Extraction and Concentration of Macrophages
from Murine Lymph nodes.

A Senior Honors Project
(ID 499)

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Abstract

This project involved the development of a protocol for releasing culturing adherent macrophages from marine lymph nodes. The process was based on methods used in extracting macrophage cells from the red pulp of spleens and liver granulomas using collagenase treatment. Some data on macrophage Ia expression in mouse models of leishmaniasis was collected in experiments leading to the development of the protocol.

Introduction

Experimental leishmaniasis in mice has been used as a model for human diseases such as Kala Azar and cutaneous leishmaniasis. The Balb/c mouse strain has been shown to be extremely vulnerable to infection by *Leishmania major* (1-5). The infected mice show an enlargement of spleens and regional draining lymph nodes; swelling and eventual ulceration of footpad (site of infection) also occurs (6-9). Research in our lab has also shown that the mice show "hyper-expression" of Ia on macrophages extracted from the peritoneal cavity (cells known as PECs) (10). Ia is a cell surface protein that is involved in the immune response by "presenting antigen" in such a way as to stimulate lymphocytes to act against a specific foreign body. Experimental leishmaniasis in the C57BL/6 strain of mouse leads to a slight enlargement of spleens and lymph nodes, but no ulceration of footpad occurs. These mice do not exhibit the hyper-expression of Ia
on their PECs (6-10). These mice mount an effective immune response and recover from the *L. major* infection. Balb/c mice that receive prophylactic treatment with the immunosuppressive drug Cyclosporin A (CsA), have been shown to mount an immune response similar to the C57BL/6 mice. The mice lack the severe pathogenic response of the untreated mice, and they also lack the hyper-expression of la on their PECs.

As mentioned above, unprotected Balb/c mice develop an increased numbers of la+ peritoneal macrophages as the infection progresses. Research has suggested that the ineffective immune response of these mice may be due to hyper-activation of some inappropriate T cell populations (11). We know that cytokines such as IL1, gamma interferon, and macrophage activating factor (MAF) create an interdependent relationship between macrophages and T cells. In our lab, we have been trying to determine what role macrophages play in the effective or ineffective immune response to leishmaniasis.

Macrophages populate various parts of the body. Their morphologies and functions can differ according what areas these cells populate. The microglia cells of the brain, Kupffer cells in the liver, PECs of the peritoneal cavity, and red pulp macrophages of the spleen are populations of macrophages that have been extensively studied. Due to the difficulty in releasing macrophages from lymph node tissues, little is known about the specific roles of these cells. In
our lab, the majority of our macrophage research has been
done on PECs and macrophages extracted from the spleen. By
developing a protocol for releasing macrophages from the
lymph nodes, we hope to better determine their role in the
immune response to leishmaniasis.

This protocol is based on procedures used to release
adherent macrophages from spleen red pulp tissue and liver
granulomas by treatment of tissues with collagenase enzymes
(12,13)

Materials Methods

Mice- All Balb/c and C57BL/6 mice were bred in our own
breeding facilities.

Leishmania major infection- Protozoa were isolated from the
spleens of infected mice. The L. major were grown in vitro
in RPMI with 20% Fetal Calf Serum. Animals were infected
with $1 \times 10^6$ stationary stage promastigotes injected
subcutaneously in the left hind footpad. The stationary
stage promastigote has been shown to be the most infective
stage in the Balb/c mouse strain (14). Leishmania were
suspected in PBS and delivered in a 0.05 ml dose with a 27
gauge needle.
Cyclosporin A treatment - Balb/c mice were treated orally with CsA in oil. Mice were given doses of 150 mg/kg of body weight twice a day at 12 hour intervals. Treatment with CsA began 1 day prior to L. major infection.

**unless noted otherwise all solutions used and stored at 4°C.**

**Reagents**

Modified Dulbecco's PBS (DPBSG) - This solution contained:

A. 131.7 mM NaCl  
B. 8.06 mM Na₃H₂PO₄ · 7H₂O  
C. 1.5 mM KH₂PO₄  
D. 5.5 mM D-glucose  
E. 2.7 mM KCl  
F. 0.9 mM CaCl₂  
G. 0.5 mM MgCl₂ · 6 H₂O  
H. Brought to a pH of 7.3  
I. Brought to an end volume of 1.00 liter

Ca²⁺/Mg²⁺ Free DPBSG - Prepared as above, but without steps F and G.

Enzyme Solutions - All solutions were prepared no more than 30 minutes before use, by dissolving enzyme in DPBSG previously warmed to 37°C. Collagenase I, II, IV, and VII and DNase I (Sigma Co.) were used as noted in results section.
Percol Gradient - A 90% solution was prepared by dissolving 10 ml of $10^x \text{Ca}^{2+}/\text{Mg}^{2+}$ Free DPBSG in 90 ml Percol (Sigma Co.). Then a 63.6 gradient solution was prepared by combining:

A. 70.6 ml stock 90% Percol solution
B. 20.4 ml 1x Ca/Mg Free DPBSG
C. 9.0 ml heat inactivated Fetal Calf Serum

Macrophage adherence incubation - 2 hour incubations of macrophage suspensions were performed in 24 well cell culture plates. Previous to use, a square glass microscope cover slip (Belco) was placed in each well to be used. The cover slips were used, because macrophages have been shown to selectively adhere to glass. Unless otherwise stated, 1 ml of cell culture was added to each well used. After a 2 hour incubation ($37^\circ \text{C} 5\% \text{CO}_2$), slips were gently washed with saline solution to remove non-adherent cells. All adherent cells remaining after wash were assumed to be macrophages. Previous studies have shown that more than 95% of these cells are esterase positive, a macrophage specific characteristic.

Paraformaldehyde Fixative - A stock solutions was prepared fresh every two weeks. The stock solution was made by dissolving 1g paraformaldehyde (solid) in 20.0 ml 0.1M $\text{PO}_4$ buffer plus 1.25 ml 0.001M $\text{CaCl}_2$ at $70^\circ \text{C}$. The solution was gravity filtered through filter paper to remove solid particles. 3.7 ml of 0.1M $\text{PO}_4$ buffer was added to filtrate. Stock solution was stored at $4^\circ\text{C}$ until used.
A 1% paraformaldehyde solution was made just prior to use by diluting stock 4% solution 1:4 with 0.1M PO₄ buffer (pH 7.3).

**Indirect fluorescent antibody stains** - Anti-IA⁺,⁺⁺⁺⁺ antibodies were produced by ascites growth of the D3137 hybridoma cell line (kindly donated by Roger J. Kurlander of the Duke Medical Center, Durham, NC). This cell line was used because the D3137 antibody has been shown to bind to the Balb/c IA⁺⁺⁺⁺ and the C57BL/6 IA⁺⁺⁺⁺ halotypes of the IA MHC molecule. Immunoglobulin was concentrated using caprilic acid purification (15). Antibody solution was dissolved 1:10 in rabbit serum diluent just prior to use. This was done to prevent nonspecific binding of immunoglobulin.

The rabbit serum diluent contained 10 mg/ml bovine serum albumin (BSA) and 10% normal rabbit serum in RPMI 1640 media (Sigma Co.).

An anti-mouse Fab'₂ antibody Fluorescein Isothiocyanate conjugate (purchased from Pel-freez) was used to tag cells marked by D3137 antibody. This antibody binds to the Fc portion of the anti-IA antibody. The Fab'₂ fragment antibody does not nonspecifically bind to macrophage cells. Just prior to use, the Fab'₂ was diluted 1:10 in Rabbit Serum Diluent.
Indirect Fluorescent Antibody Staining of Adherent Macrophages (IF stain)—The D3137 antibody was used to identify adherent Ia+ lymph node macrophages. Our lab's standard procedure for this staining was used (9).

Microscopic Examination—Coverslips were examined at 40X both under visible light and UV light using a UV microscope (Zeiss). Slips were first examined under normal light to determine total number of macrophages in examined area. Then under UV light fluorescent cells were counted. All fluorescent cells were considered Ia positive.
**Procedure for releasing adherent macrophages from lymph nodes**

Exact details of each experimental method are described under **Results** section of paper. Experimental groups of cells were collected from one or two lymph nodes as noted.

**A.** Mice were sacrificed by CO₂ asphyxiation.

**B.** Draining popliteal lymph nodes (located behind knee of left leg) were removed and transferred to petri dish containing 10 ml DPBSG at 4°C.

**C.** If lymph node was perfused with enzyme (see specifics of each experiment under **Results**), then individual nodes were injected with 5 ml of 800 units/ml Collagenase II with syringe and 22 gauge needle.

**D.** Nodes were ground using frosted portions of glass microscope slides into 10 ml of DPBSG to release cells. In experiments JPT 1-7 and JPT 10, Collagenase II, IV, or VII was added to DPBSG before grinding in those groups noted as Collagenase treated. In experiments JPT 8 and 9, lymph nodes were ground in fluid released during perfusion of tissue. Tissue was ground until majority of cells were released into suspension. In experiments JPT 11-14, lymph nodes were ground in 10 ml DPBSG, Centrifuged to pellet at 4°C, and resuspended in 1000 units/ml Collagenase I solution.

**E.** Cells were incubated at 37°C in a 5% CO₂ environment.
F. Following the release of cells the suspensions were washed using Ca/Mg free DPBSG by centrifuging down cells in DPBSG. The pellet was resuspended in 10 ml Ca/Mg free DPBSG. Then these cells were centrifuged down again. Collagenase enzyme reaction is dependent on the presence of Ca\(^+\) or Mg\(^+\) ions. Ca/Mg free DPBSG was used in wash to halt enzyme activity.

G. If cells were to be separated on percol gradient, then each test group of cells was resuspended in 1 ml Ca/Mg free DPBSG. The suspension was carefully layered on 8-10 ml of percol gradient solution. The gradient with the cover layer was centrifuged at 1800 g at 4\(^\circ\)C. The cover layer and cells resting on surface of percol were removed using transfer pipette. Cells were washed twice with Ca/Mg free DPBSG and resuspended in RPMI 1640 + 10% Fetal Calf Serum (FCS) for counting.

H. If cells were not to be separated by gradient, then pellet was resuspended in RPMI 1640 + 10% FCS for counting.

I. Cells were counted on hemocytometer, diluted to desired concentration, then samples were added to welled plates for adherence incubation.
Results

Specific guidelines of each experiment are listed with the results.

Experiment JPT 1

Mice:
2 groups of 2 unprotected Balb/c mice in week 4 of L. major infection were used.

Procedure:
The lymph nodes were ground without enzyme treatment or incubation, and placed on Percoll gradient. Cells were incubated, washed, fixed with 1% paraformaldehyde, and IF stained.

Results:
No macrophage cells were observed under microscopic examination.

Experiment JPT 2

Mice:
2 groups of 2 unprotected Balb/c mice in week 5 of L. major infection were used.

Procedure:
In this procedure Collagenase VII at 170 units/ml + DNase 1 20 units/ml in 8 ml DPBSG. Suspension was incubated for 10 minutes before Ca/Mg free wash. Otherwise identical to Ex. JPT 1.

Results:
A gelatinous mass developed after first Ca/Mg free DPBSG washing. No cells observed with microscopic examination.

Experiment JPT 3+4

Mice:
Each experiment used 4 groups of 2 mice in week 4-6 of L. major infection.

Procedures:
In these experiments, 170 units/ml Collagenase II substituted for Collagenase VII.
Results:

Gel formed as in JPT 2. The Ca/Mg free DPBSG solution erroneously was found to be stock 10^x solution. The solution had been labeled 1^x, and the hypertonic solution caused cell lysis and the formation of the gel.

Experiment JPT 5:

Mice:
- 2 groups of 2 unprotected Balb/c mice at week 5 of L. major infection.

Procedure:
In this experiment one group of lymph nodes was treated with Collagenase II 1/0 U/ml and the other treated with Collagenase IV (note: neither solution contained DNase I) at same dilution. Each test group was ground, treated with enzyme solution, and incubated for at 37°C for 10 minutes. Then groups were washed, cultured at 3.0x10^5 cells/ml RPMI + 10% FCS in 24 well cell well plate; then stained.

Results:
Collagenase II treatment produced an average of 3 adherent cells/field with 68.9% of cells being 1a+.
Collagenase IV treatment produced an average of 4.2 adherent cells/field with 65.3% of cells being 1a+.

Experiment JPT 6:

Mice:
- 2 groups of 2 unprotected Balb/c mice at week 6 of L. major infection were used.

Procedure:
In this experiment, lymph nodes were treated identical to those used in Ex. JPT 5; except, collagenase IV was used to treat both groups of mice. This was done to determine if the process could deliver consistent results.

Results:
When cells examined under fluorescent microscope, insufficient cells for accurate data collection were found. All slides contained less than 1 adherent cell/field on average.

Experiment JPT 7:

Mice:
- 2 groups of 2 unprotected Balb/c mice at week 8 of L. major infection.
Procedure:
In this experiment lymph nodes were removed, treated with Collagenase IV as in Ex. JPT 5, incubated for 10 minutes, ground again using clean frosted slides. The remainder of the procedure was identical to that of Ex. JPT 5.

Results:
Again insufficient numbers of cells could be observed for collection of La expression data.

Experiment JPT 8:

Mice:
2 unprotected Balb/c mice, 2 CsA protected Balb/c mice, and 2 C57BL/6 mice were used. All mice were in week 8 of L. major infection.

Procedure:
Lymph nodes were removed, and grouped by type:
Unprotected, CsA, or C57. In this experiment lymph nodes were perfused with 5 ml Collagenase II at 800 U/ml. Then ground suspensions were incubated for 10 minutes and then washed with Ca/Mg free DBPSG. Cells were layered on Percoll, and centrifuged. The concentration of cells in each RPMI solution was diluted to 1.8 x 10^6 cells/ml. 1 ml samples were transferred to prepared wells in 24 well plate. Plates were centrifuged at 750 gravities to sediment cells. Plates were incubated for 2 hours, fixed to slides using 1% paraformaldehyde solution, and then covered with PBS for later examination. The cells were not IF stained.

Results:
**Unprotected Balb/c** mice were counted at an average of 3.2 adherent cells/field, in 50 fields.
**CsA protected Balb/c** mice were counted at an average of 8.0 adherent cells/field in 50 fields.
**C57BL/6** mice were counted at average of 9.1 adherent cells/field in 50 fields.

Experiment JPT 9

Mice:
2 Groups of 2 CsA protected mice in week 6 of L. major infection were used.

Procedure:
Procedure identical to Ex. JPT 8 was used until dilution of cells. Suspensions of two groups were pooled and then diluted to 2.0 x 10^4 cells/ml RPMI solution. 6 slides were prepared.
Results:

Average cell count of 4.3 adherent cells/field, but 50 field counts of different slides averaged from .8 adherent cells/field to 5.3 adherent cells/field.

Experiment JPT 10

Mice:

2 groups of 2 unprotected Balb/c mice in week 9 of infection were and 1 group of CsA protected mice at week 7 of L. major infection were used.

Procedure:

Lymph nodes were removed, ground in 10 ml of DPBSG + Collagenase IV 800 U/ml. Suspensions were incubated for 10 minutes, reground and incubated for 10 more minutes. Cells were then washed with Ca/Mg free DPBSG. All suspensions were combined, and washed with Ca/Mg free DPBSG. Pellet was resuspended in 2 ml Ca/Mg free DPBSG. 1 ml of suspension was layered on percol and centrifuged. After washing cells twice, pellet was resuspended in 4 ml RPMI + 10% FCS. Suspension was diluted to 4x10^6 cells/ml. 1 ml samples were transferred to prepared cell well plate. The plate was centrifuged then incubated for 2 hours. Cells were fixed to slides by treatment with 1% paraformaldehyde, then stained using fluorescent antibody staining procedure.

Results:

Cells were observed at 11.4 - 12.9 adherent cells/field in 30 field inspections. Average cell number in 4 slides counted was 12.0 adherent cells/field.

Experiment JPT 11

Mice:

5 unprotected Balb/c mice in week 18 of L. major infection were used.

Procedure:

4 politieal lymph nodes and three lymph nodes from the lower backs of nine of the animals were removed from mice. Lymph nodes were all ground together in 10 ml DPBSG. Suspension was centrifuged to pellet. The pellet was resuspended in 1.5 ml DPBSG + 1870.5 units of collagenase I (This treatment was approximately 1000 U Collagenase/ml of pelleted cells). Cells were incubated for 30 minutes at 37°C. At 5 minute intervals, the cultures were removed from incubator, vortexed for 2 seconds and replaced in incubator. After incubation, 8 ml of chilled Ca/Mg free DPBSG was added to culture and washed. Cells were washed again with 10 ml Ca/Mg free DPBSG. Then cells were resuspended in 12 ml RPMI
+ 10% FCS. Suspension was transferred to previously prepared cell culture plate in 0.95 ml aliquots. Cells were centrifuged for 5 minutes to settle cells on glass, then incubated for 2 hours. After incubation cells were washed and fixed using 1% paraformaldehyde solution.

Results:
Cells in RPMI solution were counted at 2.0x10⁶ cells/ml. One cover slip was completely counted at 625 cells. The other slips were not completely counted, but appeared to have similar counts.

Experiment JPT 12:

Mice:
2 CsA protected and 2 unprotected Balb/c mice in week 8 of L. major infection were used.

Procedure:
4 popliteal lymph nodes were pooled, ground, centrifuged as in Ex. JPT 11. Cell suspension was treated with collagenase 1 solution at 1000 U/ml pellet volume. After 30 minute incubation with vortexing at 5 minute intervals, liquid was transferred to fresh centrifuge tube and washed 2 times with Ca/Mg free DPBSG. 2.5 ml of Ca/Mg free DPBSG was added to tissue mass remaining from enzyme treatment. The suspension was vortexed for 5 minutes, washed once and combined with other cells. Cells were resuspended in 2 ml Ca/Mg free DPBSG. Cell concentration was determined, and a 1 ml sample was layered on percol and separated. The other 1 ml sample was centrifuged to pellet and resuspended in 12 ml RPMI + 10% FCS. After percol treatment and washes, that sample was also resuspended in 12 ml RPMI + 10% FCS.

Adherence cultures were prepared: 12 ml of percol treated cells were divided, 6 wells at full 3.4x10⁶ cells/ml and 6 wells at 1.2x10⁶ cells/ml. 12 ml Sample of untreated cells were incubated, 6 wells at full 7.0x10⁶ cells/ml and 6 wells at 3.5 cells/ml.

Results:
Cover slips were counted and results listed below.

Percoll treated cells at 3.4x10⁶ concentration averaged 19 cells/field examined. Percoll treated cells at 1/2 concentration averaged 8 cells/field. 7.0x10⁶ unseparated cells averaged 18 cells/field. The untreated cells at 1/2 concentration were counted at 11 cells/window. Due to the similarity of results of cells separated on Percoll and those not treated, it was decided not to use Percoll separation in future experiments.
Experiment JPT 13

Mice:
3 groups of mice in week 4 of *L. major* infection were used. 2 C57BL/6, 2 CsA treated Balb/c, and 2 untreated Balb/c.

Procedure:
Lymph nodes were removed and pooled by type. Combined weights of lymph node groups were determined. Each test group of lymph nodes was ground in 10 ml DPBSG and centrifuged to pellet. Each pellet was resuspended in 1 ml of 1000 unit/ml Collagenase 1 for every mg of lymph node weight. Cells were cultured in enzyme solution for 30 minutes and vortexed at 5 minute intervals during the incubation. After incubation the cell suspensions were washed twice with Ca/Mg free DPBSG. Pellet was resuspended in 2 ml RPMI + 10% FCS and vortexed for 5 minutes. 2 ml more of RPMI + 10% FCS was added to each sample. Cells were counted and each sample was diluted to $2 \times 10^5$ cells/ml. 0.95 ml aliquots of samples were transferred to prepared wells in cell culture plate, centrifuged then incubated for 2 1/2 hours. Non-adherent cells were washed from slips. Remaining cells were fixed to slips using 1% paraformaldehyde then IF stained.

Results:
Counts of total cell in 2 lymph node samples
Unprotected = $9.6 \times 10^6$
CsA protected = $8.0 \times 10^6$
C57BL/6 = $3.8 \times 10^7$

<table>
<thead>
<tr>
<th>Group</th>
<th>number of wells</th>
<th>adherent cells in 25 fields</th>
<th>$\times$ Ia+ cells</th>
<th>cells/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>unprotected</td>
<td>4</td>
<td>5</td>
<td>20.0%</td>
<td>0.2</td>
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<tr>
<td>Balb/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CsA protected</td>
<td>4</td>
<td>17</td>
<td>35.2%</td>
<td>0.68</td>
</tr>
<tr>
<td>Balb/c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>4</td>
<td>63</td>
<td>78.0%</td>
<td>2.52</td>
</tr>
</tbody>
</table>

Due to an accident, only one slip/sample could be counted and data should not be considered extremely reliable.

Experiment JPT 14

Mice:
3 groups of mice in week 5 *L. major* of infection were used. 2 C57BL/6, 2 unprotected Balb/c, 2 CsA protected Balb/c.
Procedure:
Procedure identical to Ex. JPT 13 was used except, suspensions were all diluted to $2.5 \times 10^6$ cells/ml in the RPMI + 10% FCS instead of $2.0 \times 10^6$.

Results:
Counts of total cells in 2 lymph node sample:
Unprotected Balb/c = $5.0 \times 10^6$
CsA protected Balb/c = $2.7 \times 10^6$
C57BL/6 = $2.2 \times 10^6$

<table>
<thead>
<tr>
<th>Group</th>
<th>number of wells</th>
<th>adherent cells in 25 fields</th>
<th>% Ia+ cells/field</th>
</tr>
</thead>
<tbody>
<tr>
<td>infected</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Balb/c</td>
<td>1</td>
<td>27</td>
<td>66.7% 1.1</td>
</tr>
<tr>
<td>CsA treated</td>
<td>1</td>
<td>65</td>
<td>96.9% 2.6</td>
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<tr>
<td>Balb/c</td>
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<td></td>
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</tr>
<tr>
<td>C57BL/6</td>
<td>1</td>
<td>67</td>
<td>94.0% 2.68</td>
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</table>
Discussion

Problems in developing the protocol:

1. Miss labeled solutions.

2. Later studies suggest that Balb/c mice infected with L. major produce very few macrophages. These mice were used in control model of protocol. The low numbers of cells in experiments, might have been due to the low numbers of cells available.

3. Insufficient treatment of tissue with collagenase, due to low concentration of enzyme or insufficient time allowed for incubation, might have caused the inconsistent results.

4. Vortexing suspensions in later experiments, might have improved homogeneity of enzyme treatment. This could explain better results.

Data suggests that the protocol used in Experiments JPT 12-14, allows for the extraction and selective study of adherent macrophages of the murine popliteal lymph nodes. Note that the consistent release of relatively large numbers macrophages from the lymphoid tissue seems to be dependent on treatment with collagenase. Although only collagenase i was
used in the final developed protocol, other collagenase types might produce similar results if used in higher concentrations, and with longer treatment than they were allowed in earlier experiments.

Not enough data on Ia expression in lymph node macrophages was collected to draw any conclusions; however, the preliminary data suggests significant differences in levels of Ia expression and macrophage number between leishmaniasis resistant and susceptible mice. This protocol should allow for a more in depth study of the lymph node macrophages' role in leishmaniasis and other diseases. The macrophage releasing portion of the protocol could also be used in flow cytometry and in vitro studies of lymphoid tissue and cells.
Thanks

A special thank you to Dr. Nancy Behforouz. You made this project a success and helped me in so many other ways. You put heart in both your teaching and your science. Thanks again.

Jay