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**FLOW-SYSTEM ANALYSIS OF EXFOLIATED PULMONARY CELLS:
RESULTS OF INITIAL CHARACTERIZATION STUDIES IN HAMSTERS**

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FLOW-SYSTEM ANALYSIS OF EXFOLIATED PULMONARY CELLS: RESULTS
OF INITIAL CHARACTERIZATION STUDIES IN HAMSTERS

Running title: Flow Analysis of Exfoliated Hamster Lung Cells

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ABSTRACT

This paper summarizes results of preliminary experiments to develop cytological and biochemical indicators for estimating damage to respiratory cells in test animals exposed by inhalation to toxic agents associated with nonnuclear energy production, the specific goal being the application of advanced multiparameter flow-systems technologies to the detection of early atypical cellular changes in lung epithelium. Normal Syrian hamster lung cell samples composed of histiocytes, leukocytes, macrophages, ciliated columnar cells, and epithelial cells were stained with fluorescent dyes specific for different biochemical parameters and were analyzed in liquid suspension as they flowed through a chamber intersecting a laser beam of exciting light. Multiple sensors measured the total or two-color fluorescence and light scatter on a cell-by-cell basis. Cellular parameters proportional to optical measurements (i.e., cell size, DNA content, total protein, non-specific esterase activity, nuclear and cytoplasmic diameters) were displayed as frequency distribution histograms. Lung cell samples were also separated according to various cytological parameters and identified microscopically. The basic operating features of the methodology are discussed briefly, along with specific examples of preliminary results illustrating the initial characterization of exfoliated pulmonary cells from normal hamsters. As the flow technology is adapted further to the analysis of respiratory cells, measurements of changes in physical and biochemical properties as a function of exposure to toxic agents will be performed.

INTRODUCTION

The application of advanced flow-systems technologies to measure physical and biochemical properties in respiratory cells provides a new approach for assessing early damage to the lung epithelium of mammals exposed by inhalation to toxic by-products from nonnuclear energy production. Specifically, our efforts are directed toward developing automated cytological and biochemical methods for estimating early atypical cellular changes in exfoliated lung cells,^{1,2} the end objective being examination of human sputum samples from occupationally exposed populations. To develop analytical techniques for quantitative assessment of damage, automated cell analysis and sorting instrumentation³ designed and constructed at the Los Alamos Scientific Laboratory (LASL) is being applied presently to characterize initially respiratory cells from normal Syrian hamsters, which have been selected as the experimental test system in the beginning phase of the program.

The initial goals are to adapt cell preparation and staining techniques developed for flow-analysis systems⁴ to characterize lung cells using the multi-parameter cell separator⁵ and multiangle light-scatter⁶ systems. These include acquisition of adequate numbers of exfoliated cells for flow analysis by lavaging the lungs with saline, adaptation of cytological techniques designed for dispersal of human gynecological specimens to hamster lung epithelium for obtaining single-cell suspensions, adaptation of existing staining techniques for measurement of cellular biochemical parameters (e.g., DNA content, total protein, etc.), and initial characterization of lung cells using flow-analysis instrumentation. A brief description of the cell analysis and sorting techniques used to determine simultaneous fluorescence and light-scatter properties of respiratory cells, with examples of results from preliminary studies

involving characterization based on cell size, DNA content, total protein, non-specific esterase activity, nuclear and cytoplasmic diameters, and multiangle light-scatter properties, are presented herein. As the flow-systems technology is adapted further to analyze exfoliated lung cell samples from normal hamsters and subsequent characterization studies are completed, measurements of early changes in the various physical and biochemical cellular properties as a function of exposure to toxic agents associated with energy production will be attempted.

MATERIALS AND METHODS

Cell Sample Acquisition and Staining

Syrian hamsters were sacrificed by administering 0.3 mg of sodium pentobarbital intraperitoneally. The trachea was surgically exposed and intubated with a 19-gauge blunted stainless steel needle bent 45° at the midpoint and attached to a syringe containing 3.0 cc of phosphate-buffered saline. The trachea was secured around the needle by tying with chromic gut, and the lungs were lavaged⁷ to obtain exfoliated respiratory cells which were fixed in 35% ethanol. Clumps and sheets of epithelial cells were disaggregated into single cells for forcing through a 25-gauge needle. Samples were evaluated by staining with the routine Papanicolaou method and were examined microscopically to determine the cell types present. Differential counts, summarized in Table 1, indicate that washings are composed of macrophages, leukocytes, ciliated columnar cells, epithelial cells, histiocytes, and erythrocytes plus a considerable amount of small debris.

To demonstrate cell viability, unfixed samples (2.5 ml) were stained with fluorescein diacetate [FDA (10 µl. of 0.5 g FDA/100 ml acetone)] at

0°C for 30 min using the "fluorochromasia" procedure⁸ and were then analyzed for fluorescence. Live cells accumulate fluorescein intracellularly, since FDA readily enters the cell to be hydrolyzed by esterases but cannot then pass out through the intact cell membrane. Fixed lung cells were stained for DNA using mithramycin^{9,10} and then analyzed for total DNA content and cell size by measuring fluorescence and light scatter, respectively. Nuclear and cytoplasmic diameter distributions were determined similarly from analog signal time durations, as described below. Both FDA- and mithramycin-stained samples remained in the stain solution during analysis. Two-color fluorescence analysis of DNA content and total protein in respiratory cells was accomplished by staining the fixed samples with propidium iodide (PI) and fluorescein isothiocyanate (FITC) which fluoresce red and green, respectively.^{10,11} These samples were rinsed and then suspended in phosphate-buffered saline for two-color fluorescence analysis. Nuclear and cytoplasmic diameters were measured from analog red (nucleus) and green (cytoplasm) fluorescence signal time durations, respectively, as described below.

Instrumentation

The principle of measurement is as follows. Lung cells stained in liquid suspension, as described above, are introduced into a flow chamber at approximately 10^3 /sec (Fig. 1) where they flow across a fluid-filled viewing region (constant velocity) and intersect a narrow, elliptically shaped, argon-ion laser beam.⁵ As the cells cross the narrow wall of laser illumination, bound dyes are excited to fluoresce and the cells scatter light, the laser wavelength settings being 457 and 488 nm for excitation of mithramycin and FDA/PI-FITC, respectively. Fluorescence provides quantitative information on constituents

to which the dyes are bound (e.g., DNA content, total protein), whereas light scatter yields data on size¹² and internal structure.^{13,14} Both are electro-optically measured, the fluorescence sensor being a dual photomultiplier tube array which determines total or two-color fluorescence of selectable color regions. Small-angle light scatter (whole-cell size) is measured by focusing the forward light scattered onto a photodiode detector having a collection angle of 0.7° to 2.0°.¹⁵

Nuclear and cytoplasmic diameters also can be measured by determining the time required for the cell nucleus and cytoplasm to pass across the "thin wall" of laser excitation. This technique, as reported originally,¹⁶ is similar in concept to the "slit-scan" principle^{17,18} but relies on quantitative two-color fluorescence staining of the nucleus and cytoplasm using PI and FITC. As doubly stained cells cross the laser beam, analog red (nucleus) and green (cytoplasm) fluorescence signal time durations are converted electronically into signals, the amplitude of which is proportional to the respective diameters.¹⁶ In a more recent development, nuclear and cytoplasmic diameter measurements are based on staining the cell nucleus alone using mithramycin and determining the respective diameters from nuclear fluorescence and light-scatter signal time durations.¹⁹

After optical measurement, the stream carrying the cells emerges into air as a liquid jet from the flow chamber exit nozzle (Fig. 1). A piezoelectric transducer mechanically coupled to the chamber and electrically driven at about 40 kHz produces uniform liquid droplets (40 000/sec) by regularly disturbing the emerging jet (vibrational energy), thus causing the cells to be isolated into droplets,²⁰ with approximately 2% containing a single cell.

Optical signals proportional to the measured cellular parameters are processed electronically on a cell-by-cell basis as single parameters, ratios,

and gated single parameters using a hardwire signal processing unit (Fig. 1). A multichannel pulse-height analyzer accumulates and displays the processed signals as pulse-amplitude frequency distribution histograms. Single-parameter analysis is performed by selecting a cellular parameter and displaying its distribution. Ratios of parameters are computed on a single-cell basis and also displayed as histograms. Gated single-parameter analysis permits the examination of particular subclasses of cells within selectable ranges on different cellular parameter values. For example, the protein content distribution for G_1 -phase cells can be obtained by analyzing only those protein signals from cells having G_1 DNA content.¹¹ Gated analysis techniques similarly permit distributions to be determined from weakly fluorescing cells by requiring coincidence with light-scatter²¹ or surface-area signals.²²

When experiments require that cells be separated, processed signals activate sorting by comparing their amplitude with preselected ranges of a single-channel, pulse-height analyzer (SCA) located within the cell separation logic block (Fig. 1). The SCA range is chosen to correspond to a distribution region in which cells to be separated are located. If the signal amplitude then falls within the selected SCA range, an electronic time delay is activated which, after an appropriate time period between optical sensing and droplet formation (about 1000 μ sec), triggers a charging pulse as the cell arrives at the droplet formation point. This causes a group of droplets, one of which contains the cell, to be charged and deflected electrostatically into a collection vessel.²⁰ Cells not meeting the preset criteria do not trigger the above sequence of events and pass undeflected into a different vessel. In a typical experiment, lung cells were sorted at rates of a few hundred per second. The sorted suspension was then deposited onto a microscope slide using

centrifugal cytological methods (Shandon Scientific, Sewickley, Pa.) for counterstaining and microscopic examination.

In addition to measuring small-angle light scatter, a new flow instrument capable of determining light scatter at 32 angles simultaneously⁶ has been used also to analyze respiratory cells. As the cells pass through a flow chamber and are illuminated with a helium-neon laser, scattered light is detected using a circular photodiode array, is stored in a computer, and is then processed as a scatter diagram of light-scatter intensity vs angle. The scatter diagram is converted into a cluster diagram which permits individual light-scatter patterns from each cell to be grouped according to a mathematical clustering algorithm.²³

RESULTS AND DISCUSSION

Figure 2 shows a fluorescence distribution recorded on a hamster lung washing after treatment with FDA. The distribution, which is a measure of intracellular enzymatic activity (nonspecific esterases) and integrity of the cell membrane (i.e., cell viability), shows three distinct regions of cells consisting of peaks 1, 2, and 3. Lung cells corresponding to combined peaks 1 and 2 (channels 10-50) have been identified initially by sorting and microscopic examination as being composed principally of leukocytes. Respiratory cells corresponding to peak 3 have been identified similarly as primarily histiocytes, with a small percentage of macrophages present. Data from other hamsters also indicate that the total number of cells within the different regions (peaks) varies considerably from animal-to-animal. Further tests are planned to determine where epithelial and ciliated columnar cells are located and to correlate FDA activity with cell size using the gated analysis

techniques described above. Quantitative analysis of other cell enzyme activities using different fluorogenic substrates will be attempted also.

The measurement of DNA content and cell size is illustrated in Fig. 3 for a lung cell sample stained with mithramycin. Figure 3A shows the DNA distribution which is unimodal, indicating cells having 2C diploid DNA content. The cell size (small-angle light scatter) distribution (Fig. 3b) shows a heterogeneous population of cells, differing considerably in size with much overlap. Gated single-parameter analysis methods, coupled with cell sorting, were used to identify partially the individual size distributions for nucleated and non-nucleated cells in the following manner. The size distribution of nonnucleated cells (Fig. 3C) was determined by recording the light-scatter signals from only those cells (including debris) which scattered light but which did not fluoresce. Preliminary results, based on gated analysis and subsequent separation (channels 5-80 of Fig. 3C), indicate that the distribution is made up of erythrocytes and small debris. The size distribution of nucleated respiratory cells is shown in Fig. 3D and indicates much larger sized cells. This distribution was obtained by recording light-scatter signals from only those cells which both fluoresced and scattered light. These cells were also separated (channels 30-100 of Fig. 3D) and identified microscopically as consisting of histiocytes, leukocytes, macrophages, etc. Therefore, by requiring or not requiring coincidence of light scatter with nuclear fluorescence signals, size can be measured from a heterogeneous mixture of nucleated and nonnucleated cells. The individual size distributions (Figs. 3C and 3D) also demonstrate the regions to which they correspond in the total cell-size distribution (see Fig. 3B).

Flow-analysis methods can be used also to characterize cells according to nuclear and cytoplasmic diameters based on quantitative fluorescence

staining of either the nucleus alone or in combination with the cytoplasm. For example, Fig. 4 shows the nuclear and cytoplasmic diameter distributions for a lung washing stained with mithramycin. The nuclear diameter distribution (Fig. 4A), obtained by measuring the fluorescence signal time durations, is unimodal and indicates cells of similar sized nuclei. However, the cytoplasmic diameter distribution (Fig. 4B), determined from light-scatter signal time durations, shows that diameters vary over a considerable range. To find what portion of the cytoplasmic diameter distribution consisted of nucleated and nonnucleated cells, gated analysis techniques again were used. The cytoplasmic diameter distribution of nonnucleated cells (Fig. 4C), obtained by recording the diameter of only those cells (including debris) which did not fluoresce but which scattered light, is composed primarily of small debris and erythrocytes. The cytoplasmic diameter distribution of nucleated cells (Fig. 4D) was determined by recording diameter signals from only those which fluoresced and scattered light in coincidence. This distribution indicates a minimum of two populations differing in diameter by about 1.3. Similar data recorded on another lung washing indicated three distinct cell types. Experiments are under way to verify what cells correspond to the different peaks in the diameter distributions and to improve instrumentally the resolution of the diameter measurements.

Two-color fluorescence analysis of DNA content, total protein, and nuclear and cytoplasmic diameters in respiratory cells stained with PI-FITC is shown in Fig. 5. The DNA content distribution (Fig. 5A) is unimodal and similar to that recorded using mithramycin (Fig. 3A). The total protein distribution (Fig. 5B), which was obtained by recording only those green fluorescence (protein) signals coincident with red fluorescence (DNA content) signals, is broad and indicates a wide range of cellular protein values consisting of

major (peak 1) and minor (peak 2) regions. The nuclear diameter distribution (Fig. 5C), which was determined by fluorescence signal time duration measurements, is unimodal (uniformly sized nucleus) and similar to the distribution recorded in Fig. 4A using mithramycin as a nuclear stain. However, the cytoplasmic diameter distribution (Fig. 5D), as derived from green fluorescence signal time durations in coincidence with DNA content signals, is bimodal and shows a minimum of two cell populations differing in diameter by a factor of about 2.0. Future experiments will involve separating cells based on cytoplasmic diameter using this technique.

The two-color PI-FITC method permits DNA content, total protein, and nuclear and cytoplasmic diameters in lung cell samples to be determined. Except for analysis of total protein, the newer and simpler mithramycin staining procedure allows all the above cell parameters to be measured. It is also possible to combine these parameters into ratios on a cell-by-cell basis and to display them as frequency distribution histograms. Nuclear-to-cytoplasmic ratios have been shown previously to be an important parameter in automated analysis of abnormal gynecological specimens.¹⁷ Future experimentation will include measurement of nuclear-to-cytoplasmic size relationships combined with DNA, protein, and cell size measurements and sorting to correlate cell types with instrumental analysis.

In preliminary studies designed to measure subtle differences in lung cell morphology, flow multiangle light-scatter techniques have been used to classify exfoliated hamster respiratory cells based on differences in scatter patterns. Figure 6 shows an example of a cluster light-scatter diagram measured on an unfixed and unstained lung washing in which at least three groups can be distinguished based on differences in angular light-scatter intensity. Recent data indicate that possibly four to five cell types can be

detected. Future experiments will involve separating cells differing in light-scatter patterns and identifying what types correspond to the various regions of the cluster diagrams. These experiments will depend upon the addition of a cell-sorting capability to the multiangle light-scatter flow system.⁶

CONCLUSIONS

These preliminary results demonstrate the potential capability of applying advanced multiparameter flow-systems technology to analyze and separate exfoliated respiratory cells, the specific goal being development of cytological and biochemical indicators for future use in estimating early damage to the lung epithelium of mammals exposed by inhalation of toxic agents associated with nonnuclear energy production. Lung cells from normal hamsters will continue to be analyzed using the various flow-analysis methods for initial characterization purposes. These will include DNA content, total protein, esterase activity, whole-cell size, nuclear and cytoplasmic size relationships, and multiangle light-scatter properties. Emphasis will continue to be placed also on new methods which may be potential indicators of early atypical cellular changes. For example, quantitation of specific cell enzyme activities using fluorogenic substrates for possible determination of alkaline and acid phosphatase, lipase, peroxidase, esterase, etc., and others such as antigenic properties may provide methods for assessing early damage. Not only will lung cells be characterized further based on the above parameters but will be separated routinely and examined microscopically. This will permit a correlation of measured parameters with morphological observations. As experimentation proceeds, it may be necessary also to modify the present cell preparation and staining procedures and instrumental

analysis methods developed in the initial characterization studies.

To determine what cytological and biochemical differences can be measured quantitatively between control and experimental animals using flow-analysis techniques, it is planned initially to expose test animals to high concentrations of known toxic by-products from nonnuclear energy production and to stress the animals into an acute response. These initial experiments, which are being planned by the LASL Mammalian Biology Group (H-4) to determine toxic effects on the entire respiratory tract (including carcinogenesis), are to begin in the near future. Animals will be exposed by inhalation to aerosols of raw and spent oil shale or by other routes to chemical compounds associated with the fuel extraction process.

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TABLE 1 Differential Cell Counts of Normal Hamster
Lung Cell Samples Obtained by Lavaging the Lungs with Normal Saline*

Hamster Number	Macrophages (%)	Leukocytes (%)	Ciliated Columnar Cells (%)	Basal Epithelial Cells (%)	Histio- cytes (%)	Erythro- cytes (%)
1	4	2	29	37	13	15
2	1	3	2	3	22	69
3	1	19	5	4	34	37
4	4	9	4	5	20	58
5	3	3	1	3	13	77

*Determined microscopically.

FIGURE LEGENDS

FIG. 1. Diagram of the multiparameter cell separator system, illustrating laser excitation, flow chamber, fluorescence and light-scatter sensors, signal processing and cell separation electronics, and droplet charging and deflection scheme.

FIG. 2. Frequency distribution histogram for a normal hamster lung washing stained with fluorescein diacetate (FDA) and analyzed for fluorescence. The horizontal axis is proportional to the logarithm of intracellular fluorescence signal amplitude and covers a three decade range.

FIG. 3. Frequency distribution histograms of DNA content and cell size for a normal hamster lung washing stained with mithramycin and analyzed for fluorescence and small-angle light scatter, respectively: (A) DNA content distribution; (B) cell size distribution; (C) cell size distribution of non-nucleated cells and debris obtained by recording only light-scatter signals from nonfluorescing cells and debris; and (D) cell size distribution of nucleated cells obtained by recording only light-scatter signals from fluorescing cells. The horizontal axes of the three cell size distributions are proportional to the logarithm of light-scatter signal amplitude and cover a three decade range.

FIG. 4. Frequency distribution histograms of nuclear and cytoplasmic diameters from a normal hamster lung washing stained with mithramycin and analyzed for fluorescence and small-angle, light-scatter, signal time durations, respectively: (A) nuclear diameter distribution; (B) cytoplasmic diameter distribution; (C) cytoplasmic diameter distribution of nonnucleated

cells, including debris, obtained by recording only cytoplasm diameter signals from nonfluorescing material; and (D) cytoplasmic diameter distribution of nucleated cells obtained by recording only cytoplasm diameter signals from fluorescing cells.

FIG. 5. Frequency distribution histograms of DNA content, total protein, and nuclear and cytoplasmic diameters from a normal hamster lung washing stained with PI and FITC and analyzed for two-color fluorescence properties: (A) DNA content distribution (red fluorescence); (B) total protein distribution obtained by recording only green fluorescence signals from nucleated cells; (C) nuclear diameter distribution obtained from red fluorescence signal time durations; and (D) cytoplasmic diameter distribution obtained by recording only green fluorescence signal time durations from nucleated cells.

FIG. 6. Cluster diagram derived from the multiangle light-scatter pattern (three decade log intensity vs angle) recorded on a normal hamster lung-cell sample. Each cluster, which represents a cell size grouping, is enclosed by a broken or solid line and shows an excursion of one standard deviation from the mean. The percentages represent the approximate number in each size class (cluster).











