disrupted, and then the cells begin to die. When the cells are this confluent, the results obtained are scattered, not in the expected increasing linear pattern. It was experimentally determined that HEK should not be above 80% confluent for this assay. The best results were obtained from coverslips that were between 70 and 80% confluent.

Problem. Questions about uniform dye loading with the coverslips.

Resolution. The method of applying the loading dye was altered to ensure better loading. In the past the dye had been pipetted into the media inside a 6-well plate containing a coverslip. In the new method, all the media was removed, and the dye was applied directly to the coverslip. This method ensured a more uniform loading, where all the dye was applied to the coverslip and not diluted in the well.

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