Introduction

• CBD is a delayed-type hypersensitivity reaction due to a cell-mediated immune response in the lung.

• Lymphocyte recognition of beryllium presented in association with MHC II molecules on APCs results in the generation of beryllium-specific effector lymphocytes.

• Exposure of these lymphocytes to beryllium in vitro causes them to undergo cell division, as determined by incorporation of $^3$H-thymidine, BrdU or 5,6 carboxyfluorescein diacetate succinimidyyl ester (CFSE) into cellular DNA.

• This incorporation into dividing cells constitutes the basis of the lymphocyte proliferation test (LPT) used to identify Be-sensitized people.
Background

• Information provided by the traditional LPT limited to estimation of “stimulation index” due to 3H-thymidine incorporation only during the S phase in stimulated lymphocyte cultures, limiting number of responsive cells.

• Non-proliferating “bystander” cells may confound results.

• Fluorescent markers used with flow cytometry can test for beryllium sensitization, identify and quantify the specific cell types responding to beryllium, and can be linked with analyses of cell cycle and functional protein production, generating additional useful information compared with standard LPT.

• Flow cytometry with multiparametric analysis provides an alternative LPT method for highly heterogeneous cell populations in cell-mediated immune responses at the single cell level.
Background (2)

- Flow technique first reported use in CBD by LANL Biosciences in 2000*.
- BrdU incorporated into DNA (T → U) as cells proliferate, then stained w/ anti-BrdU FITC, and additional stains as desired for phenotyping and cell cycle analysis.
- 7 CBD and 120 beryllium exposed workers tested. 4 BeS detected by both standard and flow methods, as were CBDs.
- CBD cases exhibited strong CD4+ (T-helper) and CD8+ (T-killer) proliferation, while BeS showed mainly CD8+ proliferation.
- Authors recommended that this “immuno-Be-LPT be used to confirm results of standard clinical BeLPT.

BrdU Incorporation Method

Another flow cytometry-based method uses labeling with BrdU and BrdU-mediated stoichiometric quenching of the DNA intercalating dye Hoechst 33258.

Proliferating cells with incorporated BrdU identified by decreased Hoechst fluorescence compared to non-proliferating cells, with each cell division producing less fluorescence intensity due to quenching.

The position of the cells within the cell cycle identified by counter staining with a second DNA dye (e.g. 7-AAD, PI, EB) whose fluorescence is unaffected by BrdU.

The BrdU-Hoechst assay can also be combined with immunolabeling using monoclonal antibodies to cell surface receptors and intracellular cytokines for simultaneous analysis of proliferation, cell phenotype and effector cytokine production.
BrdU-Hoechst Methods

- Isolate Peripheral Blood Lymphocytes (PBL) from CBD patients (Ficoll-Paque density gradient centrifugation)
- Culture PBL in RPMI medium + 10% Fetal Bovine Serum + 150 µM BrdU at 5 X 10⁶ cells/ml
- Expose to either BeSO₄, PHA or Tetanus toxin
- Incubate cells in the dark for either 3 days (PHA), or 4 - 6 days (Be or TT)
- Harvest and fix cells (saponin + 0.25% PFA)
- Immunolabel cells with subtype-specific fluorescent Mab
- Stain nuclei with Hoechst 33258 + Ethidium Bromide (EB) or 7-AAD
- Multiparameter flow cytometry for simultaneous analysis of cell proliferation and immunophenotype
FACS 4-Channel Flow Cytometer
Simultaneous immunophenotyping and proliferation analysis of PBL stimulated in vitro with tetanus toxin
BrdU/Hoechst- EB flow cytograms of PBL from a CBD patient.

PHA

Tetanus Toxin

FBS Control

0.5 uM Be

5 uM Be

50 uM Be

Hoechst Fluorescence

EB Fluorescence
# Quantitation of cells exiting G0/G1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Proliferating Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 3</td>
</tr>
<tr>
<td>FBS Control</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>PHA</td>
<td>52±1.5</td>
</tr>
<tr>
<td>Tetanus</td>
<td></td>
</tr>
<tr>
<td>BeSO₄ 0.5 uM</td>
<td></td>
</tr>
<tr>
<td>5.0 uM</td>
<td></td>
</tr>
<tr>
<td>50 uM</td>
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</table>
Be Stimulation Index
(ratio of Be stimulated proliferation to FBS control)

![Graph showing the Be Stimulation Index over different concentrations of BeSO₄ on Day 4 and Day 6.]
Kinetic analysis of the proliferative response to beryllium

<table>
<thead>
<tr>
<th></th>
<th>DAY 4 % CELLS ENTERING 1^{ST} CYCLE</th>
<th>DAY 6 % CELLS ENTERING 1^{ST} CYCLE</th>
<th>DAY 4 % CELLS COMPLETED 1^{ST} CYCLE</th>
<th>DAY 6 % CELLS COMPLETED 1^{ST} CYCLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrdU</td>
<td>0.4±0.1</td>
<td>0.5±0.3</td>
<td>0.3±0.2</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>0.5 µM BeSO₄</td>
<td>0.6±0.2</td>
<td>0.3±0.2</td>
<td>1.2±0.1</td>
<td>3±0.2</td>
</tr>
<tr>
<td>5 µM BeSO₄</td>
<td>0.9±0.5</td>
<td>0.5±0.3</td>
<td>3.3±0.4</td>
<td>5.3±0.5</td>
</tr>
<tr>
<td>50 µM BeSO₄</td>
<td>1.9±0.2</td>
<td>0.7±0.2</td>
<td>4.3±0.9</td>
<td>7.4±0.6</td>
</tr>
</tbody>
</table>
Immunophenotyping of lymphocytes from CBD patient

![Graph showing immunophenotyping of lymphocytes from CBD patient.](image)
Rossman Lab Methods *

- Isolate Peripheral Blood Lymphocytes (PBL) from BeS patients (Ficoll-Paque density gradient centrifugation)
- Culture PBL in RPMI medium + Heat inactivated human serum Following 10 min. staining with CFCE
- Expose to either BeSO$_4$ (10 or 100 uM), PHA or Candida
- Incubate cells 37°C for 7 days
- One set pulsed with $^3$H thymidine in standard fashion
- One set harvested and fixed (saponin + 2% formaldehyde)
- Immunolabel cells with subtype-specific fluorescent monoclonal antibody
  - CD3-Tri-color (TC)  CD4- Phycoerythrin (PE)  CD8- allophycocyanin (AC)
- FACS 4-color flow cytometer (CFSE FL1, CD4 FL2, CD3 FL3, CD8, FL4)
- 50,000 events collected and analyzed using CellQuest software

5,6 Carboxyfluorescein Diacetate Succinimidyl Ester (CSFE)

Proliferative ratio calculated on Day 7:
% divided cells/ % undivided cells

9 normal unexposed controls
24 BeS (two pos BeLPTs)
   4 CBDs, 7 Be alveolitis, 13 non-diseased
GATING:

R1 light scatter to determine lymphocyte population

R2 live CD3+ cells

R3 CD3+,CD4+ cells
Milovanova, et al. 2004

BeS individual, with CFSE labeled CD3+, CD4+ T-cells cultured for 7 days.
Milovanova, et al. 2004

<table>
<thead>
<tr>
<th>Mitogen and Antigen</th>
<th>CD3⁺/CD4⁺ T-Lymphocyte Responses From Beryllium-Sensitized Subjects and Beryllium-Unexposed Donor Controls Measured by CFSE Dye Dilution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subset/treatments</td>
<td>Normal controls (n = 9)</td>
</tr>
<tr>
<td>Stimulants</td>
<td>CD3⁺/CD4⁺</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>0.023 ± 0.005                                                                   †</td>
</tr>
<tr>
<td>PHA</td>
<td>10.064 ± 2.753                                                                  †</td>
</tr>
<tr>
<td>Candida</td>
<td>0.076 ± 0.035                                                                   †</td>
</tr>
<tr>
<td>Be 10 μM</td>
<td>0.027 ± 0.005                                                                   †</td>
</tr>
<tr>
<td>Be 100 μM</td>
<td>0.029 ± 0.010                                                                   †</td>
</tr>
</tbody>
</table>

† p < 0.05 BeS vs. controls
Beryllium Responses of CD3⁺, CD3⁺/CD4⁺, and CD3⁺/CD8⁺ Subpopulations From Control and Beryllium-Sensitized Subjects as Measured by CFSE Dye Dilution

<table>
<thead>
<tr>
<th>Subsets/treatments</th>
<th>Normal controls (n = 6)</th>
<th>Be-sensitized subjects (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3⁺</td>
<td></td>
<td></td>
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<tr>
<td>Be 10 µM</td>
<td>0.012 ± 0.002⁺</td>
<td>0.156 ± 0.114⁺</td>
</tr>
<tr>
<td>Be 100 µM</td>
<td>0.013 ± 0.002</td>
<td>0.188 ± 0.137⁺</td>
</tr>
<tr>
<td>CD4⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Be 10 µM</td>
<td>0.015 ± 0.003</td>
<td>0.164 ± 0.112⁺</td>
</tr>
<tr>
<td>Be 100 µM</td>
<td>0.015 ± 0.002</td>
<td>0.210 ± 0.114⁺</td>
</tr>
<tr>
<td>CD8⁺</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Be 10 µM</td>
<td>0.013 ± 0.003</td>
<td>0.016 ± 0.003</td>
</tr>
<tr>
<td>Be 100 µM</td>
<td>0.011 ± 0.004</td>
<td>0.019 ± 0.002</td>
</tr>
</tbody>
</table>
Limitations

- Nucleotide analogue incorporation not always correlated well with increased DNA content and cell proliferation due to incomplete incorporation.

- These flow cytometric techniques performed only under research conditions to date.

- Full validation studies need to be done before questions of improved performance over traditional LPT can be answered.
Conclusions

• These non-radioactive assays of beryllium sensitization can measure cell proliferation in response to beryllium using continuous BrdU or CFSE labeling of cultured cells with standard clinical laboratory cytometers.

• Results suggest that continuous labeling could be more sensitive than the standard LPT assay since all cells that incorporate BrdU throughout the culture period are measured rather than those that just happen to be in S phase at the time of $^3$H-thymidine pulse-labeling. Dead cells are not counted, and multiple cell divisions can be measured.

• Knowing specific proliferating cell-types can add specificity to the test.

• In addition, the flow cytometry method incorporates additional mechanistic and kinetic information. Discrimination of the responding population can be simultaneously determined using cell surface or cytokine immunophenotyping.

• Flow cytometry can be successfully performed using about 1/3 number of cells than traditional BeLPT
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Effects of exogenous IL-2 on beryllium-induced PBL proliferation

Day 4

Day 6

- IL2

+IL2

BeSO₄ (µM)