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Measurement of Cellular Responses to Toxic Agents using a Silicon Microphysiometer

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The silicon microphysiometer monitors cellular metabolism *in vitro* and has been used to detect and study biological responses to xenobiotics. This instrument uses a light addressable potentiometric sensor to measure millipH changes in micro-flow chambers maintained at 37°C. Cells are immobilized in the flow chambers. Cell proton excretion can be measured as the acidification of the medium when medium flow through the chamber is stopped. The rate of acidification is a measure of catabolism, which produces lactic and carbonic acids. Through continual cycling of on and off periods of flow, non-destructive metabolic measurements may be made every few minutes. When a cell affecting agent is introduced into the fluid stream a change in acidification rate indicates either the stimulatory or toxic effect of the agent on the cells. Thus, rapid cellular responses to chemical agents can be detected within minutes and quantitated by the magnitude of acidification rate change.

Receptor-mediated cell activation by specific ligands, such as hormones, neurotransmitters, and growth factors, causes increases in acidification rate within minutes of receptor-ligand binding. This provides a receptor-specific response that can be monitored during exposure to materials as a possible indicator of cell-type specific toxicity. Receptor specific toxicity in hippocampal neurons has also been tested using extracellular acidification as the measurement of the toxicity of glutamate receptor overstimulation.

In tests for chemotherapeutic efficacy, antiviral drug activity and toxicity, acidification rates have been used as the cell activity and viability indicator. Agents, such as detergents, that produce irritancy or other non-specific toxicity have been evaluated for their effects on extracellular acidification rate and found to decrease acidification rate. Concentrations at which acidification rates are reduced by 50% were determined for test substances and correlated with animal ocular irritation test results. Recovery after insult is readily measured and may be an important index of irritancy or toxicity. These examples illustrate the broad range of cell affecting agents that cause cellular responses detectable in the microphysiometer using extracellular acidification rate.

Introduction

Toxicologic assays and other bioassays seek to test, predict and characterize the effects of substances upon organisms. *In vitro* cellular assays simplify this inherently complex task by measuring single, defined endpoints and by reducing the number of cell types tested. Since the object is to mimic events taking

place in the intact organism, both the cell type studied and the endpoint measured may impose significant limitations on the assay system if prediction of irritancy is the object. One solution is to assay cells by a wide variety of endpoints. A recently developed instrument system, the Cytosensor™ silicon microphysiometer provides a novel means to monitor cellular responses to test substances. This system is based on a biosensor that measures a parameter that is central to the complex network of metabolic pathways[1,2]. Thus, it provides a broad screen for diverse agents that alter cellular metabolism by varied mechanisms.

The silicon microphysiometer measures changes in the metabolic rate of cells upon exposure to xenobiotics. Principal excreted products of carbohydrate, fat and amino acid metabolism are acids, and the rate of acidification of the minimally buffered culture medium reflects the rate of metabolic energy production[3]. Initial studies showed that the effects of agents such as hormones, neurotransmitters, growth factors, and toxic compounds on acidification rate could be detected rapidly and directly[1,4,5,6]. Activation of receptors by agonists increases metabolic activity, which is detected as increased acidification rate. The physiological changes associated with this increased metabolic activity are yet to be identified conclusively, but they are thought to include alterations of ionic homeostasis and proliferation rate[3]. In contrast to receptor activation, exposure to chemicals at levels that are toxic by other criteria, such as decreased viability and proliferation, usually decreases metabolic activity in the microphysiometer.

The advantages offered by the silicon microphysiometer include speed, precision, reproducibility, detection of a broad spectrum of effects of chemical agents on metabolically active cells, and suitability for virtually any cell capable of growth or maintenance in culture. Specificity is introduced through selection of appropriate blocking agents such as antibodies, antagonists, specific transduction pathway blockers, or by the use of cells with transfected receptors. Since the technique is nondestructive, cells may be removed from chambers and subsequently tested by other assays. In addition, information on the recovery of cells from initial metabolic response to effector agents can be obtained[4,6,7]. The time required to recover to basal metabolic activity from treatments with agents that either activate or reduce metabolic rate may be a useful parameter for characterizing cell-affecting agents.

Materials and Methods

The Silicon Microphysiometer:

Cells are grown and tested in a compact flow chamber into which metered volumes of liquids can be introduced and then removed. The pH of the culture medium is measured with a light addressable potentiometric sensor (LAPS), which serves as one wall of the flow chamber. The LAPS is a silicon semiconductor device that detects changes in the surface potential at the interface between electrolyte and an insulating layer on the surface of the silicon chip[8]. This insulating surface contains silicon nitride and silicon oxide groups that can be protonated; thus, the pH of the culture medium affects surface charge. The sensor detects changes in surface potential, which depends on pH in a Nernstian way (61 mV per pH unit at 37°C), similar to a glass pH electrode. Data are obtained only from illuminated regions of the sensor, which permits spatial selectivity in some applications.

Figure 1 shows the components of the system. The fluidics system includes a pump to deliver medium to the low-volume culture chamber via a debubbling device. The temperature of the culture chamber is regulated. Fluid flow, electronics for the LAPS, and data acquisition and computation are controlled by a microcomputer.

The microphysiometer measures net metabolic activity of small numbers of cells in real time. The cell chamber contains 10^5 to 10^6

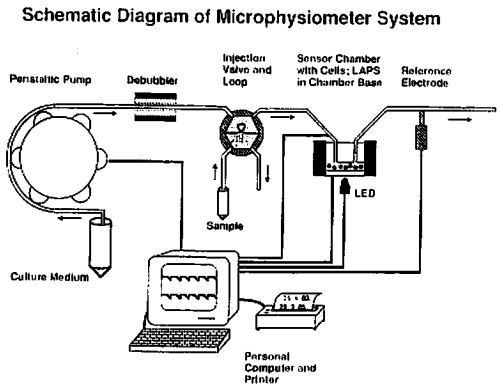

Fig. 1. Diagram of the microphysiometer system. A personal computer controls the on/off cycle of the peristalic pump and acquires data from the light addressable potentiometric (LAP) sensor in the sensor chamber. The debubbler removes bubbles and excess gas from the culture medium so that bubbles do not form in the warm (37°C) flow chambers. An injection loop valve is provided for the introduction of samples [after H.M. McConnell, et al.,[4]].

cells, although only a fraction, as few as 3000 cells, are actually monitored in the instrument. The time necessary to introduce a compound and measure the cellular response can be on the order of a few minutes. The flow of medium alternates on and off, and one determination of acidification rate is made during each flow-off period. When flow is on, the pH at the sensor is close to that of the fresh medium entering the sensor chamber. When flow ceases, the lactic and carbonic acids secreted by the cells build up in the chamber; the pH drops at a rate that is proportional to the number, size, and metabolic activity of the cells.

Cells and chemicals:

Human keratinocytes (Clonetics, San Diego, CA) were cultured using the vendor's growth medium. Cells from the human uterine sarcoma lines, MES-SA and Dx5 (from Branimir Sikic, Stanford University, Stanford, CA) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 mg/ml streptomycin (pen/strep). Mouse fibroblastic L 929 cells from Frank Lee (DNAX Research Institute, Palo Alto, CA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and pen/step. CD4-transfected HeLa cells, HT4–6C, from William Robinson (Stanford University, Stanford, CA) were cultured in RPMI 1640 supplemented with 10% FBS, pen/strep, and 50 μg/ml geneticin. Hippocampal neurons were prepared from fetal rat brains as described by Raley-Susman, et al.[9] and maintained in high glucose HEPES-buffered DMEM supplemented with 5% FBS. TF-1 cells were from Toshio Kitamura (DNAX Research Institute, Palo Alto, CA). These nonadherent cells were cultured in RPMI 1640 supplemented with 2 mM sodium pyruvate, 50 μM β-mercaptoethanol, 1 ng/ml granulocyte/macrophage colony stimulating factor (GM-CSF) from Sandoz/Schering-Plough (Bloomfield, NJ), pen/strep, and 10% FBS.

Certain responses may be enhanced by replacing complete medium with serum-free medium 12–24 hours prior to placement into the microphysiometer. Serum contains growth factors, insulin, and other cytokines that can elevate basal metabolic rate and decrease the net metabolic response to stimulus. Low buffering tissue culture medium is use in the microphysiometer to make acidification rate measurements; we routinely use a buffer at approximately 1–2 mM buffering capacity such as sodium bicarbonate-free RPMI 1640, 1 mM phosphate (Irvine Scientific, Irvine, CA) supplemented with pen/strep. Bicarbonate buffer is avoided to reduce buffering and simplify pH control.

Loading of cells into cell capsules; metabolic measuerments:

The Cytosensor silicon microphysiometer and associated cell capsule parts and reagents are manufactured by Molecular Devices Corporation, Menlo Park, CA. Cells are confined to the sensor chamber by growth on membranes in disposable cell capsules (Figure 2). Adherent cell scan be seeded onto these surfaces and maintained in a 12-well cell culture plate in an incubator until needed. The diameter of the membrane is 12 mm, but

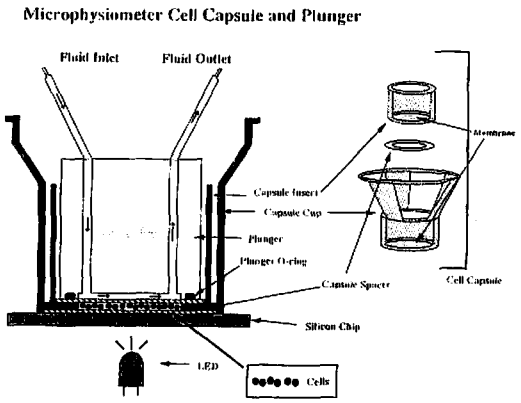

Fig. 2. Diagram of the disposable cell capsule used to immobilize cells in the microphysiometer sensor chamber. Cells are grown on the lower capsule cup membrane and enclosed using a capsule spacer with a capsule insert membrane. Non-adherent cells require an immobilizing matrix such as a fibrin gel to hold the cells onto the lower capsule membrane.

the sensor only collects data from a central region of 2 mm diameter. An added capsule spacer ring separates the cells growing on one surface from the other surface, which in the preferred method is a second microporous membrane on a capsule insert. The capsule insert is sunk over the cells and enclosed by a sealing plunger, thereby creating the low volume culture chamber.

Cells are at about 60–95% confluence when the chambers are used. Early versions of the microphysiometer, not commercially available, employed indium tin oxide coated coverslips on which adherent cells were grown. Non-adherent cells, such as the TF-1 cells, can also be confined using the double membrane system described above. These cells are embedded in a collagen particle matrix or a fibrin gel to immobilize them in the cell capsule. Many elements of the Cytosensor (LAPS, tubing, and cell capsule housing) are reusable. The system is decontaminated by cycling Cytosensor sterilant, an oxidant plus detergent solution, through the assembled system (minus the cell capsule assembly), to sterilize and depyrogenate the system, and then rinsing with sterile distilled water followed by sterile phosphate buffered saline or medium.

Sample handling:

Samples are usually water-soluble substances. Water-insoluble or viscous materials pose delivery problems; however, dimethylsulfoxide has been used to increase solubility since it is non-toxic at relatively high doses (1%). Samples are diluted in the same low buffered medium used for acidification-rate measurements. Matrix controls must be run to estimate the contribution of the vehicle in medium to the cytotoxicity observed.

Determination of the 50% Metabolic Reduction Dose (MRD50):

The concentration of test substance that reduces the metabolic rate to 50% of the initial rate (MRD50) has been used to characterize the irritancy of test substances. The MRD50 is measured using the protocol previously described by Bruner et al.[6] and depicted in Figure 3, which shows one cycle of the assay protocol. The flow rate of medium, the introduction of test substance into the cell culture chamber, and the net change in medium pH are displayed. A 300 μl sample bolus is introduced in a sample loop using injection valves. The sample flows into the chamber for 120 sec, flow halts for a 200 sec incubation, and the sample is washed out for 380 sec. The nominal time that the cells are exposed to the sample is 380 sec (300 μl/100 μl/min+200 sec). Following washout, the acidification rate is determined while flow is halted for 200 sec. It is simple to vary sample exposure time, the time of subsequent wash with fresh medium, and the time for reading acidification rate. Typically, in this protocol acidification rate is measured only after the sample is washed out to avoid possible changes in buffer capacity and pH of the medium due to the sample itself. Endpoints are calculated after testing a series of dilutions of the test substance, starting with the greatest dilution and continuing until the acidification rate (measured in μV/s) is reduced by >50%. The *in vitro* irritancy is often expressed as −log (MRD50), or pMRD50.

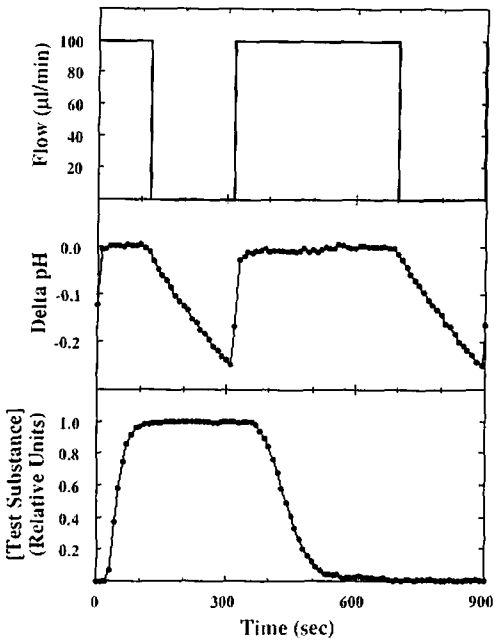

Fig. 3. One cycle of the assay protocol for ocular irritancy. The top figure shows the liquid flow rate over the 900 sec cycle. The net change in medium pH is shown in the middle figure. The second stopped flow time period is used to measure the acidification rate. The bottom figure shows the actual introduction of test substances into the cell culture chamber [after L.H. Bruner, et al., [6]].

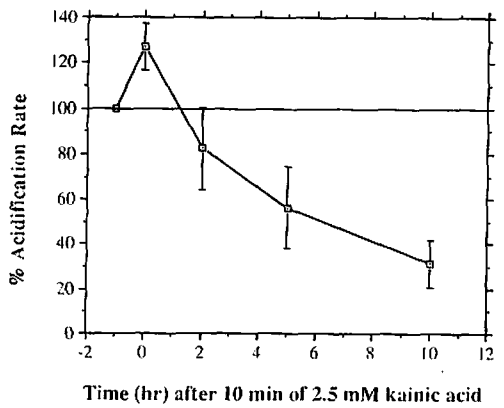

Fig. 4. Effect of glutamate receptor stimulation of mixed hippocampal cultures. Hippocampal cultures were exposed to 2.5 mM kainic acid for 10 min at time 0, then returned to normal perfusion medium. Data represents the average acidification rate (µV/sec) of 3 experiments. The average, initial acidification rate was 213+-29 µV/sec, and expressed as 100% acidification rate. Statistical analysis of the decline in rate after kainic acid stimulation indicated high degree of significance, $p<0.0037$ level, using the Fisher PLSD test [after Raley-Susman et al., [9]].

Results

Stimulus-induced neurotoxicity:

Glutamate is an important excitatory neurotransmitter in the central nervous system. There is much evidence that overstimulation of the glutamate receptor leads to the neurotoxicity associated with a host of metabolic insults, including seizures and hypoxia-ischemia, because of the disruption of metabolism-dependent homeostatic mechanisms[10]. The microphysiometer was used to monitor the toxicity of glutamate receptor stimulation by the agonist kainic acid, using co-cultures of hippocampal neurons and glia that were isolated from fetal rats and maintained in culture as previously described[11]. These cells were tested in the microphysiometer after serum starvation for 24 hr. Exposure to kainate caused a prompt (<1 min) increase in acidification rate of up to 100%, with half-maximal stimulation at 2 µM kainate. As is shown in Figure 4, transient (10 min) exposure to 2.5 mM kainate, a level known to be neurotoxic, led to a depression of acidification rate within 5 hr. This decrease in metabolic activity was not due to cell loss since cell-associated lactate dehydrogenase was not significantly decreased in cells treated with kainate, in parallel experiments, and untreated control cells did not show the decrease in metabolic activity (data not shown).

Inhibition of receptor-activated cellular response by a protein kinase C inhibitor:

Another type of receptor-specific toxicity that can be measured by the microphysiometer is the interference with intracellular signal transduction pathways. The effect of an enzyme inhibitor on the activation of TF-1 cells by granulocyte-macrophage colony stimulating factor (GM-CSF) was examined. Exposure of TF-1 cells to GM-CSF causes a rapid increase in extracellular acidification rate[12]. Protein kinase C (PKC) is activated by a number of colony stimulating factors[13], including GM-CSF, and depletion of PKC

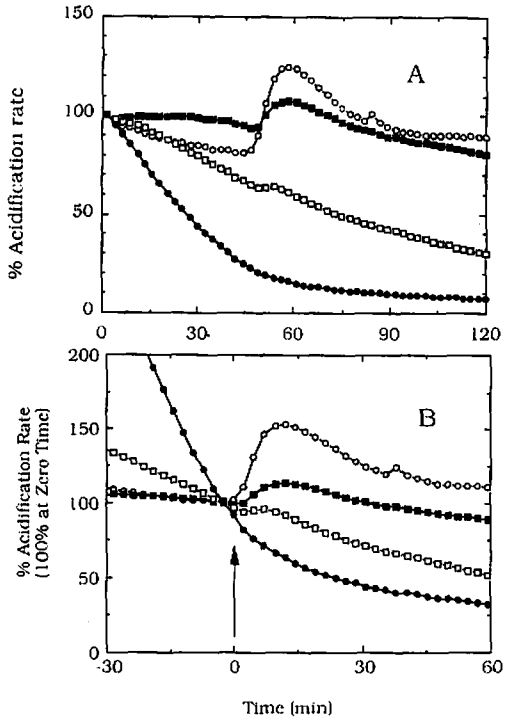

Fig. 5. Calphostin C inhibits the GM-CSF acidification response in TF-1 cells. Panel A shows the acidification rate of cells treated with 0 (open circles), 12.5 nM (closed squares), 25 nM (open squares), and 50 nM (closed circles) of calphostin C for 1 hr under fluorescent lighting prior to loading into the microphysiometer chamber using the fibrin gel method. At about 50 min, the cells were exposed to GM-CSF at 5 ng/ml for 6 min. Panel B shows the acidification rates of the same TF-1 cells in panstetl A, normalized to 100% at the time of GM-CSF exposure.

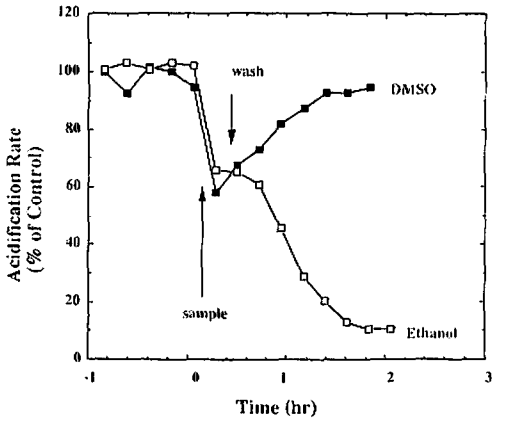

Fig. 6. Recovery of human keratinocytes from exposure to irritating test materials. Keratinocytes were exposed to DMSO or to ethanol at concentrations corresponding to the MRD50 for 5 minutes. The first arrow indicates the time of exposure to test sample, and the second arrow indicates the start of sample washout. The cells treated with DMSO (solid squares) and ethanol (open squares) were monitored for acidification rates for an additional 90 min [after J.W. Parce et al., [7]].

blocks GM-CSF dependent cell proliferation as well as other cellular responses. The effects of calphostin C, an irreversible photo-activated protein kinase C inhibitor[14], on the GM-CSF activation of TF-1 cells was evaluated as was calphostin general toxicity. Cells were exposed to calphostin C under fluorescent lights for 1 hr prior to being loaded into the microphysiometer. Figure 5A shows that cytotoxicity was observed at calphostin concentrations as low as 25 nM, as measured by the decrease in acidification rate monitored for 2 hr after treatment. The effect of calphostin on the GM-CSF response was observed at a lower concentration, 12.5 nM, which caused a 66% decrease in the activation of acidification rate (Figure 5B). The onset of cellular toxicity could in this way be differentiated from the inhibition of GM-CSF signal transduction. This example shows how receptor activation of acidification rate may be used to test for agents that interfere with cellular signaling pathways.

Recovery from toxic insult:

Physiological responses to toxic insult can be characterized both by intensity and duration. most *in vitro* toxicological assays measure intensity. The microphysiometer is well suited to measure not only intensity, but also duration of alterations of cellular metabolic activity. Two pilot experiments in the microphysiometer have tested the relationship between *in vivo* irritancy and recovery from insult.

The *in vivo* irritancy of dimethylsulfoxide (DMSO) is less than that of ethanol, and DMSO accordingly has a lower pMRD50 than does ethanol (0.1 vs. 0.8)[1]. Human keratinocytes were exposed to each compound for 5 minutes at its MRD50 concentration, to equalize acute effects, and then metabolic activity was monitored for 2 hours (Figure 6)[15]. The acidification rate of keratinocytes

exposed to DMSO returned to its initial control value within the observation period, suggesting complete recovery. In contrast, the acidification rate of the cells exposed to ethanol continued to decline, reaching about 10% of the control value by 2 hours after exposure.

Another experiment of similar design compared recovery from exposure to triethanolamine and heavy-duty laundry detergent[6]. Triethanolamine is the less irritating *in vivo* and in the microphysiometer (pMRD50=1.77 vs. 3.78 for the detergent). Keratinocytes exposed to triethanolamine recovered their initial acidification rates, while those exposed to the detergent did not.

In vitro irritancy testing

Initial studies using human keratinocytes grown on coverslips tested half-log serial dilutions of eight irritants previously characterized as having *in vivo* ocular irritancy ranging from mild to severe[1]. Cells were exposed to irritants for 5 min in a protocol similar to that described in Materials and Methods, and MRD50 was determined as shown in Figure 7. There was high rank correlation between the *in vivo* irritancy and the MRD50. There was a 4 log range of MRD50 values between the most mild (DMSO) and most severe (benzalkonium chloride) chemicals (Table 1). Reproducibility of the MRD50 value was within a factor of 2 over a period of several months using different lots of primary keratinocytes.

Bruner et al[6] tested seventeen product formulations and chemicals well characterized with historical data obtained at Procter and Gamble Co. using the rabbit Low Volume

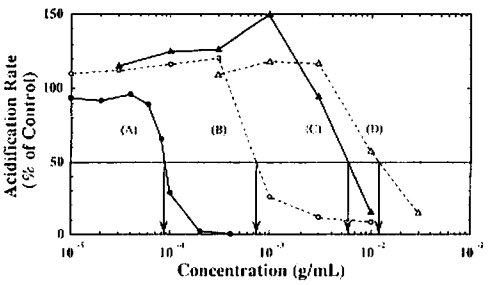

Fig. 7. Analysis of test substances for irritancy using reduction of metabolic rate. Two-fold serial dilutions of materials were sequentially tested, starting with the least concentrated samples. As the concentration of irritating substances was increased the acidification rate decreased. The concentration causing a 50% decrease in rate (MRD50) was interpolated for each test substance. In this study, heavy-duty laundry detergent (A) was the most irritating, MRD50=8.6×10^{-5} g/ml, and triethanolamine (D) was the least irritating, MRD50=1.2×10^{-2} g/ml [after L.H. Bruner, et al., [6]].

Eye Test (LVET). These materials represent the range of activities commonly encountered in ocular irritancy testing of cleaning products: soaps, shampoos, detergents, fabric softener, and 4 single chemicals. Keratinocytes grown on coverslips were also used in the microphysiometer to determine MRD50. MRD50 ranged from 2.50 10^{-4} g/ml [most irritating] to 1.8×10^{-2} g/ml [least irritating]. The correlation was r=0.86, $p<0.0001$ with the LVET maximum average scores (MAS), illustrated in Figure 8. It was noted that the metabolic rate of the cells increased at sub-inhibitory concentrations for some of the test materials, before higher concentration caused decreased rates. It was also noted that metabolic rate recovered to the basal rate after removal of some irritants which decreased rate. In a later study, Catroux et al., using L

Table 1. Comparison of the in vivo ocular irritancy to MRD50

ID#	Substance	-log MRD_{50}	In vivo irritancy
1	Dimethyl sulfoxide	0.1	Mild
2	Propylene glycol	0.5	Mild
3	Methanol	0.7	Moderate to mild
4	Ethanol	0.8	Moderate to mild
5	Acetone	0.9	moderate to mild
6	n-Butanol	1.7	moderate
7	Na dodecyl sulfate	3.9	Severe to moderate
8	Benzalkonium Cl	4.1	Severe

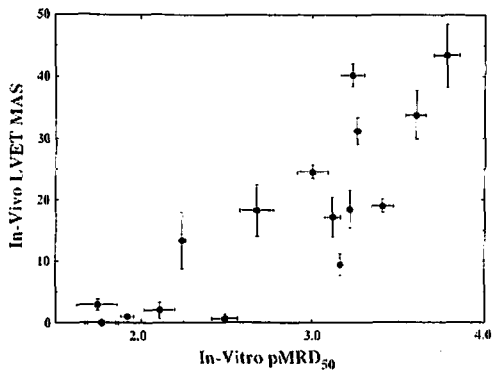

Fig. 8. Correlation of *in vitro* and *in vivo* results. The *in vivo* test was the Low Volume Eye Test (LVET); Maximum Average Scores (MAS) were determined for 17 test substances. The arithmetic mean of the negative log of the MRD_{50}, the $pMRD_{50}$, is plotted versus the LVET MAS value. There is significant positive correlation between the two data sets (correlation coefficient $r=0.85$, $p<0.0001$). The vertical and horizontal error bars show the SEM from at least three replicates for both assays [after L.H. Bruner. et al., [6]].

929 cells grown on the polycarbonate membrane of a capsule cup, evaluated the MRD50 of 19 surfactants (representing the cationic, anionic, nonionic and amphoteric classes) using a sample exposure time of 380 sec[16]. This panel of substances had been characterized by *in vivo* ocular irritancy testing and had maximum average scores (MAS) ranging from 3.8 to 54.0. Microphysiometer MRD50 values ranged from 40 to 200,000 μg/ml. A plot of MAS versus the pMRD50 yielded $r=0.89$, which was comparable to the Bruner study[6] using keratinocytes grown on coverslips.

Chemotherapeutic susceptibility testing:

Closely related to toxicity testing is chemotherapeutic susceptibility testing. A human uterine carcinoma cell line, MES-SA, which is sensitive to doxorubicin and vincristine, and a cell line, Dx5, derived by selection to be resistant to these drugs [17] by virtue of the P-glycoprotein transporter were tested in the microphysiometer for susceptibility to 1 μM doxorubicin and 100 μM vincristine. Figure 9A[1,7] shows that compared to MES-SA cells, which show 50% reduction in net metabolic activity in response to doxorubicin after 15 hr,

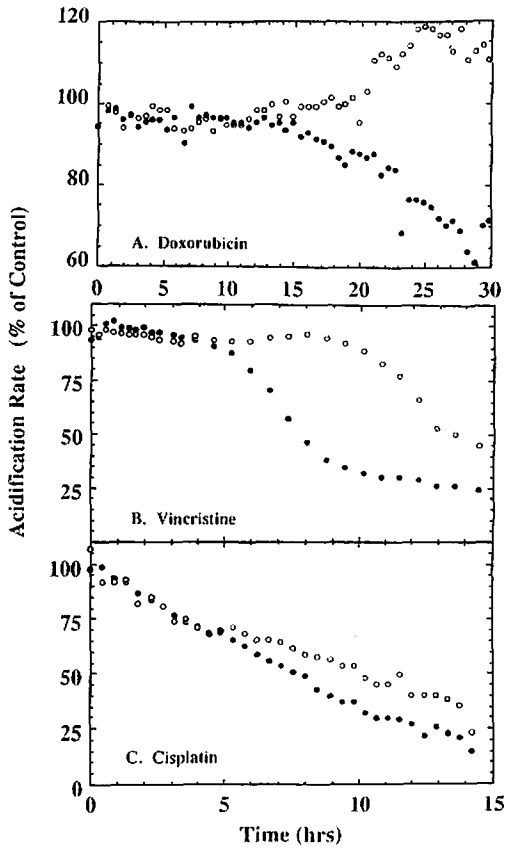

Fig. 9. The sensitivity of two human sarcoma cell lines to chemotherapeutic drugs. Acidification rates are presented as percent of rates before the drug was introduced into the microphysiometer. In panel A, MES-SA cells (solid circles) and Dx5 cells (open circles) were treated with 1 μM doxorubicin beginning at time 0. In panel B, these same cells were treated with 100 μM vincristine. In panel C, the cells were treated with 100 μM cisplatin [after Parce et al., [1,7]].

the Dx5 cells are resistant to this chemotherapeutic drug. Vincristine at 100 μM shortens the time to cytotoxic effects to 5 hr for MESSA cells. The Dx5 cells were shown in Figure 9B to be differentially sensitive in a much shorter time. Figure 9C shows that both cell lines are similarly sensitive to cisplatin.

Anti-viral drug testing:

The detection of viral ytopathic effects with the microphysiometer was first demonstrated using mouse L cells infected with vesicular stomatitis virus[1]. Ribavirin treatment of infected cells inhibited the viral cytopathic effect of decreases in acidification rate and

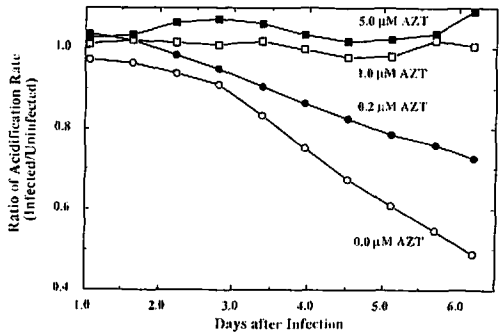

Fig. 10. The metabolic consequences of infection of cells with human immunodeficiency virus (HIV-1). Acidification rate data are presented as the ratio of the acidification rates of infected and uninfected cells. CD4 transfected HeLa cells were infected on coverslips by incubation for 1 hr at 37°C with 100 μl of viral innoculum, 1×10^5 infectious units/ml, prior to loading into microphysiometer. The perfusing medium contains the indicated concentrations of AZT [after Wada et al., [17]].

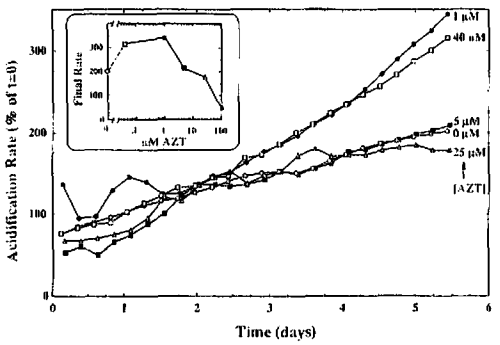

Fig. 11. Toxic effect of AZT on uninfected cells. CD4 transfected HeLa cells were perfused with medium containing various concentrations of AZT. A stimulation of acidificaton rate growth is seen at low concentrations of AZT. The insert figure shows the final acidification rate as a function of AZT concentration [after Wada et al., [17]].

delayed their on-set. Later, the infection of CD4 transfected HeLa cells, HT4–6C, with HIV-1 was detected in the microphysiometer (Figure 10)[7,18]. Six days were required for the HIV effects to be fully manifest, as a drop in the ratio of acidification rates of infected versus uninfected cells. The inhibition of viral cytopathic effects was demonstrated using 0 to 5 μM concentrations of 3'-azido-3'-deoxythymidine (AZT). The dose causing 50% inhibition of the HIV cytopathic effects (ED50) was 0.23 μM. The toxicity of AZT on the same CD4 transfected, uninfected cells was evaluated in the microphysiometer. As shown in Figure 11, AZT caused an increase in rate at low levels and a decrease at higher levels in uninfected cells relative to untreated controls over the 6 day treatment period. The dose causing a 50% reduction in acidification rate (TD50) was 67 μM. Testing of a panel of four compounds showed a rank order of anti-HIV potency of AZT ED50=0.23 μM<2', 3'-dideoxyinosine (DDI) ED50=8.3 μM<chloramine T ED50=19.2 μM<mannitol (no activity), which is in agreement with their known *in vitro* anti-HIV properties[17]. The ratio of TD50/ED50 was determined as a measure of *in vitro* anti-HIV selectivity, and the rank order was the same, AZT = 291 > DDI = 66 > chloramine T = 30>mannitol ≈ 0.

Discussion

In vitro toxicology can be considered to comprise two types of activities: safety assessment screening and studies of mechanism. The former seeks replacements for animal (or human) testing, typically for ocular and skin irritation. The most critical attribute of any *in vitro* alternative must be high predictive value: results for an unknown compound should provide an index of irritancy *in vivo*. The latter activity, mechanistic study, seeks to identify the molecular mechanism of toxicity. In this type of study, toxicity is already demonstrated and one investigates the target of the toxic effect, as toxicokinetics and toxicodynamics. The silicon microphysiometer is suitable for both types of study, as demonstrated by the above examples. Basic research in signal transduction has broadened the areas of toxicology.

An important parameter of both irritancy screening and mechanistic study, recovery from toxic insult, is uniquely measurable in the silicon microphysiometer. Results in three studies show good correspondence between pMRD50 and rabbit eye irritation scores. Two prelimary tests of recovery using essentially identical protcols were each able to demons-

trate recovery (DMSO and triethanolamine) and failure to recover (ethanol and heavy duty laundry detergent) after a toxic insult corresponding to the MRD50. For this small study, the pMRD50 did not correlate unequivocally with inability to recover: the ranking of pMRD50 (with recovery in parentheses) is DMSO (yes) < ethanol (no) > triethanolamine (yes) < detergent (no). Recovery apparently does measure a different property from MRD50, and more work is necessary to relate it to the behavior of irritants *in vivo*. This is a little investigated area of *in vitro* toxicology and promises to be a powerful tool for screening and mechanistic studies.

The prospects for advanncing *in vitro* toxicology include the monitoring of receptor-specific toxic events, and elucidating mechanism(s) of cytotoxicity by studying cell-affecting agents in the microphysiometer. An illustration of neurotoxicity mediated through the glutamate receptor was provided by the work of Raley-Susman, et al.[9], who demonstrated the toxic effects of kainic acid, a glutamate receptor agonist, on hippocampal neurons using acidification rate to monitor the metabolic activity of the neuronal cell cultures as well as detect the rapid activation of glutamate receptors in these cells. The decreased metabolic activity is thought to be due to depletion of the cellular energy stores preceding overt cell degeneration[10]; alternatively, it may be due to leakage of essential low molecular weight metabolites through damaged membranes. Other tissue specific toxicity mediated through either inappropriate receptor activation or interruption in normal receptor mediated signaling processes could be evaluated using metabolic monitoring. Calphostin C inhibition of GM-CSF signaling provides an example of this type of toxicity. The interruption of the GM-CSF activation of bone marrow cells was detected by acidification rate monitoring, and this form of toxicity was also kinetically distinguished from the general cytotoxicity of higher levels of calphostin C. It should be possible to select other receptor systems characteristic of various tissue types to establish tests for other forms of tissue specific toxicity.

The measurement of toxicity is an important aspect of drug testing. In the case of chemotherapeutics, the efficacy of the drug is measured by the sensitivity of tumor cells to the therapeutic agent. This measurement can be made on relatively few cells which may make testing of tumor biopsies possible. By using elevated concentrations of the chemotherapeutic agents in the microphysiometer, the time required to determine drug susceptibility of tumor cells could be shortened. Evaluation of drugs for anti-viral activity requires the evaluation of toxicity as well as the measurement of vial inhibition, since inhibition of viral replication is closely tied to host cell metabolism. The general technique of acidification rate monitoring is capable of making both measurements, using infected or uninfected cells in the same series of assays.

The use of acidification rates to monitor the metabolism of cells exposed to irritating substances has been evaluated using human keratinocytes and mouse L cells and found to correlate well with *in vivo* measurement of ocular irritancy (r=0.86 and 0.89). Bruner et al. also compared 7 *in vitro* methods for ocular safety testing[19] including the microphysiometer, neutral red assay, total protein assay *Tetrahymena thermophila* motility assay, bovine eye/chorioallantoic membrane assay, and the EYTEX system and found significant correlation between *in vivo* ocular irritation and all *in vitro* methods except the EYTEX system. These authors noted that the microphysiometer method of monitoring recovery of cells after exposure to irritating test substances may be useful in differentiating innocuous irritants from more irritating materials.

Several features suggest that the silicon microphysiometer will be useful for irritancy testing as well as for mechanistic studies of toxicity. These factor include screening for multiple sites of toxic actions, real time

results, the possibility of both dosage and recovery studies, and suitability for a broad range of cell types.

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