

HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS
CULTURED IN NORMAL AND
HYPERGLYCEMIC MEDIA IN SIMULATED MICROGRAVITY
USING NASA BIOREACTORS

Final Report

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ABSTRACT

We sought answers to several questions this summer at NASA Johnson Space Center. Initial studies involved the in vitro culture of human peripheral blood mononuclear in cells in different conditioned culture media. Several human cancer clones were similarly studied to determine responses to aberrant glycosylation by the argon laser. The cells were grown at unit gravity in flasks and in simulated microgravity using NASA bioreactors.

The cells in each instance were analyzed by flow cytometry. Cell cycle analysis was acquired by staining nuclear DNA with propidium iodide. Responses to the laser stimulation was measured by observing autofluorescence emitted in the green and red spectra after stimulation.

Extent of glycosylation correlated with the intensity of the laser stimulated autofluorescence.

Our particular study was to detect and monitor aberrant glycosylation and its role in etiopathogenesis.

Comparisons were made between cells known to be neoplastic and normal cell controls using the same Laser Induced Autofluorescence technique.

Studies were begun after extensive literature searches on using the antigen presenting potential of dendritic cells to induce proliferation of antigen specific cytotoxic T-cells. The Sendai virus served as the antigen.

Our goal is to generate sufficient numbers of such cells in the simulated microgravity environment for use in autologous transplants of virally infected individuals including those positive for hepatitis and HIV.

INTRODUCTION

Carbohydrates are ubiquitous in living systems. Glycosylation is the most extensive of all the posttranslational modifications and has important functions in secretion and antigenicity. (1) Carbohydrates account for the structural diversity of organisms and for differentiation and development. Several factors influence the extent of glycation; one is the ambient glucose concentration. Long term hyperglycemia is a factor contributing to the accumulation of Advanced Glycosylated End Products (AGEs) on tissue macromolecules. (2) AGEs are the result of non-enzymatic glycosylation of proteins. The interaction between reactive terminal amino group and the carbonyl group of a reducing sugar results in a Schiff base adduct, which can undergo an Amadori rearrangement to form a ketoamine adduct (3) These products can undergo multiple rearrangements to become the irreversibly bound, chemically reactive AGE. These insoluble products are responsible for the symptomology associated with the aging process and with diabetes. Aberrant glycosylation in some instances is involved in the formation of tumor antigens., (4)

A physical property associated with AGEs is the emission of 570 nm or 630nm light energy (autofluorescence following the absorption of 448 nm energy associated with argon laser. The induction of Laser Induced Fluorescence (LIF) was compared in tumor cell lines of various lineages, and found to be distinguish lymphoid tumor cells from normal. Notable variations in induced fluorescent intensity were noted in normal murine lymphocytes cultured in excess glucose. Human peripheral blood mononuclear cells (PBMC) demonstrated similar fluctuations in autofluorescence emission spectra when grown in the presence of excess glucose at either unit gravity or in simulated microgravity.

Our results indicate that cells altered by aberrant glycosylation can be distinguished by LIF. It follows that various therapies to reverse or prevent aberrant glycosylation can similarly be monitored in a brief time with the flow cytometer and its laser energy source. In previous studies we demonstrated that AGEs responsible for some of the diabetic symptomology can be reversed using hyperbaric oxygen protocols. .

MATERIALS AND METHODS

Cells and Media

Normal human blood was obtained from the Gulf Coast Regional Blood Center, Houston, Texas. The peripheral blood mononuclear cells were isolated on a Ficoll-Hypaque gradient (Pharmacia LKB, Piscataway, N.J.), washed three times in PBS and resuspended in complete RPMI-1640 (GIBCO-BRL), Grand Island, N.Y. supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, Utah) and penicillin (100 U/ml)-streptomycin (100 ug/ml, GIBCO-BRL). Cell counts were determined with a hemacytometer and cell viability by trypan blue exclusion. Glucose, Na⁺, K⁺, Cl⁻ concentrations in the media were checked using the Portable Clinical Analyzer, I-Stat from I-STAT Corporation, Princeton, N.J. Cell concentrations were adjusted to 1 x 10⁶ cells/ml. The glucose concentration was 100 mg% in the control study and 400 mg% in hyperglycemic medium. Cell conditions in both instances were maintained at 37°C in 5% CO₂.

RWV Bioreactor

One of the bioreactors devised by NASA scientists is the Rotating Wall Vessel (RWV) Bioreactor known as the HARV (High Aspect Rotating Vessel). This instrument creates an environment for cells which simulates some aspects of microgravity. The HARV with a volume of 10 ml and a rotation speed of 14 rpm was used in these studies.

Flow Cytometry Analysis

Cell data were acquired on a Becton Dickinson FACS-Calibur Flow Cytometer and analyzed with the ModFit Software program (Verity, Inc., Maine). 10,000 events were recorded as dot plots with side scatter vs forward scatter ordinates. In all experiments, cells exhibiting 90 degree scatter were eliminated; dead and large granular cells were excluded from analysis by gating.

Altered Glucose Concentrations

Cell concentrations were adjusted to 1×10^6 cells/ml. Cells in control studies were cultured in complete medium which contained approximately 100 mg% glucose. In the test study, glucose concentrations were adjusted to 400 mg% glucose. We wanted to learn if the hyperglycemic medium affected cell cycle. All cell suspensions were maintained at 37° C in 5% CO₂ atmosphere. Data were analyzed on a Becton Dickinson FACS-Calibur Flow Cytometer from aliquots using side scatter vs log FL-1 (green) emitted fluorescence. Overlay histograms plots of FL-1 values for control vs test indicated the extent that cells absorbed the laser energy and retransmitted it as light in the green spectrum. Our data measured Mean Fluorescence Intensity (MFI) in response to argon laser stimulation for the suspensions at normal and at reduced gravity and at both normal and elevated glucose.

Cell Cycle Determinations

The procedure of Shapiro for DNA staining was followed. (5) Cells were pelleted, washed and resuspended in 70% cold ethanol overnight. The ethanol fixative was removed and 100 ul Propidium Iodide in buffer was added for ten minutes. Residual RNA was eliminated by ribonuclease contained in the PI buffer. The cells were then stimulated by the argon laser of the flow cytometer. Fluorescence intensity was recorded in histogram graphs of side scatter vs linear FL-2. The linear scale permitted us to read 2N (diploid) and 4N (tetraploid – dividing) cells. Cells undergoing apoptosis (programmed cell death) appeared as hypodiploid. Software statistical analysis was completed with the ModFit Software Program (Verity, Inc., Maine) and yielded cell cycle percentages including diploid, tetraploid cells and percentage of cells in apoptosis.

Distinguishing malignant Cells from Normal Cells

When Jurkat Cancer Cells (Clone EC-1) were stimulated by the argon laser of the flow cytometer, they emitted fluorescent light in the green (570nm) and red (630 nm) regions of the visible spectrum. The cells were suspended in HBSS and 3% FCS. The cytometer was set at side scatter vs log FL-1 or log FL-2 and data were recorded in histograms. Normal human T-cells served as controls. Control T-cells did not show appreciable autofluorescence under laser stimulation. The results confirmed our hypothesis that LIF could be used as an early assay for malignancy and aberrant glycosylation. The conclusion was based on the assumption that a tumor glycoconjugate antigen or other aberrant glycosylation was involved in the etiopathogenesis of the cancer. Other cancer cell lines that were found to autofluoresce under LIF were Daudi (B Lymphoblasts from human Burkitts Lymphoma), Raji, (Human Lymphoid-like line, K-560, Human melanogenous leukemia), and HL-60, Human Lymphoma.

The Raji B cell line demonstrated significantly higher emission in both spectra than any other cell line in this panel. Jurkat demonstrated lowered emission within the green spectra when compared to the B and myeloid lineage tumors, but higher emissions as compared to normal lymphocytes. Cellular autofluorescence has been attributed primarily to the presence of reduced flavins and pyridine nucleotides. We therefore attribute the reported differences in autofluorescence to be representative of the higher metabolic activity characteristic within this tumor panel may be indicative of additional differences in cellular glycosylation

Cell Cycle Analysis by Flow Cytometry of Human Peripheral Blood Lymphocytes in Media of Normal glucose concentration and hyperglycemia.

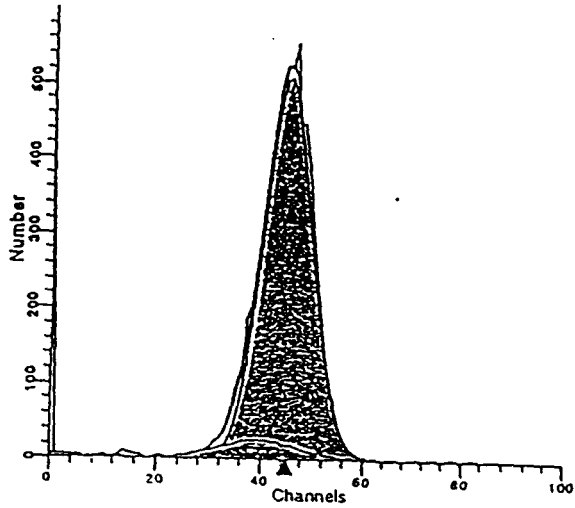


Fig 1
Cells in unit gravity - high glucose
0.45% Tetraploid 99.5% Diploid
0.00% Apoptosis

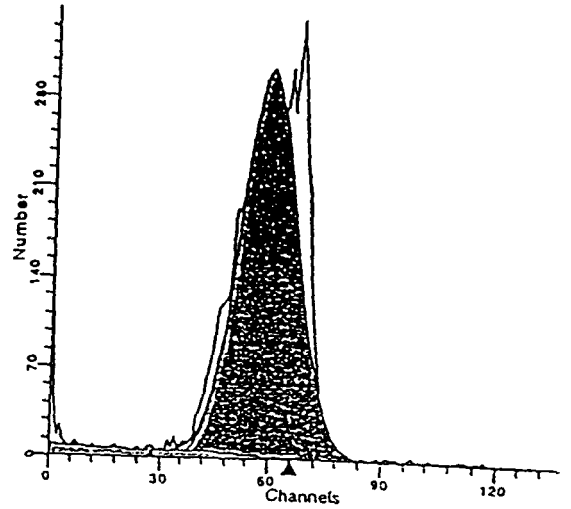


Fig 2
Cells in unit gravity - normal glucose
0.59% Tetraploid 99.28% Diploid
0.12% Apoptosis

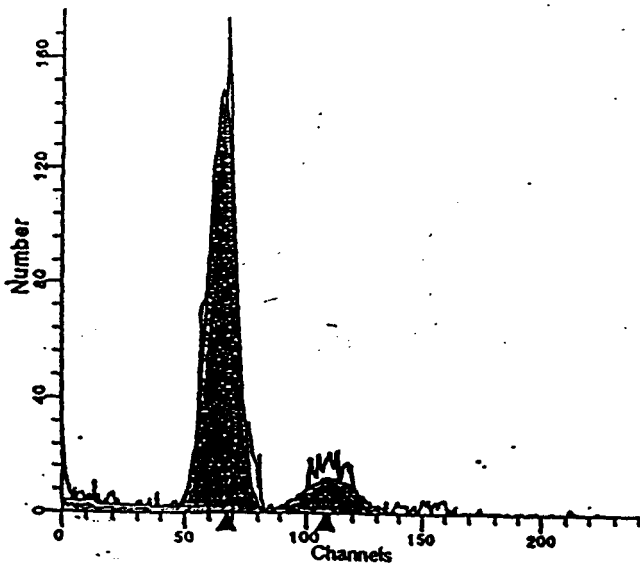


Fig 3
Cells in simulated microgravity - normal glucose
14.05% Tetraploid 85.95% Diploid
0.00% Apoptosis

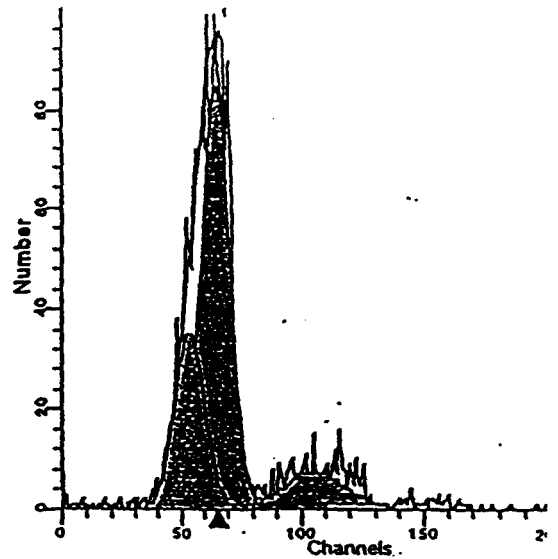


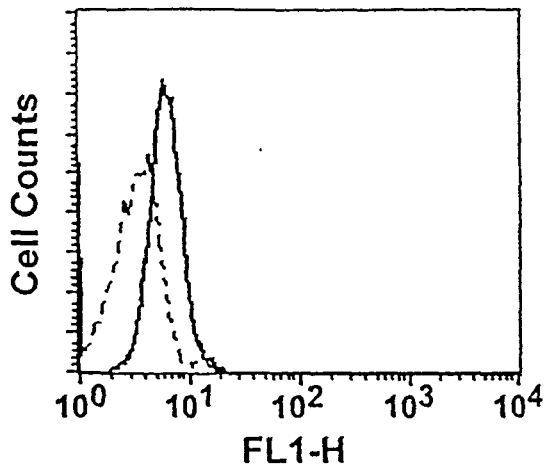
Fig 4
Cells in simulated microgravity - high glucose
14.99% Tetraploid 85.01% Diploid
27.04% Apoptosis

Argon laser stimulation of PBMCs at unit gravity and in simulated microgravity.

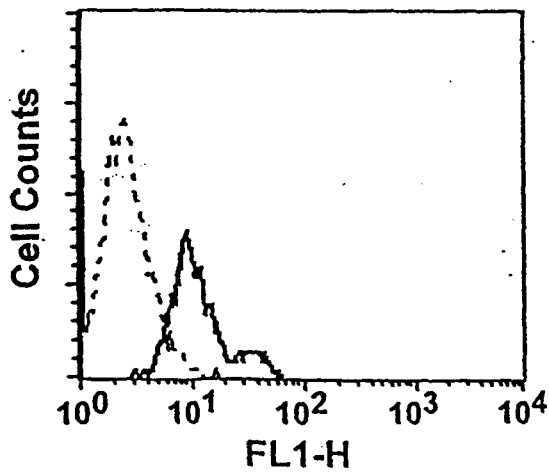
Green fluorescence of PBMCs after stimulation by the argon laser of the flow cytometer.
Fluorescence intensity is proportional to the glucose concentration and is more pronounced
in microgravity.

- = normal glucose concentration (100 mg%)
- = elevated glucose concentration (400 mg%)

PBMC at unit gravity



PBMC in simulated microgravity



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DISCUSSION

In the present work, the inherent autofluorescent characteristic of tumor cells was measured as well as autofluorescence of cells affected by environmental influences. All cells autofluoresce to some degree when stimulated by the appropriate electromagnetic energy. Laser-induced autofluorescence has been attributed to the emission of excitation energy by substances such as flavins, porphyrins and aromatic structures within the cell. The absorption of 360 nm energy by the coenzyme NADH₂ results in an emission maxima at 460 nm. Riboflavin emission has been reported at 530 nm. Thus, differences in autofluorescent spectra have been used to appraise the oxidative state of the cell.

Recent investigations have demonstrated the usefulness of this distinction in diagnosis of tumors in situ, eliminating the need for biopsy in some instances. Thus, early detection of colon cancer cells has been detected by LIF on in situ tissues. (Dus). Bladder cancer has likewise been recognized (Koenig).

These assays were performed histochemically. Cellular biopsies could be performed on in vitro cell suspensions as well as by a single investigation with flow cytometry.

Cells that autofluoresce by absorbing the laser energy can be separated from cells that absorb the energy to a lesser extent. The exploitation of this characteristic provides the basis for the characterization of distinct cell types. Researchers have separated dendritic cells from macrophages in this manner. (10)

Splenic macrophages and murine Kupffer cells have also been separated. (11)

Aberrant glycosylation in the formation of AGEs has been firmly implicated in the symptomatology of diabetes and the aging process (2) The presence of AGE formation is readily detected with LIF. The effectiveness of methodologies which prevent or reverse glycosylation can likewise be monitored since the intensity of emitted fluorescence from simulated laser energy is proportional to the extent of glycosylation.

In a previous investigation we showed hyperbaric oxygen therapy can be used to treat non-healing wounds of diabetics by reversing AGE formation. Others have used aminoguanidine or butyrate successfully. (9)

The deglycosylation in these procedures can be monitored by LIF.

All cells autofluoresce to some degree when stimulated by appropriate electromagnetic energy. In addition to cell surface glycoproteins, LIF detects the presence of the reduced flavin and nicotinamide coenzymes, $FADH_2$ and $NADH_2$. These compounds appraise the oxidate state of the cell. They are pronounced in cancer cells.

Cells that autofluoresce by absorbing various amounts of laser energy can be separated from cells that absorb such energy to a lesser extent. Researchers have separated dendritic cells from macrophages in this manner. (10). Splenic macrophages and murine Kupffer cells have been also separated using the technique. (11)

Early detection of colon cancer cells has been detected by LIF (12). Bladder cancer has also been recognized (13). Cellular biopsies can be performed on in vitro cell suspensions by a single investigation with flow cytometry.

When we stimulated Jurkat Cells (Clone E6-1 acute T Cell Leukemia, human) with the argon laser, they emitted fluorescent light in the green, (570 nm) and red, (630nm) regions of the visible spectrum. the other cancer lines which we examined in this research showed similar results. Normal human T-cells served as our controls, and they did not show appreciable autofluorescence under laser stimulation. The results confirmed our hypothesis that LIF can be used as an indicator of malignancy and aberrant glycosylation

This conclusion was based on the premise that a tumor glycoconjugate antigen or other aberrant glycation was involved in the etiopathogenesis of these cancers.

Murine splenocytes and bone marrow cells cultured in excess glucose exhibited greater autofluorescence when compared to cultures grown in normal glucose. The induction of increased metabolism was verified by quantitation of the cellular DNA. Human peripheral blood mononuclear cells (PBMC) demonstrated similar fluctuations in autofluorescence emission spectra when grown in the presence of elevated glucose in both unit gravity and in simulated microgravity. Taken together, these findings show new potentials of LIF in cell biology and flow cytometry.

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EPILOGUE

A half century ago, Vannevar Bush wrote "Science: The Endless Frontier". Alexander Pope wrote, "Hill peeps o'er hill and Alp on Alp arise". These two classics haunt me at the conclusion of these investigations. The research has revealed some answers but most importantly it has given rise to further questions. And that is science: asking the right question.

Laser Induced Autofluorescence as shown in this work has tremendous analytical potential. It monitors glycosylation and deglycosylation, measures the rate of cellular metabolism, in the instances cited distinguishes cancer cells from normal ones. It potentially can be a tool for the oncologist to show the effectiveness of particular therapies. Conceivably, it can show that deglycosylation can convert cancer cell to normalcy.

LIF has established itself in assay protocol and determining etiopathogenesis in some instances. The argon laser of the flow cytometer readily determines rates of apoptosis and cell proliferation in the cell cycles. It does more: when coupled with other techniques, it shows the influences that alter cell cycle.

The most valuable contribution of this summer research is to "ask the right question" and now to seek the answer to that question.

We are grateful for having had this opportunity.

August 4, 2000

Important background papers to support this research proposal:

GETTING THE GLYCOSYLATION RIGHT: IMPLICATIONS FOR THE BIOTECHNOLOGY INDUSTRY

Nigel Jenkins, Raj B Parekh and David C James
NATURE BIOTECHNOLOGY, Vol 14, August 1996

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17 (1994) 27-51

DIRECT ACTIVATION OF CD8+ CYTOTOXIC T LYMPHOCYTES BY DENDRITIC CELLS.

Kayo Inaba, James W. Young, and Ralph M. Steinman
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Ruta Nonacs, Cornelia Humborg, James P. Tam and Ralph M. Steinman.
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BIOASSAYS 21, 412-421, 1999

INACTIVATED INFLUENZA VIRUS, WHEN PRESENTED ON DENDRITIC CELLS ELICITS HUMAN CD8+ CYTOTOXIC T CELL RESPONSES.

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Piepmeyer, et al., FASEB JOURNAL, 12, August, 1998.