PROTEOMIC PROFILING OF MACROPHAGES INFECTED WITH *PENICILLIUM MARNEFFEI*

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Proteomic Profiling of Macrophages Infected With *Penicillium marneffei*

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Abstract

Penicillium marneffei is a pathogenic fungus with the unique characteristic of dimorphism. When grown at 25°C, it grows in the mold phase, while at 37°C it takes on characteristics of a yeast. Humans can be infected with this fungus if conidia (mold spores) are inhaled. Once inside the body, *P. marneffei* undergoes a phase change to form yeast-like cells. It is this phase transition that accounts for the pathogenic effect on the body.

Activated macrophages are capable of carrying out phagocytosis of foreign entities in the body and initiating an immune response to destroy them. *Penicillium marneffei* is one such pathogen that macrophages are known to attack. Conceivably, the proteins produced by macrophages may differ depending on whether or not they have phagocytized a foreign molecule. The purpose of my study was to compare the proteins produced by macrophages infected with the fungus *Penicillium marneffei* to those not infected with the fungus.

This objective was achieved by growing and collecting the conidia of *P. marneffei*, adding them to a solution containing live murine macrophages, and allowing for phagocytosis to occur. The proteins were then isolated from both infected and control macrophages and were visualized with the aid of 2-dimensional gel electrophoresis. This technique provided spot "maps" of the proteins present, which were then be compared and contrasted. Proteins of interest are those found in only one group of macrophages (infected or control). Further studies may include excision of these spots and sequencing in order to identify the proteins.

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Table of Contents

Chapters

List of Figures

List of Tables

Table Page

CHAPTER I

Introduction

Penicillium marneffei

Fungi belonging to the genus *Penicillium* are quite common in almost all environments. Most varieties are fairly harmless to humans (18) and actually play a beneficial role in environmental processes such as "biodegradation and modification of organic substances" (9). However, because of *Penicillium marneffei's* ability to disseminate throughout the body (11), it is one species that is pathogenic. This fungus has been proven to cause infections mainly in immunosuppressed individuals from endemic areas in Southeast Asia (9, 13).

The reservoir for *P. marneffei* has actually never been identified (9). The fungus was initially isolated from bamboo rats (*Rhizomys sinensis, Rhizomys pruinosus*), which are rodents indigenous to the Vietnam area (10, 13). More specifically, *P. marneffei* has been found in the organs of these rats much more frequently than in their feces or burrows (9). Even though bamboo rats seem to become infected by *P. marneffei* in the same way humans do (by inhalation of conidia), it is doubtful that this animal transmits the disease to humans. A study conducted by Deng and others provides evidence suggesting that "both humans and bamboo rats are probably infected from a common environmental source" (10).

Even though it is more common for immunocompromised individuals to contract infections due to *P. marneffei*, it is possible for those with normal immune systems to fall victim (2, 8, 9). As previously stated, inhalation of *P. marneffei* conidia is suspected to

be the way that humans naturally become infected with this fungus. This is especially seen in people living or visiting areas of Southeast Asia including Thailand, Vietnam, China, and Hong Kong (8, 9). Because *P. marneffei* infections were seen largely in HIV-positive patients in these regions (in fact, it has become the third most prevalent opportunistic infection found in these individuals (2, 9)), this fungus is now said to be an 'AIDS defining pathogen' (2). Conversely, the first human to contract an infection due to *P. marneffei* was a relatively healthy scientist studying the fungus. Infection occurred when he accidentally pricked his finger with a needle containing traces of *P. marneffei* (13).

The morphology of *P. marneffei* is quite unique. It is classified as a dimorphic fungus, which means it has the ability to grow as a mold at 25°C while taking on characteristics of a yeast (arthroconidia) at 37°C (9). The mold phase bears numerous spores called conidia, which allow for the growth of mold colonies (9, 10). When grown on Sabouraud glucose agar at 25°C, the colonies are a yellowish-white color and appear velvety in texture. After about a week, the mold colonies grow to a diameter of approximately 28 mm, while becoming more pinkish-brown in color. Soon the entire medium becomes a wine red color because of "the diffusion of the mold's pigments into the medium" (10) (Figure 1.1). The hyphae seen in this mycelial form are branched and septate. The smooth, oval-shaped conidia are arranged in individual, jumbled chains (9, 10, 13) (Figure 1.2). Conversely, when grown at 37°C on the same medium the colonies do not grow as quickly as the ones grown at 25°C. The colonies are about half the diameter and do not exhibit the same reddish coloring (10). The conidia swell and grow spherical, allowing a small number of undersized septate hyphae to form (8, 10, 13).

Unlike other species of *Penicillium* that cause rare infections, when the conidia of *P. marneffei* are inhaled, instead of growing in the usual filamentous pattern, they go through a morphological change after being phagocytized (9). It is this phase transition that allows for the parasitic yeast form to arise and cause destruction (9). In summary, the dimorphic nature of *P. marneffei* is a unique characteristic among the Penicillia.

Macrophage

A macrophage is a phagocytic cell capable of recognizing foreign entities in the body and initiating an immune response to destroy them (19). Normally, macrophages remain in a resting state. However, "host- and pathogen-derived signals" can act to trigger the macrophages into an active state of antimicrobial activity (20) (Figure 1.3). It is in this state that macrophages work more efficiently to phagocytize and kill pathogens. One of the main stimuli of macrophages is Interferon-γ (20, 23). It acts primarily by interacting with other components such as IFN-α, IFN-β, TNF, and CD40 ligand to activate macrophage cells (20).

Activated macrophage cells are not only more competent in phagocytosis, but also produce antimicrobial molecules such as oxygen radicals and nitric oxide (NO) (23). These responses to infection are important factors in gaining host immunity as they act to hinder the growth of many microbes, including fungi, that enter the body (6). A well-understood relationship exists between the cytotoxicity of macrophages and their production of nitrogen and oxygen intermediates in the destruction of parasites (16, 17). In order for activated macrophages to produce NO, cytokines or lipopolysaccharides such

as IFN-γ and TNFα are needed. This respiratory burst of NO is significant because NO acts as "an effector molecule for the cytotoxicity of macrophages" (14).

Activating macrophages is a necessary step in the process of phagocytosis, but this process does not occur without consequences. One result of their killing activity is a minor degree of damage to healthy tissue. Because of this, the extent of activation must be closely monitored. Regulatory proteins called cytokines are the chief regulators of this response (23). Interleukin-10 is the main cytokine to play a role in this down-regulation. It works by down-regulating the production of NO and the expression of Major Histocompatibility Complex (MHC) II while superseding the effects of IFN-γ (23).

Macrophage response to Penicillium marneffei

Studies have shown that *P. marneffei* conidia can be phagocytized by macrophages including mouse J774 and human THP1 cell lines. In fact, macrophages are thought to be the initial method of defense against *P. marneffei* (22). Once phagocytosis has taken place and the conidia are actually inside the macrophage, they undergo a phase transition. The conidia convert to the yeast form – the phase that actually flourishes within the macrophage and allows for propagation throughout the body (7).

As previously stated, NO released by macrophages plays a vital role in the destruction of host invaders. It has been seen, however, that this NO is "significantly less effective in killing conidia than arthroconidia" (7). Another study provided evidence indicating "that the yeast form of *P. marneffei* is more sensitive to the fungicidal activity of IFN-γ-stimulated macrophages than conidia" (16). This may be due to the different

mechanisms in which the two phases of *P. marneffei* trigger the production of NO by the macrophages (16). Both phases of the fungus do cause macrophages to manufacture NO, but after 48 hours of incubation it was observed that the amount of NO generated was higher when the yeast phase was used to infect the macrophages than when the conidia were tested (16). These findings indicate that macrophages respond in distinctive ways to the two different phases of *P. marneffei* (7).

When first phagocytized, *P. marneffei* conidia can be seen using light microscopy in the phagocytic vacuoles of the macrophages. "The membrane of the phagocytic vacuoles was closely attached to the fungal cell wall" (6). This finding was observed after an incubation time of two hours. However, after being allowed to incubate for 24 hours, the same phagocytic vacuoles had grown larger in size and exhibited such adjustments in structure as swelling of the mitochondria and membrane disturbances. Moreover, the *P. marneffei* cells appeared to be in the yeast form, as they were larger and more irregular in shape and had a more slender cell wall than the conidia. "Yeast-like cells dividing by fission" were even seen within the phagocytic vacuoles of the macrophage (6).

Proteomics

The term "proteomics" was coined in 1995 to describe "the study of protein properties (expression level, post-translational modification, interactions etc.) on a large scale to obtain a global, integrated view of disease processes, cellular processes, and networks at the protein level" (3, 15). Uncovering the protein profiles of a cell or organism is a valuable step in the development of new therapies for diseases. It is

important to note, however, that when a proteome is revealed, it only displays the protein expression profile at that time, under those specific conditions (15). Also, because of current limitations, duplicating results can be a challenging process. Steps are being taken to improve methods used in the study of proteins so that these limitations may be overcome.

Because it is somewhat easier to study DNA than proteins, the field of genomics has grown rapidly over the past years. However, as more knowledge pertaining to proteomics is gained, it is becoming obvious that proteomics and genomics are complementary (15). For example, the study of genomics revolves largely on the ability to sequence DNA, which can lead to the construction of chromosome maps (23). This mirrors proteomics in the sense that once a map of an organism's proteome is created, the proteins can then be visualized and identified. Being able to link a distinct protein with its corresponding gene sequence is an advantage of studying proteomics (5). When analyzing proteomes, the generated data shows "where and in which ratio and under what conditions proteins are expressed" (15). This supplements previously attained data on gene sequences of the organism.

Two fundamental processes that have allowed for such progress in proteomics are isoelectric focusing (IEF) and two-dimensional polyacrylamide gel electrophoresis (2DE). The first dimension employs isoelectric focusing, which works by separating proteins based on their isoelectric point (Figure 1.4). More specifically, a protein sample is added to an IPG strip, which is a plastic-based strip that is layered with a gel containing an immobilized pH gradient on one side. When subjected to IEF, the proteins travel across the strip and stop at the pH at which their net charge is zero. The use of IPG strips

is crucial because they allow for the technique to be reproduced easily and accurately (15).

The second dimension, 2DE, separates the molecules based on their molecular weight (Figure 1.5). Smaller proteins travel further vertically through the matrix of the gel, resulting in larger proteins near the top and smaller proteins toward the bottom. 2DE allows us to concurrently see hundreds of proteins "without the need of prior knowledge of their identities or functions" (5). This step, while beneficial in allowing many proteins to be separated at one time, is not as easily reproduced as the first dimension (15). Once the gel is run, spots are observed by staining either with Coomassie brilliant blue, silver, or a fluorescent dye. Imaging software such as PDQuest (Bio-Rad, Inc., La Jolla, CA) can then be used for automated spot detection to create a protein map and allow for comparisons between gels.

When a protein of interest is found, it can be excised from the gel and sequenced. Finely tuned mass-spectrometric techniques are the best for identifying proteins and finding post-translational modifications (3). Matrix-assisted laser-desorption-ionization-time-of-flight mass spectrometry, or MALDI-TOF MS, is one of the more common types of mass spectrometry used to identify proteins. This form of MS is generally used for peptide mass fingerprinting (3). Once the sequence is determined, databases can be searched to find exactly what the protein is. These databases are composed of annotated data acquired from 2-dimensional gels run under various conditions (1).

The latest techniques allow scientists to separate thousands of proteins in just one trial. Moreover, only tiny amounts of sample protein are needed for small gels, making it possible to visualize the proteome of organisms from which a great deal of protein would

be difficult to obtain (15). Developments in proteomics make it possible to test the hypothesis: *macrophages have developed a specific protein response when acting to phagocytize P. marneffei.* Proteomics profiles obtained from collected samples will be used to test this hypothesis by providing a spot map of the existing proteins. The protein(s) of interest can then be further studied by physically excising them and having them sequenced in order to determine the identity.

Goals of This Study

The overall aim of this study was to examine the interaction and results that occur when murine macrophages are infected with *Penicillium marneffei.* This was done by visualizing and identifying post-infection proteins (or lack thereof) specific to infected macrophages.

In order to achieve results, various smaller goals had to be reached along the way. For instance, *P. marneffei* needed to be plated, grown, and collected in sterile conditions in order to avoid contamination. The same was true for maintaining the macrophage cell line – sterile technique and conditions were a must so that contamination did not occur. If either of these cells became tainted, the experiment could not continue and had to be restarted from the beginning. Another sub-goal was running and dealing with the finished gels in such a way that they work. Working on the proteomics portion of the experiment demanded extremely careful technique and thorough knowledge of the subject so that results could actually be obtained and analyzed.

It is thought that macrophages have developed a specific response to phagocytize *P. marneffei*. The expected results of my experiments would show whether or not this is true. It is also possible that macrophages simply have a general way of carrying out phagocytosis and apply it to all pathogens that enter the body, including *P. marneffei.* However, once inside the macrophage, studies have shown that *P. marneffei* has developed means of modifying the responses of the macrophage that would allow for its elimination (7). Once protein profiles are obtained from my experiments, we will be one step closer in determining the different proteins expressed when macrophages are infected with *P. marneffei* than when they are not infected at all. Further studies using other pathogenic organisms can then be performed in order to determine whether or not the expressed macrophage post-infection proteins are specific to *P. marneffei* or if they are general proteins expressed whenever the macrophage responds to infection.

Figure 1.1: Fungal culture of *P. marneffei* displaying characteristic diffusion of red pigment into medium.

Figure 1.2: Conidia chains of *P. marneffei.*

Figure 1.1

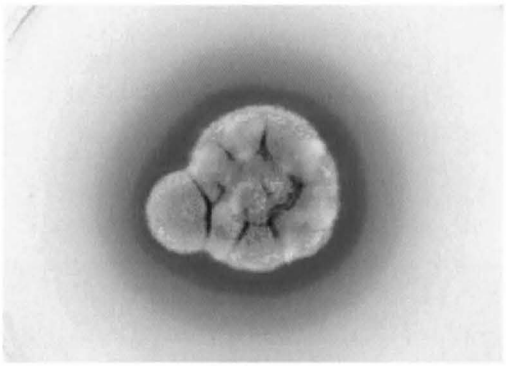
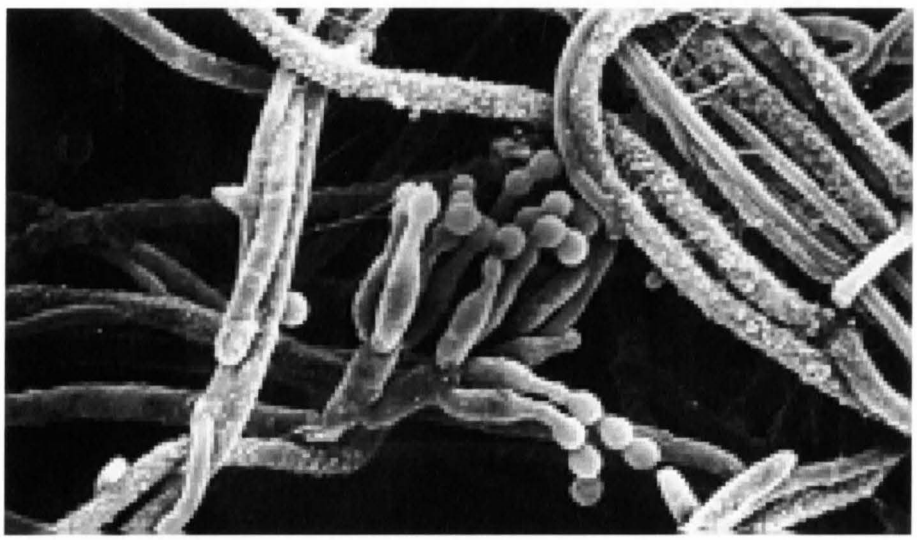


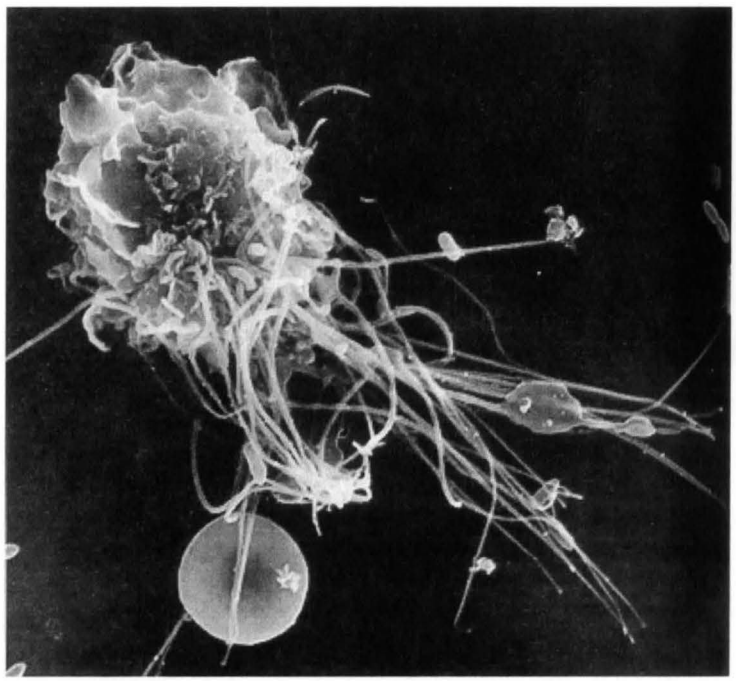
Figure 1.2



(Pictures courtesy of
http://www.mycology.adelaide.edu.au/Fungal_Descriptions/Hyphomycetes_(hyaline)/Penicillium/marneffei.html)

Figure 1.3: Activated macrophage attacking foreign cells that have entered the body.

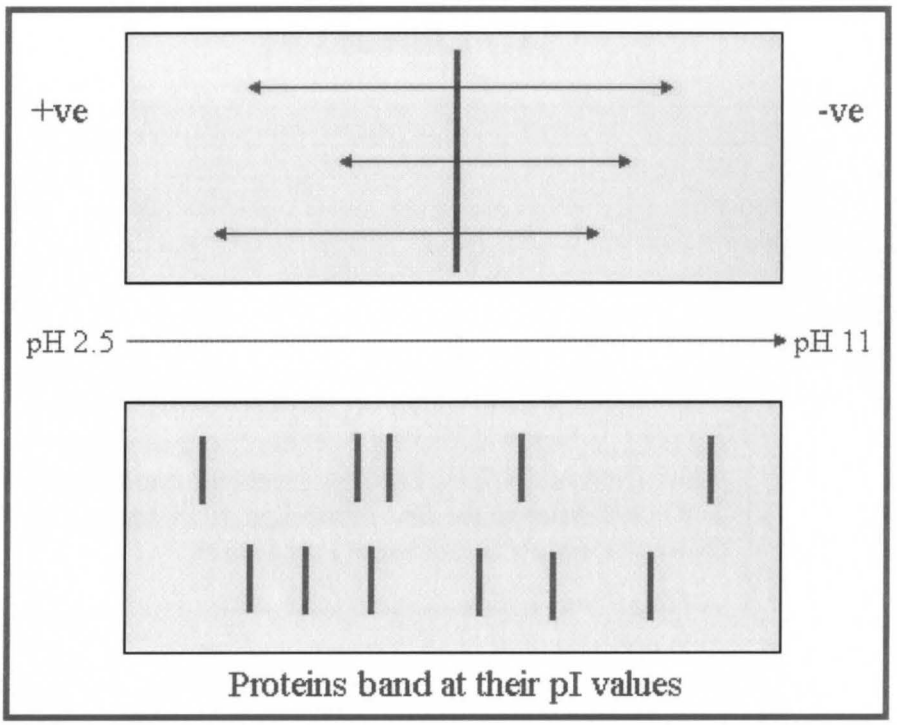
Figure 1.3



(Picture courtesy of
http://s99.middlebury.edu/BI330A/projects/Howard/Mpneumoniae.html)

Figure 1.4: Figure demonstrating the (+)/(-) orientation of immobilized pH gradient (IPG) strips, an example of a strip's pH range (2.5 – 11) and the way proteins travel across the gradient and band at their pI values.

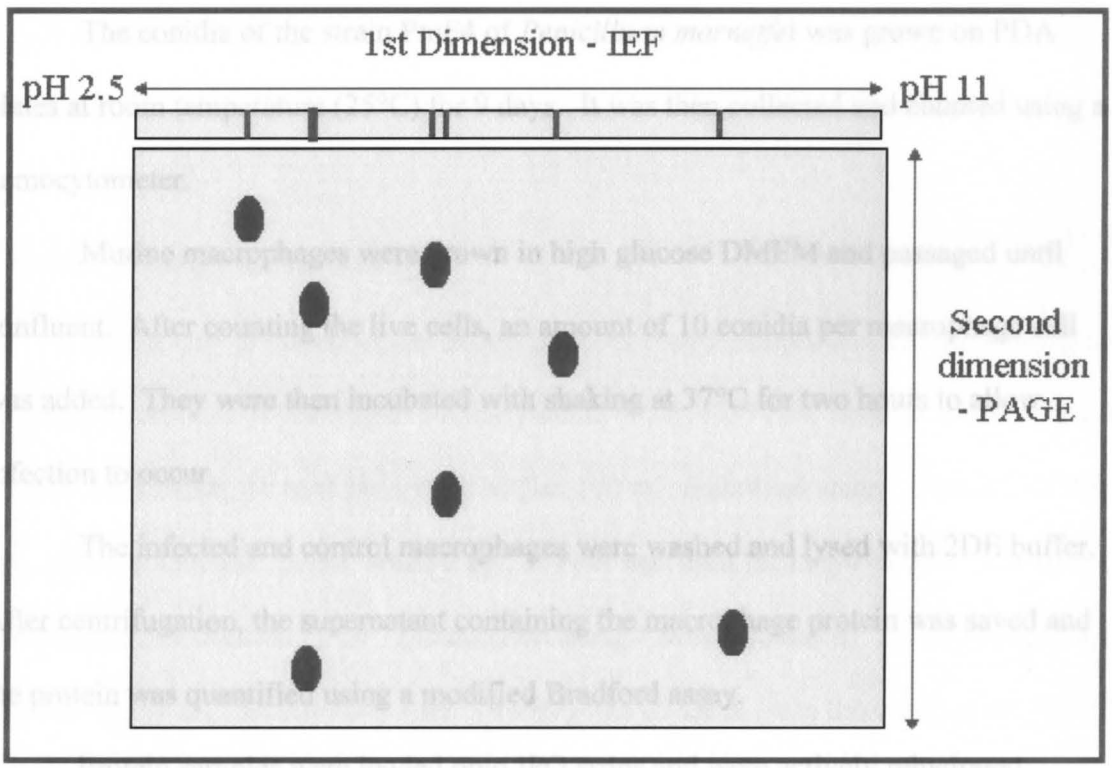
Figure 1.4



(Figure courtesy of http://www.food.rdg.ac.uk/online/fs460/lecture4/lecture4.htm)

Figure 1.5: Figure depicting both the first and second dimensions of 2-DGE. Proteins separated horizontally by isoelectric point in the first dimension and vertically by molecular weight in the second dimension.

Figure 1.5



(Figure courtesy of http://www.food.rdg.ac.uk/online/fs460/lecture4/lecture4.htm)

CHAPTER II

Materials and Methods

Overview of Methods

The conidia of the strain PmF4 of *Penicillium marneffei* was grown on PDA plates at room temperature (25°C) for 9 days. It was then collected and counted using a hemocytometer.

Murine macrophages were grown in high glucose DMEM and passaged until confluent. After counting the live cells, an amount of 10 conidia per macrophage cell was added. They were then incubated with shaking at 37°C for two hours to allow infection to occur.

The infected and control macrophages were washed and lysed with 2DE buffer. After centrifugation, the supernatant containing the macrophage protein was saved and the protein was quantified using a modified Bradford assay.

Protein samples were loaded onto IPG strips and were actively rehydrated. Isoelectric focusing allowed for proteins to be separated in the first dimension by isoelectric point. Separation in the second dimension was carried out on 17cm 10% polyacrylamide gels through electrophoresis. Gels were stained with Sypro Ruby Red fluorescent stain and analyzed using the Bio-Rad PDQuest 2-D Image Analysis Software.

Growing and collecting fungal strain

The strain PmF4 of *Penicillium marneffei* was grown on Potato Dextrose Agar (PDA; Difco, Detroit, MI) at room temperature (approximately 25°C) for 9 days. The plates were scraped with the aid of sterile water, and the liquid containing the conidia was filtered by brief centrifugation (1000 rpm) through sterile glass wool. The remaining liquid was then centrifuged at 10,000 rpm for 10 minutes so that a solid pellet of conidia was formed. This pellet was frozen at -80°C until it was used for infection.

Conidia resuspension and dye

On the day of infection, the frozen pellet of PmF4 conidia was resuspended in an appropriate amount of phosphate-buffered saline (PBS) (137 mM sodium chloride, 2 mM potassium chloride, 10 mM phosphate buffer, 100 mL deionized water). One mL of PBS was used for a smaller pellet, while 2 mL of PBS was used for larger pellets. The conidia was diluted to a dilution of 10^{-2} and counted using a hemocytometer. A 20μM amount of calcein-AM (1 mg calcein-AM, 1 mL DMSO) was added to stain the conidia and allowed to sit for 30 minutes. After staining, the conidia were washed three times using a microcentrifuge (3500 rpm for 15 minutes each at 4°C) with 1 mL PBS. After the last wash, the pellet was resuspended in 1 mL of PBS and kept on ice.

Hemocytometer Count

PmF4 conidia used for infection first had to be counted so that the appropriate amount would be added to infect the macrophage cells. The cells were diluted 1:100; 15 μL were placed under the cover slip on both the top and bottom grid sections of the

hemocytometer slide. The slide was placed under the microscope, which was set at 400X magnification and focused. The slide containing the conidia was then allowed to settle for 10 minutes before counting began.

The hemocytometer slide consisted of two separate sections of grids. Each section contained four individual grids consisting of 16 squares each. To count the conidia, the slide was focused so the top section of grids was clear. Cells in the four outer corners of each of the four grids were counted, totaling 16 squares counted altogether. This process was repeated for the bottom grid section. Once the top and bottom sections were both counted, the numbers were added then divided by two to find the average. This number was then multiplied by the dilution factor for the hemocytometer slide (10^4) and by the dilution factor for the conidia resuspension (10^2). The product provides the number of conidia cells per mL to be used for infection.

Macrophage cells were also counted using a hemocytometer. After the cells were scraped and stained, 15 µL were placed beneath the cover slip on both the top and bottom sections of grids. The slide was then placed under an inverted microscope and focused at 100X magnification.

This slide is similar to the slide used for counting conidia in the sense that it too has two sections of grids (top and bottom), both consisting of four individual grids of 16 squares. When counting macrophages, all 16 squares of two of the grids are counted; for this experiment, the top left and bottom right grids were used. The totals from the top and bottom sections are added together then divided by 200. This number is multiplied by the number of mL in which the macrophage cells are suspended (in this case, it was 3 mL). The resulting number multiplied by 10^6 provides the amount of macrophage cells

present. It is important to note that although live and dead macrophages were both counted individually, only the numbers of live macrophages were used in figuring out the total number of cells.

Macrophage infection

Murine macrophages (J774.1) were grown in high glucose Dulbecco's Modified Eagle's medium (DMEM) (24) containing 10% Fetal Bovine Serum (FBS) (12) and 1% Penicillin Streptomycin (10,000 1.U/ml and 10,000 μg/ml). They were passaged at 1/6 until confluent. Cell passaging was done by removing the old media, adding 6 mL of fresh medium, scraping the cells from the tissue culture flask using a sterile scraper, and transferring 1 mL of that solution to a clean flask. Ten mL of additional medium were then added, and the cells were incubated at 37°C with 5% CO_2 until the next transfer. Once ready for infection, the old medium of the macrophage sample was poured off and 3 mL of fresh complete medium was warmed to 37°C and added. The macrophages were then aseptically scraped using a cell scraper and transferred to 15 mL conical tubes. 100 μL of the macrophage solution from each of the samples were transferred to a microcentrifuge tube and stained with 100 μL of trypan blue (0.4% solution in 0.85% saline). This stain was used as an aid in the counting of live vs. dead macrophages using a hemocytometer, as previously described. This allowed for the number of macrophages in the 3 mL of media to be determined. An amount of 10 conidia per macrophage cell was then added into the remaining cells. It was incubated with shaking at 37°C for two hours.

Protein isolation

Post-infection, two 100 µL samples of each macrophage solution were removed and put into separate microcentrifuge tubes. One of the 100 µL samples was used for plating, and the other for counting, both of which will be later discussed in further detail. The remaining solution of infected cells was then centrifuged at 1000 rpm for 10 minutes in order to form a pellet. It was washed three times with 4 mL PBS and lysed in 1 mL 2-dimensional electrophoresis (2DE) buffer (8.4 M urea, 2.4 M thiourea, 5% CHAPS, 25 mM spermine base, 50 mM dithiothreitol). The solution was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was saved, as it contained the macrophage proteins. A modified Bradford Assay was performed protein (see method below), then isoelectric focusing and 2-dimensional gel electrophoresis were subsequently performed. As needed, the protein samples were frozen at -80°C.

Plating

Once the infection had taken place, 100 µL of the infected and non-infected (control) macrophages were removed from each sample. This was added to 16 µL of 2% para-formaldehyde ($HO(CH_2O)_nH$ (n = 6 - 100)) and was allowed to sit for 3 minutes. 50 µL of that solution from each sample was added to 50 µL DOC solution and was also allowed to sit for 3 minutes. The samples were then diluted by adding 10 µL of the macrophage solution to 2 mL PBS. 20 µL of the dilutions were placed on PDA plates, streaked, and incubated at 25°C. Conidia alone were also plated following the same technique. All of the previous procedures were completed in the biological safety hood and kept on ice until transferred to the plate.

Post-infection cell counting

The other 100 μL of the infected and control macrophages that were removed from each sample were used for counting on a hemocytometer slide under a fluorescent microscope. The same hemocytometer slide used to count the conidia was used in this instance. 15 μL of the macrophage cell solution were added under the cover slip of the hemocytometer slide, which was then placed under the microscope and allowed to sit for 10 minutes so the cells could settle. The fluorescent microscope, which had been previously turned on and allowed to warm up for 20 minutes, was focused on the slide at 400X power. The numbers of macrophage cells, conidia phagocytized by macrophages, free conidia, and total number of conidia were recorded. Cells in each of the four corners of each of the four grids (totaling 16) in the top grid section were counted, as were those on the bottom. These two numbers were averaged, multiplied by 10^4, and recorded in Table 3.2.

Modified Bradford Assay

In order to determine the quantity of protein in the protein sample collected, a modified Bradford Assay was performed (4). Before beginning, the red light of the spectrophotometer (Hewlett-Packard 8453 UV-Visible System) was turned on so that it could warm up for at least 15 minutes prior to use. Using the computer software (UV-Visible Chemstation Software Rev. A.06.04 [48]), the wavelength was set to 595 nanometers.

Standards and a blank were first prepared. The seven standard tubes contained bovine serum albumin (BSA) in various microgram amounts – 10, 15, 20, 25, 30, 35, and

40. They were prepared by adding 10 µL of 2DE buffer, 10 µL of 0.1 M hydrochloric acid, 80 µL of distilled/deionized water and the appropriate amount of BSA to different test tubes (4). The blank was prepared the same way, only without BSA. The mixture was then swirled to better mix all of the components.

The unknown protein sample tubes were prepared in a similar manner. They also were comprised of 10 µL of 2DE buffer, 10 µL of 0.1 M hydrochloric acid, and 80 µL of distilled/deionized water, but instead of BSA, 10 µL of the macrophage protein sample previously collected was added. This mixture was also then swirled to mix.

Once mixed, 4 mL of Bradford Dye (50 mL 95% ethanol, 100 mL 85% (w/v) phosphoric acid, 100 mg Coomassie Brilliant Blue G-250, dilute to 1 L) were added to each tube and inverted to thoroughly mix.

When the blank, standard, and unknown test tubes were complete and mixed, their absorbances were ready to be recorded. The spectrophotometer was first blanked, then the absorbances of the seven standards were recorded followed by the unknown samples using the above mentioned software (Tables 2.1 and 2.2).

Once the absorbances were recorded, the values of the standards were entered into a spreadsheet and plotted on a linear graph (Figure 2.1). This provided an equation which allowed for the protein concentrations of the unknowns to be determined. This was done by dividing the provided concentration by 10, as 10 microliters of the sample was the amount added to the test tube.

Isoelectric Focusing

The results from the Bradford Assay allowed for the proper amounts of protein sample to be determined for isoelectric focusing. The microliter amounts of samples needed were calculated from the concentrations for a protein load of 100 μg for 7 cm immobilized pH gradient (IPG) strips or 255 μg for 17 cm IPG strips. The protein sample and Rehydration buffer (8 M urea, 1% CHAPS, 15 mM dithiothreitol, 0.2% Biolytes (BioRad), 0.001% bromophenol blue) were combined in a microcentrifuge tube so that the total volume was 125 μL for 7 cm IPG strips and 300 μL for 17 cm IPG strips, and were swirled to mix. This sample solution was then pipetted into one row of the IEF tray, taking care to avoid creating bubbles in the sample. Using forceps, the plastic backing of an IPG strip was removed, and the strip was placed on the sample in the tray, gel side facing down. The strip was oriented so that the positive (acidic) end of the strip would line up with the red positive (anode) side of the IEF cell (Bio-Rad PROTEAN IEF system). Again, it was imperative to remove all bubbles. Once all of the samples were placed in the tray and the strips were positioned over them, care was taken to ensure the gel of the strip was in contact with the electrodes. The strips were overlaid with 2-3 mL of mineral oil in order to prevent evaporation. The tray was covered with the plastic lid and placed in the IEF machine.

The strips were then actively rehydrated. The PROTEAN IEF cell was programmed using the conditions 50 V, 20°C, 12-16 hours. Once rehydrated, isoelectric focusing automatically began, as the machine was programmed not to pause in between the two processes. For 7 cm strips, the volt hours were set to 40,000; for 17 cm strips, 60,000 volt hours. A 500V hold was programmed so that the IPG strips would be kept at

that holding voltage when they were finished until the tray was removed from the machine.

2-Dimensional Gel Electrophoresis

Once isoelectric focusing is complete, the IPG strips were removed from the tray. The mineral oil was blotted using filter paper, and each strip was placed in a separate row of an equilibration tray, gel side up. The 7 cm strips were then covered with 2.5 mL of Equilibration buffer I (6 M urea, 0.375 M Tris, pH 8.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 2% (w/v) dithiothreitol) while 17 cm strips were covered with 6 mL, and left to equilibrate for 10 minutes, gently shaking on an orbital shaker. After 10 minutes, the strips were removed from Equilibration buffer I and placed in an empty row of the equilibration tray, gel side up. The same amount of Equilibration buffer II (6 M urea, 0.375 M Tris, pH 8.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 2.5% (w/v) iodoacetamide) was then pipetted on top of the strips, which were again allowed to shake gently for 10 minutes on an orbital shaker.

Once equilibrated, the strips were rinsed with 1X Tris-Glycine-SDS (TGS) buffer (25 mM tris base, 250 mM glycine, 0.1% SDS) by dipping them into a conical tube containing the buffer then blotting the excess onto filter paper. The strip was then placed gel side out against the back (tall) plate of the previously cast gel (Table 2.3), leaving space between the strip and gel. Warmed overlay agarose (0.5% (w/v) agarose, 1X TGS buffer, touch of bromophenol blue) was then carefully pipetted into the IPG well of the gel. The strip was then quickly pushed down into the agarose-filled well so that it was in

contact with the gel itself. Any bubbles that may have formed were removed before the agarose set.

Once the overlay agarose solidified, the gel plates were arranged in cases and put into the electrophoresis cell (BioRad Mini-PROTEAN for 7 cm gels, BioRad PROTEAN II XL for 17 cm gels). Enough 1X TGS buffer was added to the inner and outer reservoirs of the cells so that short plate in the middle was completely covered with buffer. Lids were then placed on the cells, being sure to line up (+) to (+) and (–) to (–), and all electrodes were plugged into the power supply. Gels were then run at a constant current – 16 mA per gel for 7 cm gels and 10 mA per gel for 17 cm gels – in refrigerated conditions. They were closely monitored by noting the position of the blue dyefront (formed by the agarose); once the line had nearly reached the bottom of the gel, the current was shut off and the gel was removed.

Coomassie staining

Once finished, the gels were removed from the electrophoresis cells. The glass plates were carefully separated using a plastic wedge, leaving the gel resting on the back plate. The plastic IPG strip was removed and discarded. The positive side of the gel was marked by cutting off the top corner of that side. The gel was then loosened from the plate using the wedge, and placed in containers containing enough Coomassie brilliant blue to totally cover the gel. They were left to stain, each in a separate container, for approximately 24 hours. After that time, the Coomassie was poured off and high de-stain (40% methanol, 10% acetic acid) was added to the containers. The gels were left to soak in this stain-removing solution for approximately one hour. The gels were then switched

to low de-stain (10% methanol, 6% acetic acid) for another 2-3 hours. The de-stain worked to remove any background stain that would interfere with spot detection. After de-staining, the gels were stored in distilled water. Coomassie was the stain of choice for the first few trials of infections, as the goal was mainly to see if the infection protocol succeeded in providing proteins for analysis.

Sypro Ruby Red staining

Once it was clear that proteins were being obtained using the described protocol, subsequent gels were stained with the more sensitive (and costly) Sypro Ruby Red dye (21). After being run, gels were removed from the glass plates as described above. The IPG strip was removed, and corner on the positive side of the gel was sliced off. The gels were placed into containers containing enough fixing solution (40% methanol, 10% acetic acid) to cover the gel. After one hour, the fixing solution was removed and 1X Sypro Ruby Red dye was added to the containers. The gels were allowed to stain overnight, gently shaking on an orbital shaker. Once stained, the Sypro Ruby Red was poured off and de-stain (10% methanol, 6% acetic acid) was added to each container. The gels remained in this solution for two hours. After the gels were de-stained, the de-stain was discarded and the gels were stored in distilled water.

Spot detection and analysis

Visualization of spots was carried out using the BioRad ChemiDoc XRS Gel Documentation system. Early trials stained with Coomassie were photographed using a UV to white light converter plate. The plate was cleaned and moistened with distilled

water. The gel was then laid on the plate, and the image was focused using the software. Digital pictures were taken and saved on the computer. Gels stained with Sypro were imaged in a similar manner; the difference being the UV to white light converter plate is not needed. The gels were laid directly on the moistened tray of the imager, focused, and photographed. These images were also saved to the computer.

After the gels from all of the trials of infected and control macrophages had been imaged, they were ready for analysis using the BioRad PDQuest 2-D Imaging Analysis Software. Gel images were first cropped so that any outer areas not containing spots were removed. This was done by cropping one of the trials of either infected or control macrophages, saving the crop settings, and loading it onto the other trials so that they were all uniform.

Once all infected macrophage trial gels were cropped, automated spot detection and matching was used. Streaks were filtered out, and a master gel was chosen (usually the "best" gel of all the trials). The gels were then processed to create a primary matchset. Because all of the trials of infected macrophages should have ideally produced the same proteins, their spot maps should be identical. However, due to variances in conditions and human error, this was not always the case. The software automatically matched the spots that did match up among the different trials of infected cells, but some spots were still left unmatched. Edit Spot Tools and Match Tools were then used to manually match most of the remaining spots. When the matching was complete, a composite was formed of the gels of all of the trials of infected macrophage proteins. The same processes were carried out for all trials of control macrophages to create a composite of these gel images as well.

From these composites, a higher level matchset was able to be created. This matchset compared the proteins found in the infected macrophage trials with those in the control. Proteins of interest are those that are present in one condition but not the other or those that are expressed at different magnitudes between the two conditions.

Table 2.1: Example of absorbance readings of standards provided by a Bradford assay.

Table 2.1

Standard Concentration (μg)	Absorbance <595 nm>
10	0.088821
15	0.099861
20	0.13612
25	0.17914
30	0.21139
35	0.21922
40	0.24352

Table 2.2: Example of absorbance readings of 6 macrophage protein samples (3 infected trials, 3 control trials) as provided by a Bradford assay, along with protein concentration values of samples.

Table 2.2

Macrophage Sample	Absorbance <595 nm>	Concentration (μg/μl)
Infected Trial 1	0.19283	1.45525
Infected Trial 2	0.19555	1.477916667
Infected Trial 3	0.14156	1.028
Control Trial 1	0.42931	3.425916667
Control Trial 2	0.31873	2.504416667
Control Trial 3	0.44187	3.530583333

Figure 2.1: Example of a standard curve of protein concentrations provided by results of a Bradford Assay. An acceptable R^2 value is 0.97 or above.

Figure 2.1

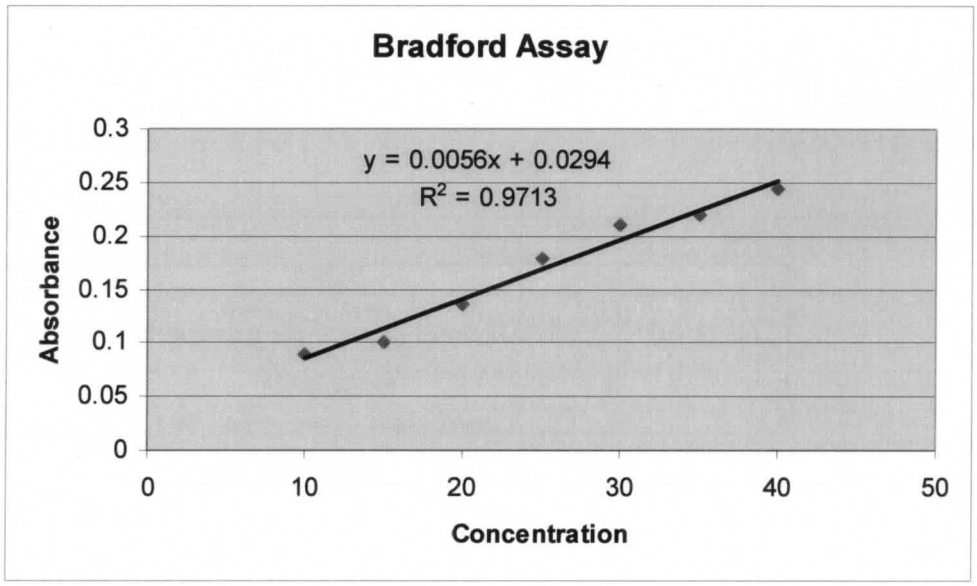

Bradford Assay
y = 0.0056x + 0.0294
R2 = 0.9713
Absorbance
0.3
0.25
0.2
0.15
0.1
0.05
0
0
10
20
30
40
50
Concentration

Table 2.3: SDS-PAGE recipes used for making 100 mL or 500 mL of solution for casting 10% gels.

Table 2.3

	100 mL	500 mL
Water	48.0	240.0
40% acrylamide	25.0	125.0
1.5 M Tris (pH 8.8)	25.0	125.0
10% SDS	1.0	5.0
10% ammonium persulfate	1.0	5.0
TEMED	0.04	0.2

CHAPTER III

Results

Once the proteins were collected from both infected and non-infected macrophage cells, they were separated based on their isoelectric point in the first dimension of a 2-D gel, then again based on molecular weight in the second dimension. Proteins appear as spots of varying size and intensity on these gels. Data has indicated that different protein spots do exist between macrophages infected with *P. marneffei* and those that are not.

Figures 3.1 and 3.2 are raw images of early gels run and stained with Coomassie. Figure 3.1 shows proteins expressed by infected macrophage cells while Figure 3.2 shows proteins from control cells. These preliminary 7cm gels utilized a pH gradient of 3-10. Visible proteins can be seen as blue spots. Not many proteins are visible in these gels due to the fact that Coomassie is not as sensitive of a stain as Sypro, which was used in later experiments.

Figures 3.3 and 3.4 are also raw images of 7cm gels (pH range 3-10), however they were stained using the fluorescent stain Sypro. Figure 3.3 reveals proteins expressed by infected macrophages; figure 3.4 shows those of control macrophage cells. Each protein can be seen as a white spot.

Figure 3.5 is the first of the images of 17 cm gels. This raw image depicts proteins expressed by macrophages infected with *P. marneffei*. The proteins were separated on a 3-10 pH range before the gel was run. When finished, the gel was stained with Sypro and photographed. Some vertical streaking is apparent, possibly because of sample overload.

Figure 3.1: Raw 2-dimensional gel electrophoresis image of **infected** macrophage proteins. The protein sample (100 μg) was actively rehydrated at 40,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 7cm 10% polyacrylamide gel in 1% TGS buffer at 16 mA. Gel was stained with Coomassie brilliant blue and photographed. Protein spots are seen in black.

Figure 3.2: Raw 2-dimensional gel electrophoresis image of **control** macrophage proteins. The protein sample (100 μg) was actively rehydrated at 40,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 7cm 10% polyacrylamide gel in 1% TGS buffer at 16 mA. Gel was stained with Coomassie brilliant blue and photographed. Protein spots are seen in black.

Figure 3.1

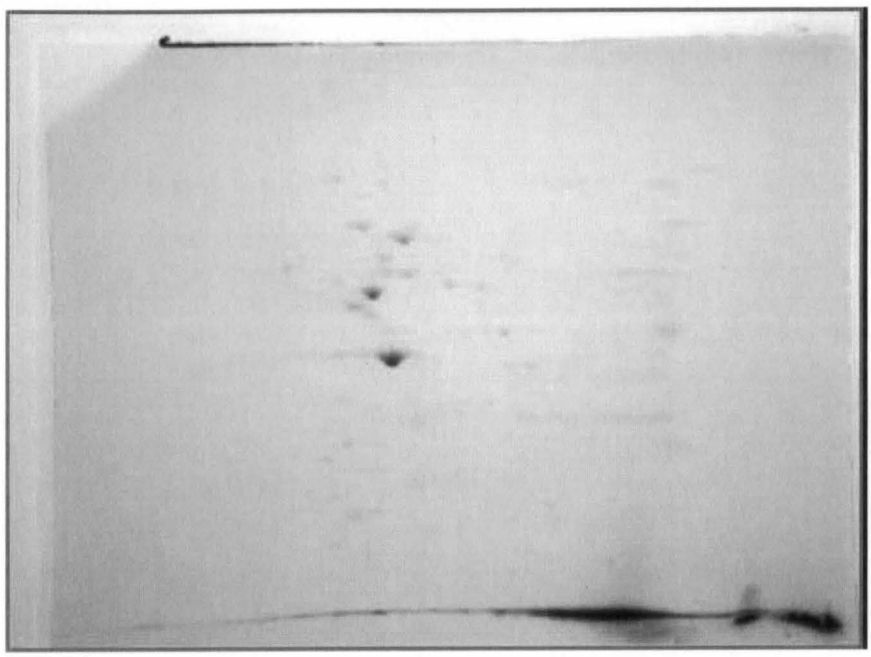


Figure 3.2

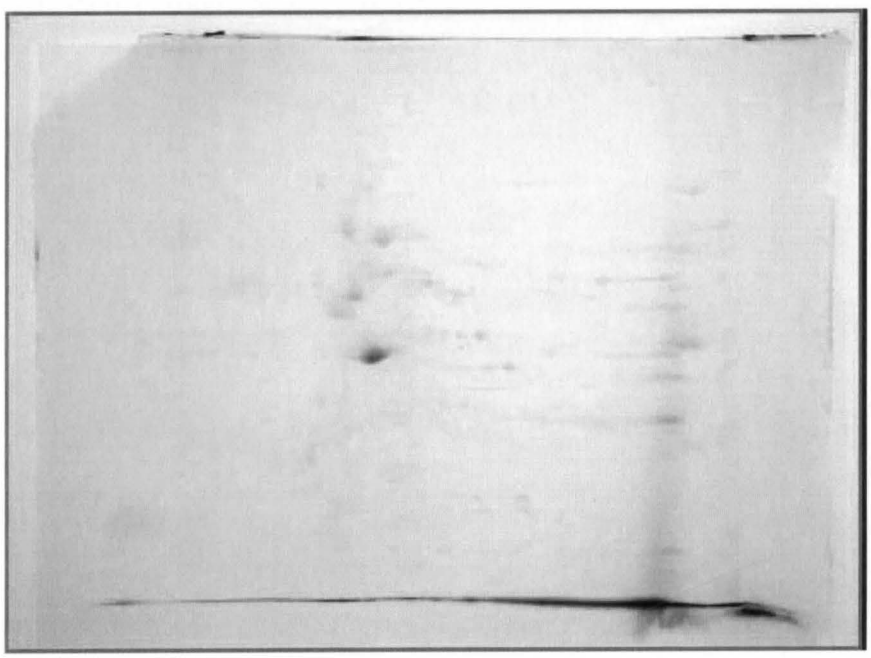


Figure 3.3: Raw 2-dimensional gel electrophoresis image of **infected** macrophage proteins. The protein sample (100 μg) was actively rehydrated at 40,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 7cm 10% polyacrylamide gel in 1% TGS buffer at 16 mA. Gel was stained with Sypro Ruby Red and photographed. Protein spots are seen in white.

Figure 3.4: Raw 2-dimensional gel electrophoresis image of **control** macrophage proteins. The protein sample (100 μg) was actively rehydrated at 40,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 7cm 10% polyacrylamide gel in 1% TGS buffer at 16 mA. Gel was stained with Sypro Ruby Red and photographed. Protein spots are seen in white.

Figure 3.3

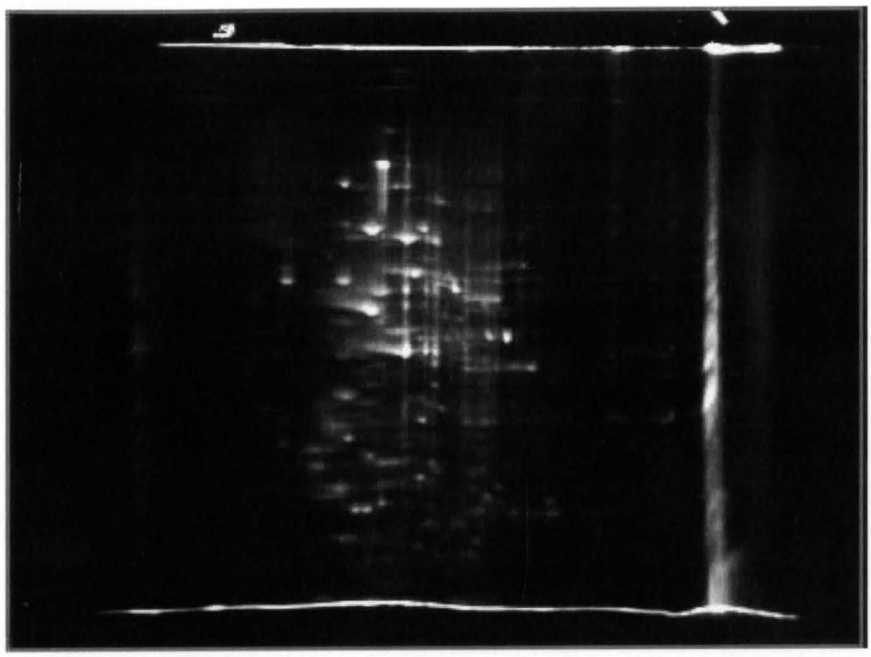


Figure 3.4

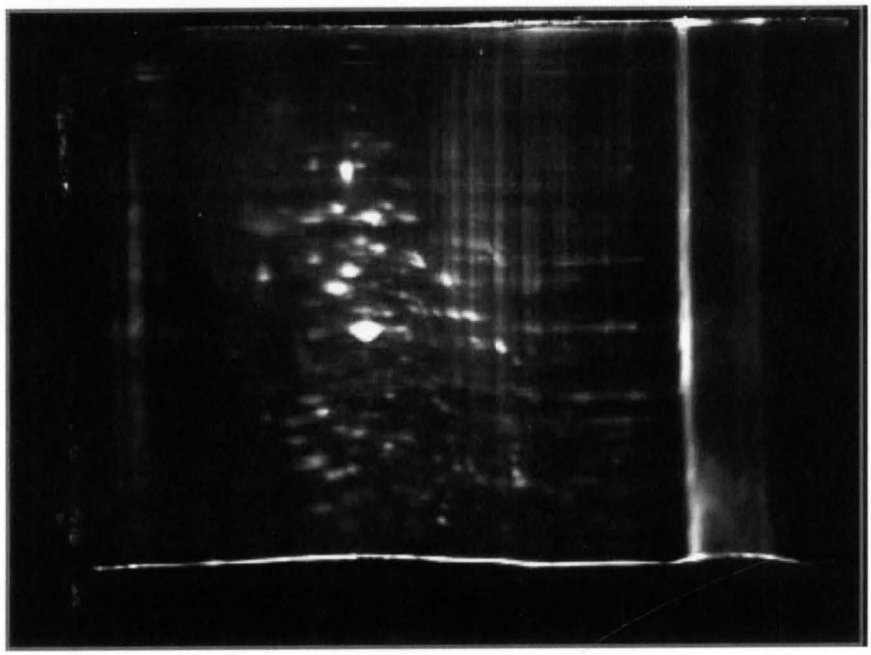


Figure 3.5: Raw 2-dimensional gel electrophoresis image of **infected** macrophage proteins. The protein sample (255 μg) was actively rehydrated at 60,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 17cm 10% polyacrylamide gel in 1% TGS buffer at 10 mA. Gel was stained with Sypro Ruby Red and photographed. Protein spots are seen in white.

Figure 3.5

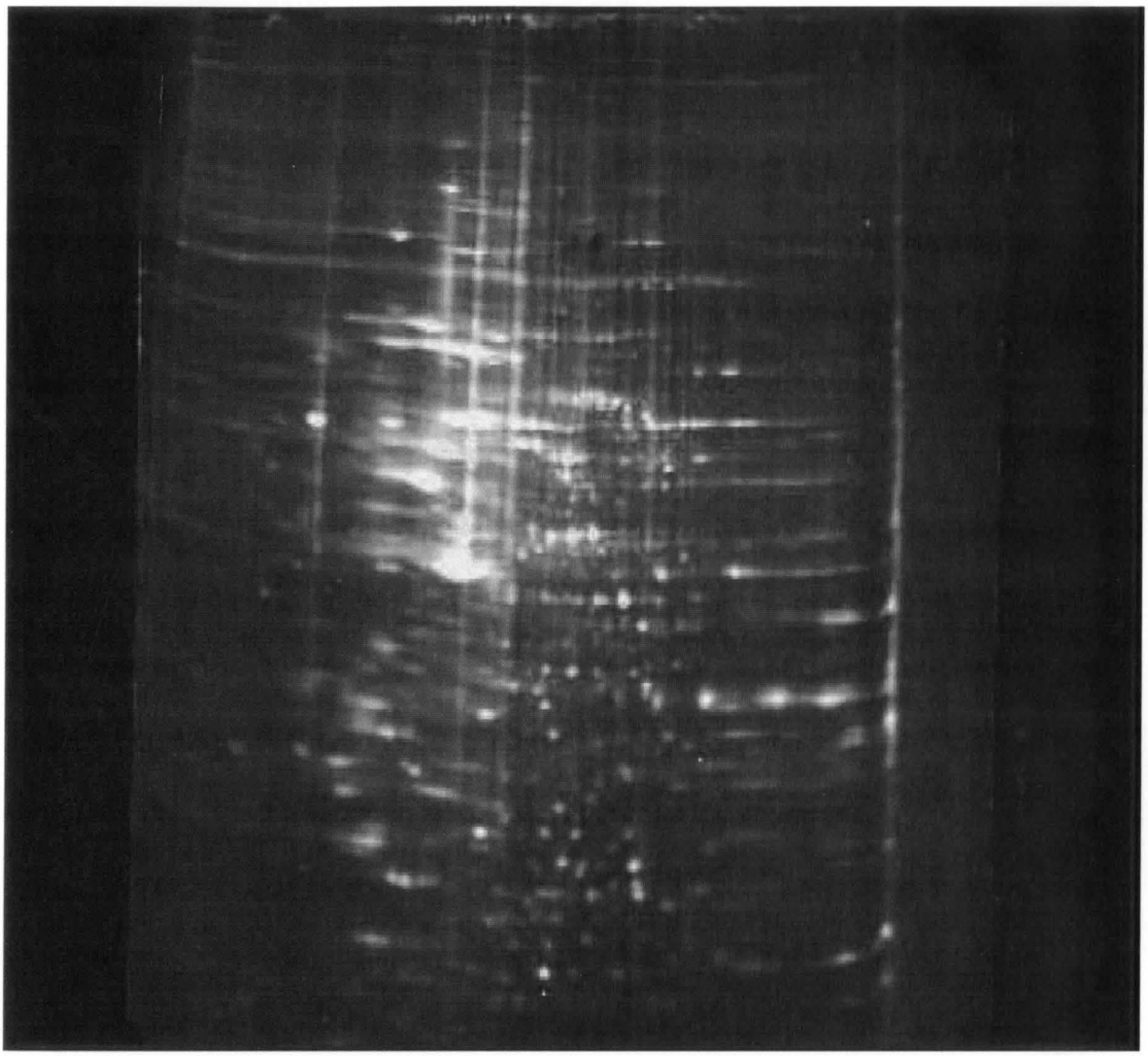


Figure 3.6 is another 3-10 pH range 17 cm gel. It is a raw image revealing proteins expressed by control macrophages not infected with *P. marneffei*. This gel was stained with Sypro and visualized. Some vertical and horizontal streaking occurred and is visible on the gels. Furthermore, some spots appear indistinct and blurred, again possibly because of sample overload.

Figures 3.7 and 3.8 were created by using the PDQuest software. Figure 3.7 displays images of gels run using samples from three different trials of macrophage infection. These three gels then formed a composite, which is shown in the figure in the top left corner. Figure 3.8 was made the same way; the only difference is that these gels are of control macrophage cells rather than infected.

Figures 3.9 and 3.10 are enlarged images of the composites seen in Figures 3.7 and 3.8. The composite of the gels from the infection trials (Figure 3.9) was created by matching spots to each other on the gels from the three trials of infections. Similarly, to create the composite of control cells (Figure 3.10), the three gels of control trials were compared and spots appearing in all of them appear on the composite.

Figure 3.11 is the composite of three trials of infected macrophage proteins with the proteins unique to the infected group encircled in red. These indicated proteins totaled 220 and are found only in gels showing proteins of infected macrophages, not in those of control cells.

Another composite image indicating unique proteins is seen in Figure 3.12. This portrays all proteins found in the control group, with proteins unique to this group encircled in red. 71 proteins unique to the control group were revealed.

Figure 3.6: Raw 2-dimensional gel electrophoresis image of **control** macrophage proteins. The protein sample (255 μg) was actively rehydrated at 60,000 volt-hours on a 3-10 pH gradient IPG strip in the first dimension. In the second dimension, electrophoresis was done using a 17cm 10% polyacrylamide gel in 1% TGS buffer at 10 mA. Gel was stained with Sypro Ruby Red and photographed. Protein spots are seen in white.

Figure 3.6

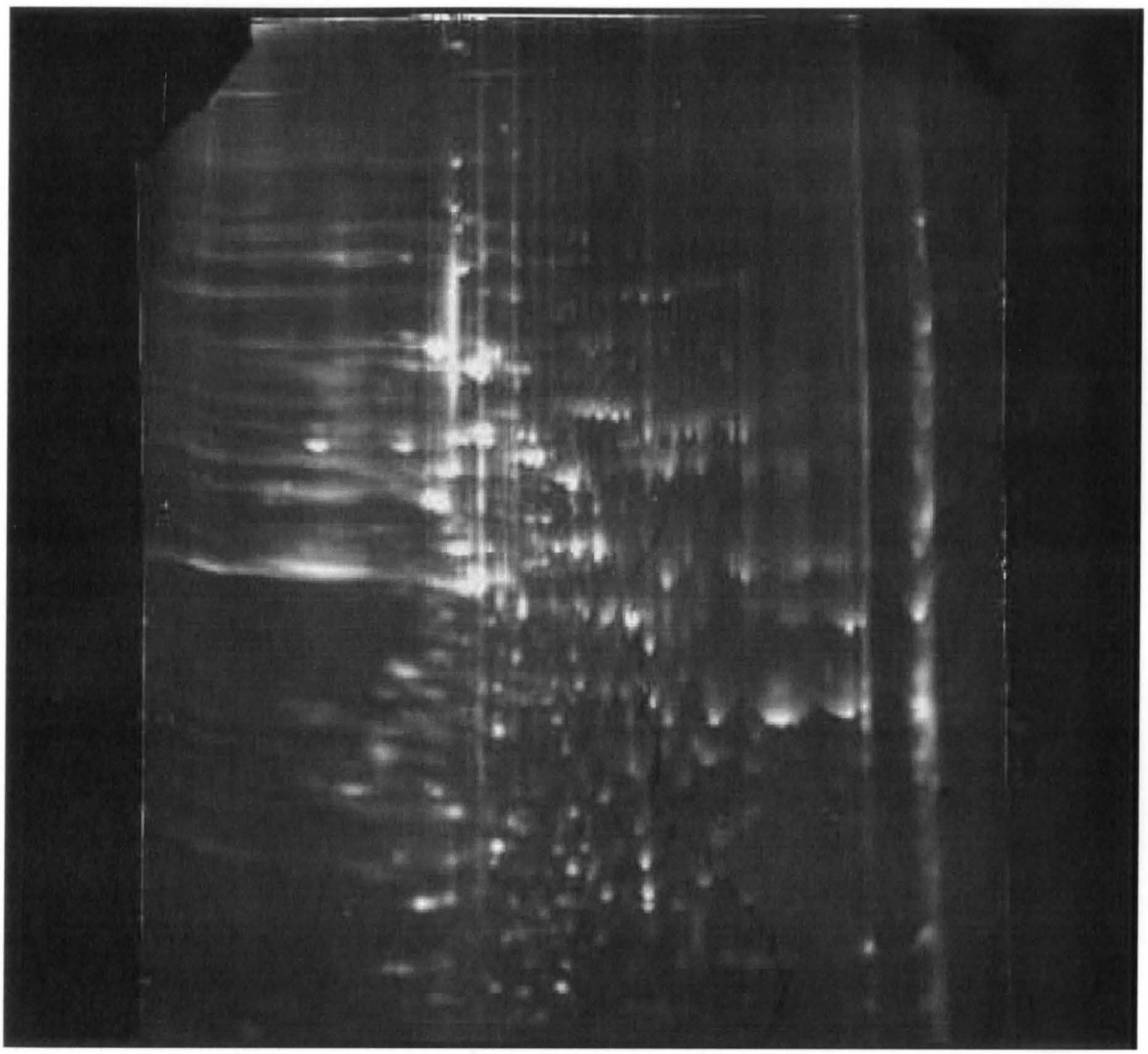


Figure 3.7: Three separate trials of macrophage infections performed; 17 cm gels were run and imaged. Figure depicts the gel images of the three trials of **infected** macrophage proteins (upper right, lower left and right images) and the composite formed from them (upper left).

Figure 3.7

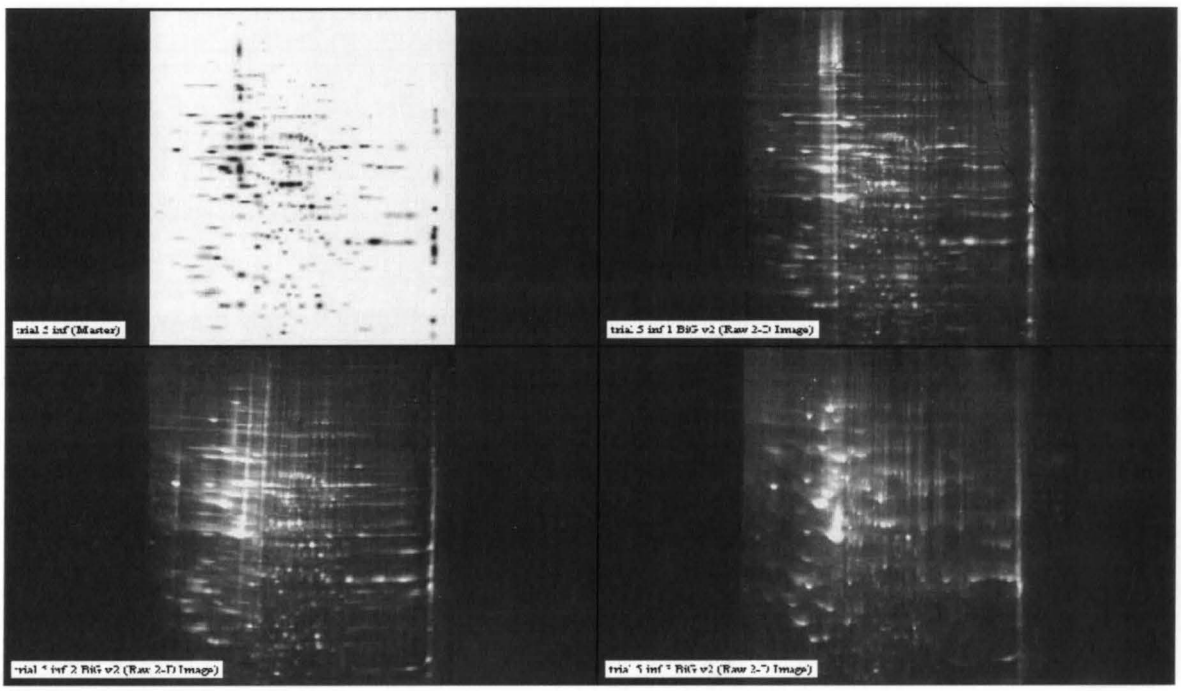

trial 5 inf (Master)
trial 5 inf 1 BitS v2 (Raw 2-D Image)
trial 5 inf 2 BitS v2 (Raw 2-D Image)
trial 5 inf 3 BitS v2 (Raw 2-D Image)

Figure 3.8: Three separate trials of control macrophages; 17 cm gels were run and imaged. Figure depicts the gel images of the three trials of **control** macrophage proteins (upper right, lower left and right images) and the composite formed from them (upper left).

Figure 3.8

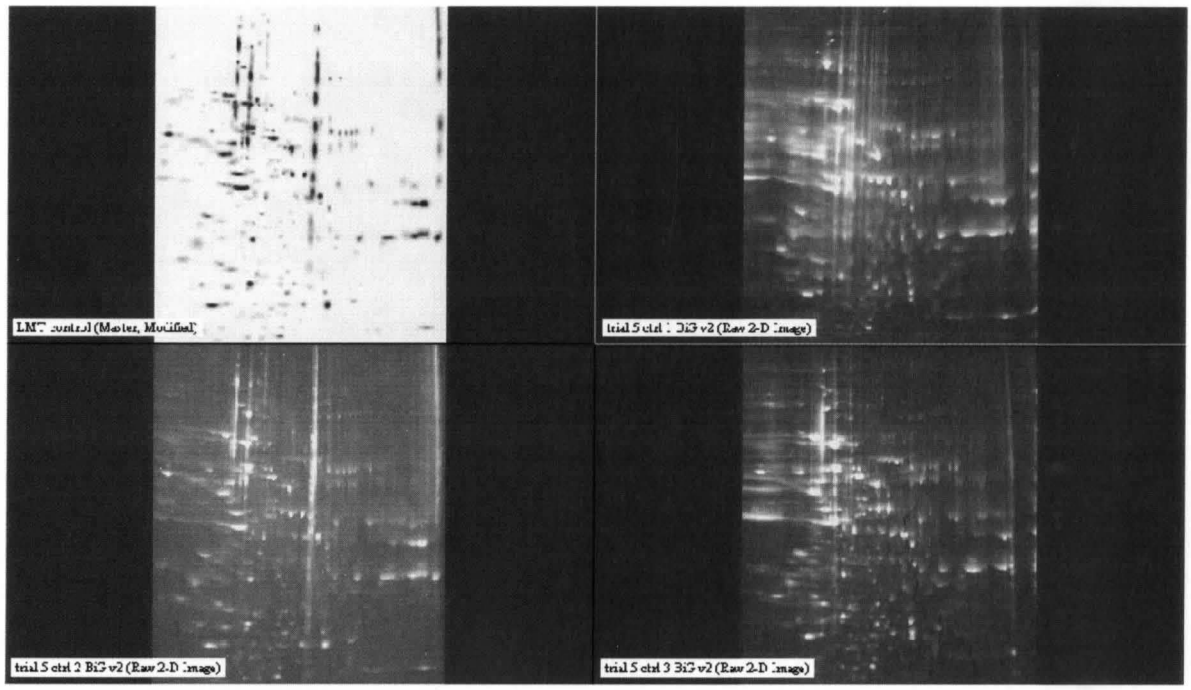


Figure 3.9: Composite image of three different images of **infected** macrophage proteins. Gels were run as described in Figure 3.5. Protein spots appear black.

Figure 3.9

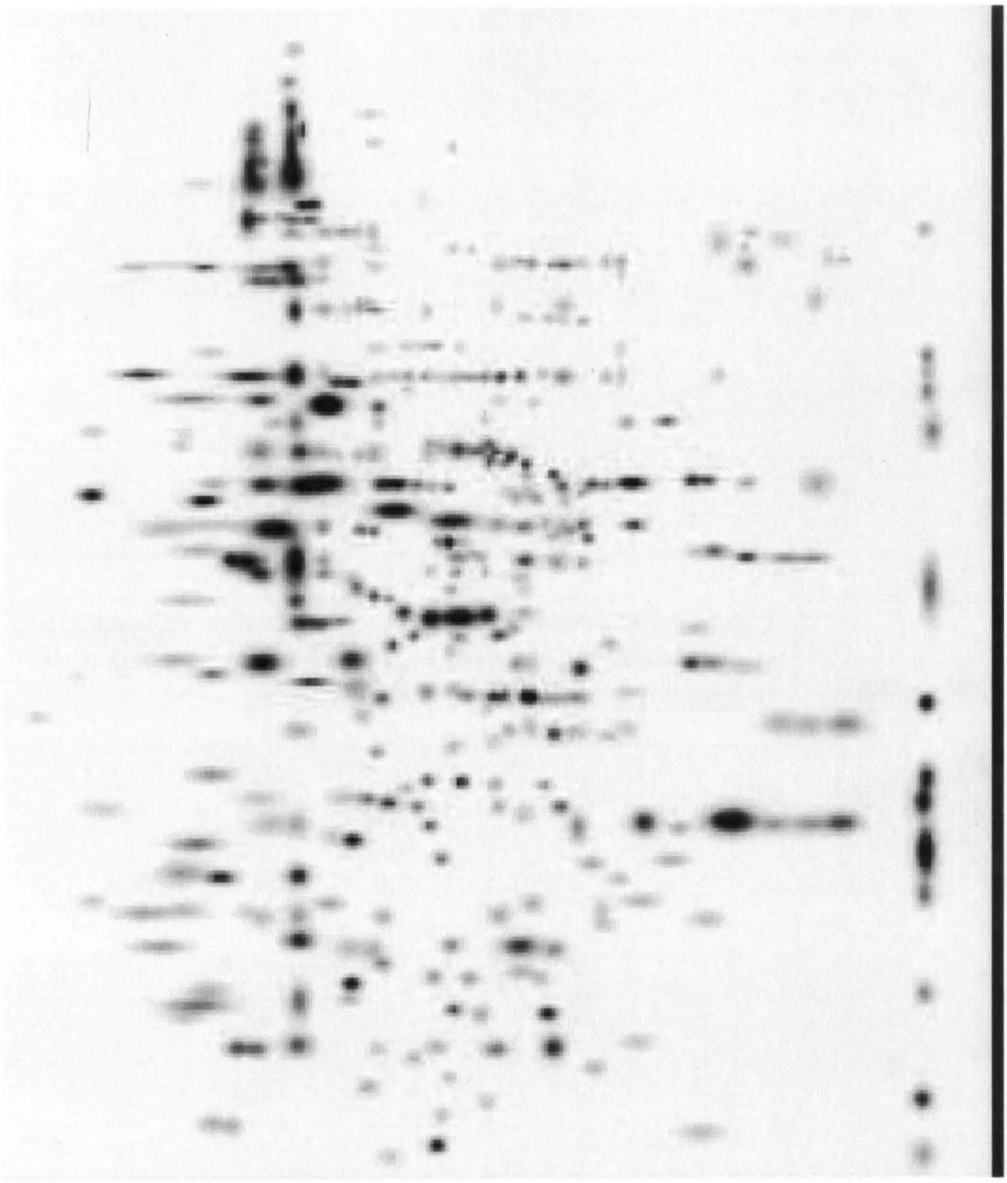


Figure 3.10: Composite image of three different images of **control** macrophage proteins. Gels were run as described in Figure 3.6. Protein spots appear black.

Figure 3.10

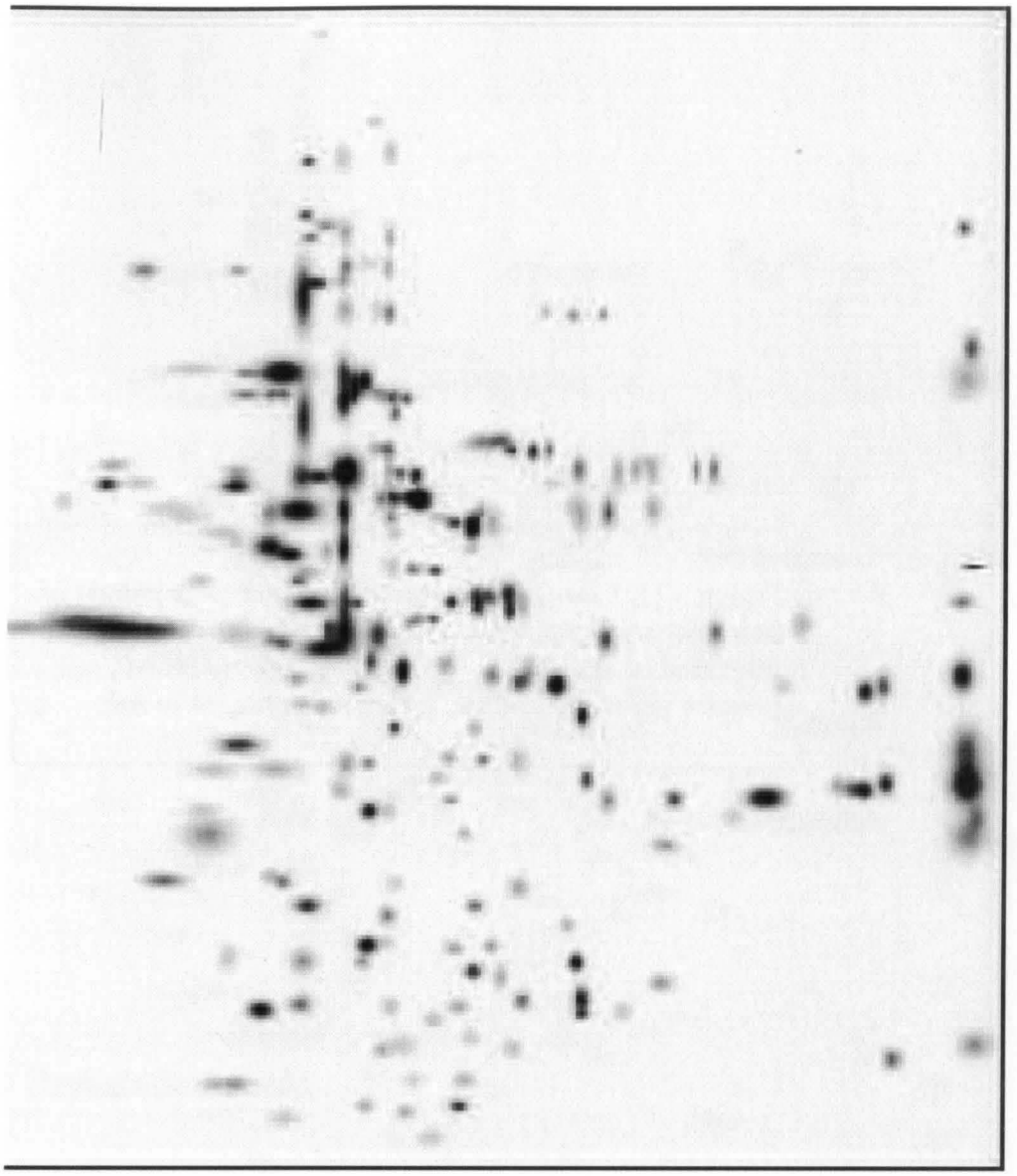


Figure 3.11: Composite image of three different images of **infected** macrophage proteins. Gels were run as described in Figure 3.5. Protein spots appear brown; proteins unique to infected group are encircled in red.

Figure 3.11

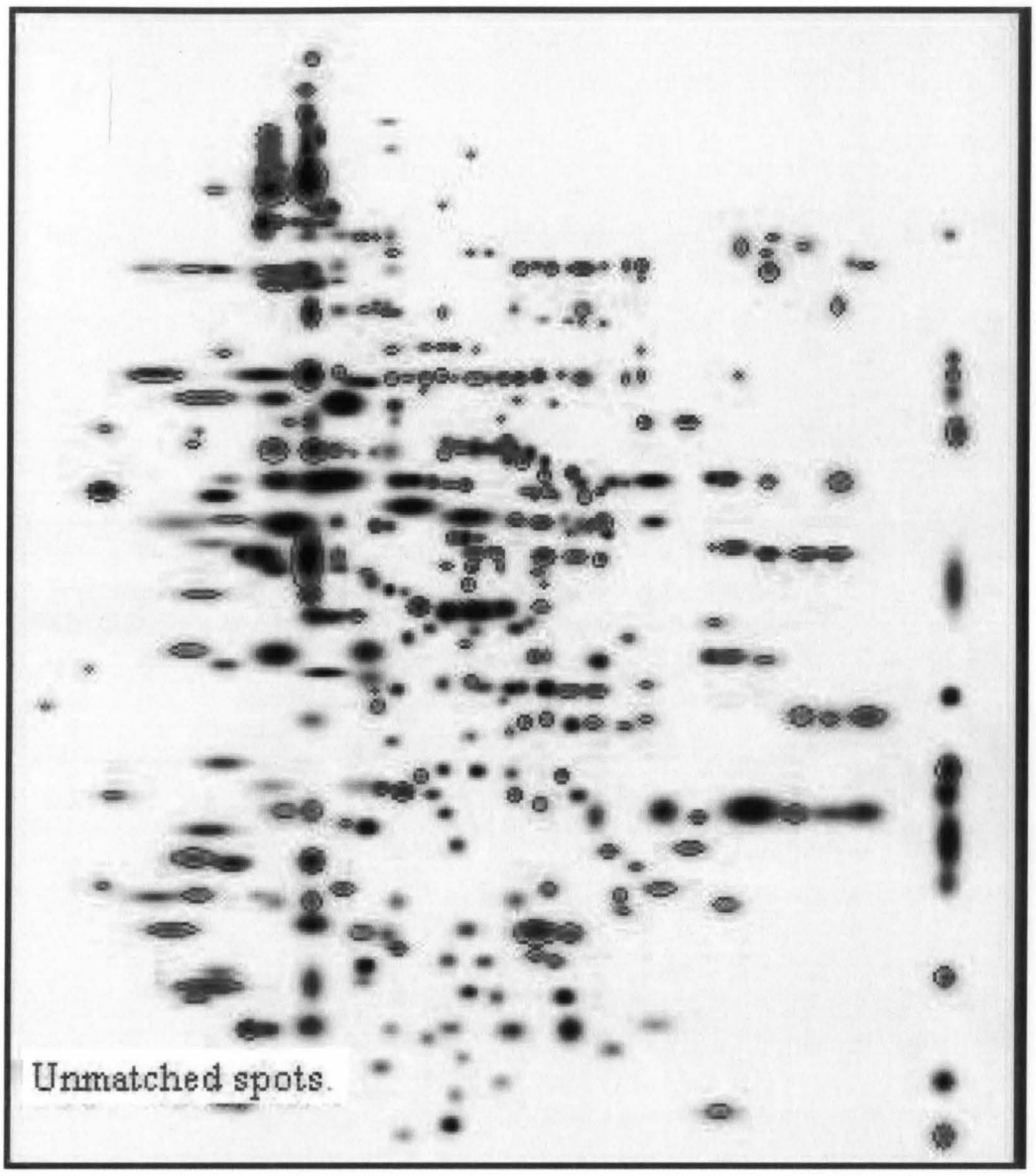
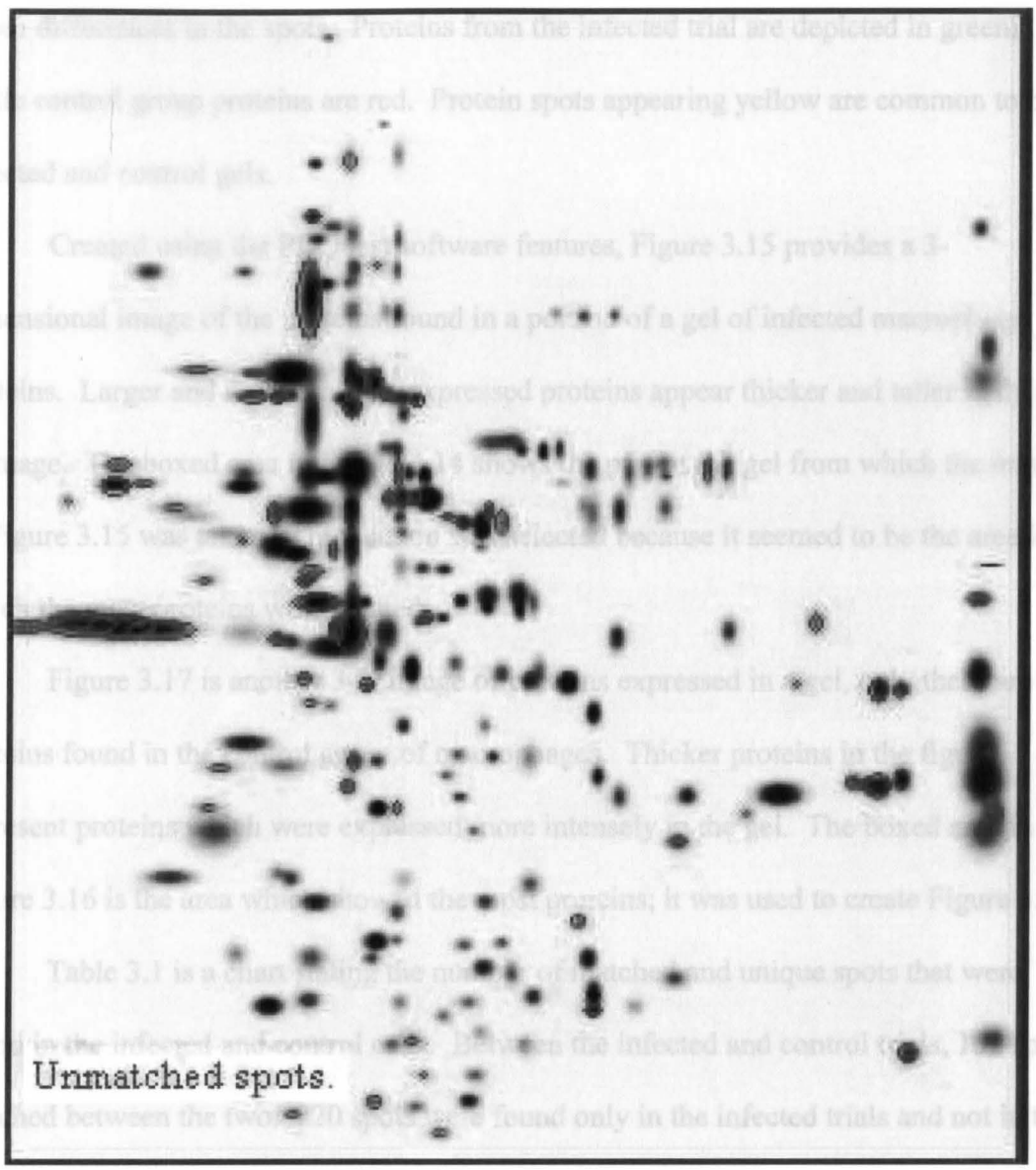


Figure 3.12: Composite image of three different images of **control** macrophage proteins. Gels were run as described in Figure 3.6. Protein spots appear brown; proteins unique to control group are encircled in red.

Figure 3.12



A gel run using the protein sample of infected macrophage cells was overlain with a gel run with a control group protein sample, as seen in Figure 3.13. Both gels were of the 3-10 pH range and were 17 cm in length. Although streaking is visible, it is possible to see differences in the spots. Proteins from the infected trial are depicted in green, while control group proteins are red. Protein spots appearing yellow are common to both infected and control gels.

Created using the PDQuest software features, Figure 3.15 provides a 3-dimensional image of the proteins found in a portion of a gel of infected macrophage proteins. Larger and more strongly expressed proteins appear thicker and taller in the 3-D image. The boxed area in Figure 3.14 shows the part of the gel from which the image in Figure 3.15 was taken. This section was selected because it seemed to be the area in which the most proteins were located.

Figure 3.17 is another 3-D image of proteins expressed in a gel, only these are proteins found in the control group of macrophages. Thicker proteins in the figure represent proteins which were expressed more intensely in the gel. The boxed area in Figure 3.16 is the area which showed the most proteins; it was used to create Figure 3.17.

Table 3.1 is a chart stating the number of matched and unique spots that were found in the infected and control cells. Between the infected and control trials, 152 spots matched between the two. 220 spots were found only in the infected trials and not in the controls, while 71 spots were present in the control group only and not the infected trials.

Figure 3.13: Overlay of gels of infected and control macrophages. Proteins found in infected gel appear green; proteins found in control gel appear red.

Figure 3.13

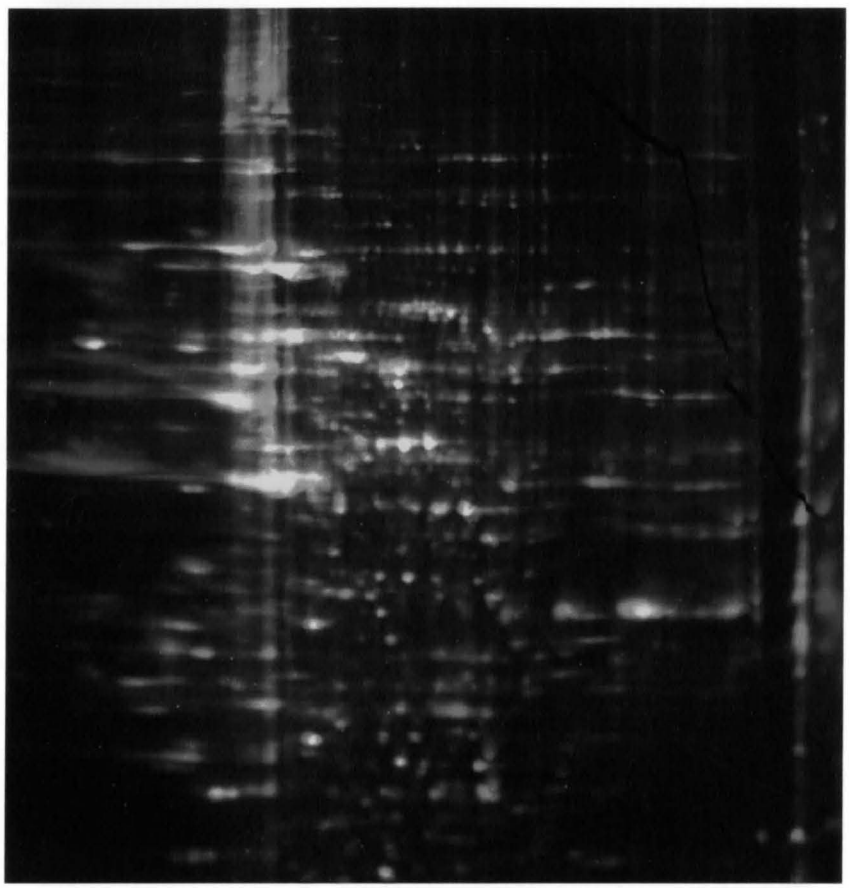


Figure 3.14: 17 cm gel image of **infected** macrophage proteins. Boxed area indicates area of interest in Figure 3.15.

Figure 3.15: 3-dimensional image of the proteins found in the boxed area of Figure 3.14. Greater height and thickness of peak indicates stronger expression of the given protein.

Figure 3.14

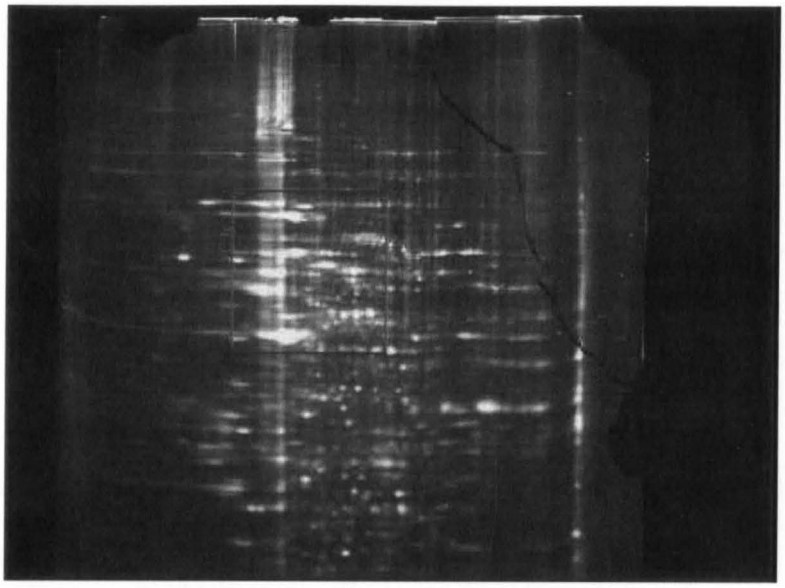


Figure 3.15

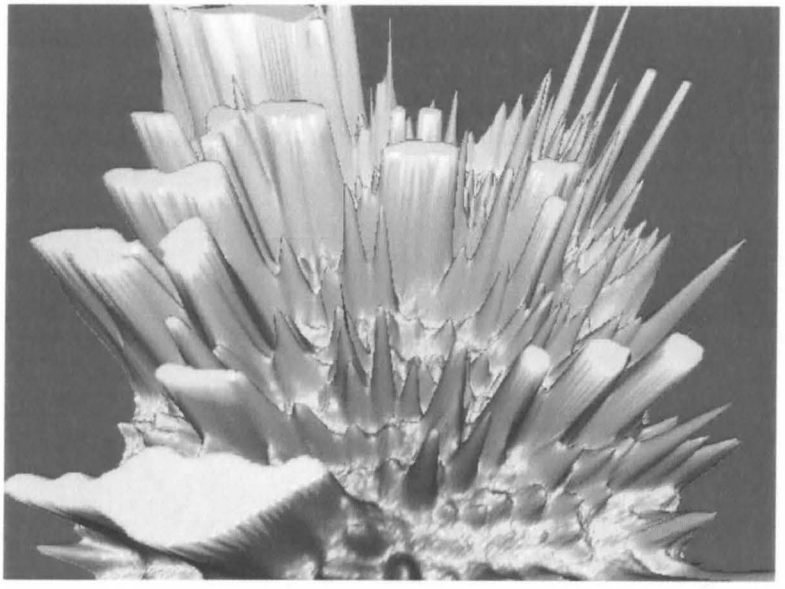


Figure 3.16: 17 cm gel image of **control** macrophage proteins. Boxed area indicates area of interest in Figure 3.17.

Figure 3.17: 3-dimensional image of the proteins found in the boxed area of Figure 3.16. Greater height and thickness of peak indicates stronger expression of the given protein.

Figure 3.16

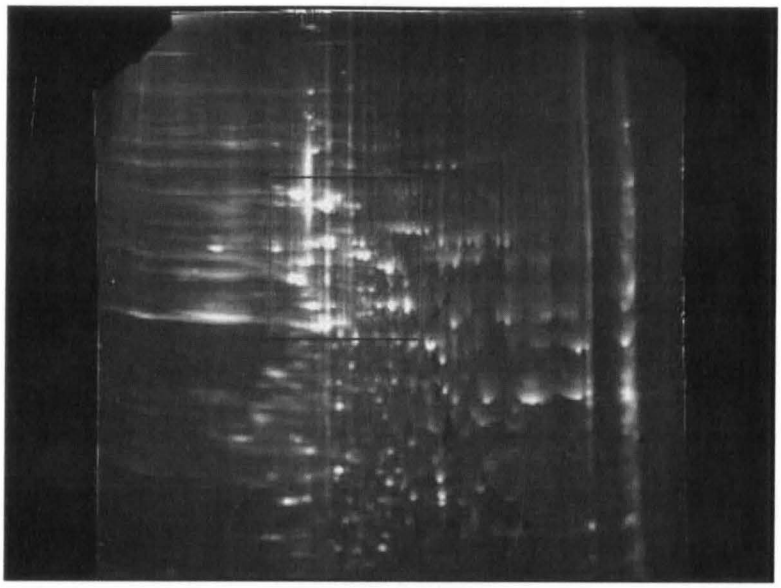


Figure 3.17

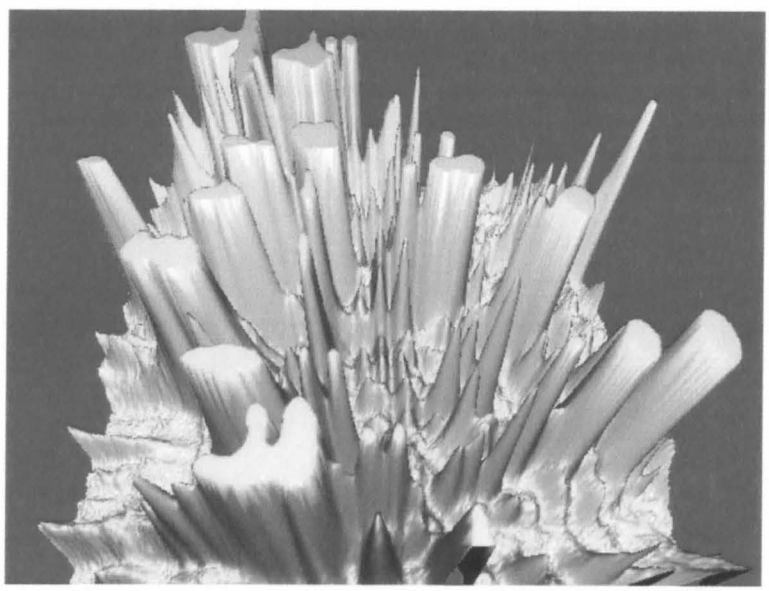


Table 3.1: Table displaying the number of unique and matched spots for infected and control macrophages. Data taken from images in Figures 3.11 and 3.12.

Table 3.1

	Matched Spots	Unique Spots
Infected Macrophage Cells	152	220
Control Macrophage Cells	152	71

Figures 3.18 – 3.20 are pictures taken of plates on which a portion of each sample was grown. Figure 3.18 shows a plate on which only the macrophage solution was plated. No conidia were in the solution. Figure 3.19 shows only a plate on which conidia were grown. The plate seen in Figure 3.20 contains the macrophages infected with conidia. Both macrophage and conidial cells can be seen on the plate. All samples were grown on PDA agar and incubated at room temperature (~25°C) for approximately two weeks, at which time pictures were taken.

Table 3.2 displays results from counting the numbers of cells after infection has occurred. The counting was done using a hemocytometer slide and a fluorescent microscope focused at 400X. Macrophage cells, free conidia, and conidia inside macrophage cells were counted, then the total number of conidia present was determined. Results were recorded in the chart.

Figure 3.18: Results of plating macrophage cells alone. Grown on PDA plate, incubated at 25°C.

Figure 3.19: Results of plating conidia alone. Grown on PDA plate, incubated at 25°C.

Figure 3.20: Results of plating macrophages infected with conidia. Grown on PDA plate, incubated at 25°C.

Figure 3.18

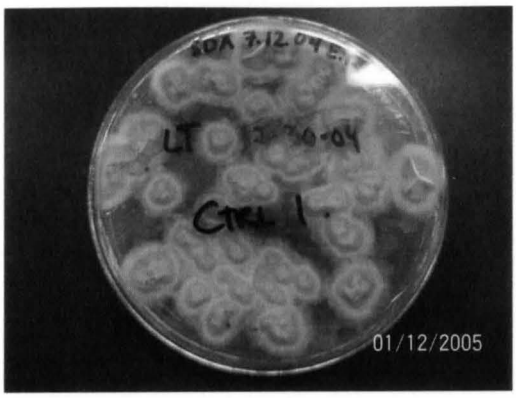


Figure 3.19

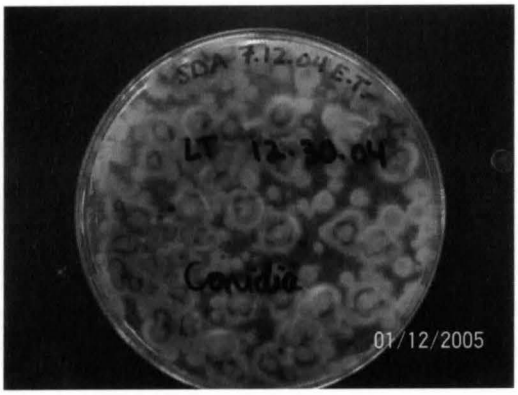


Figure 3.20

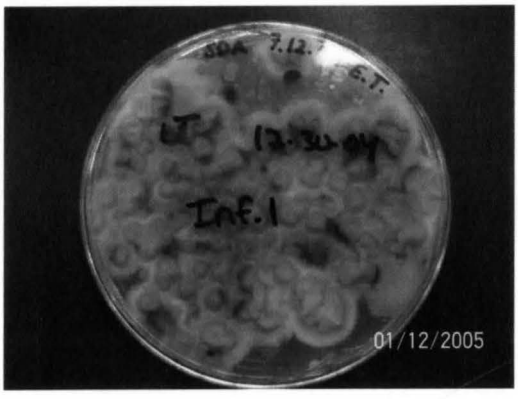


Table 3.2: Results from post-infection cell counting using fluorescent microscope.

Table 3.2

	Number of Macrophage Cells	Number of Conidia Inside Macrophage	Number of Free Conidia	Total Number of Conidia
Trial 1 Infected	$1.352 * 10^7$	$3.928 * 10^7$	$2.65 * 10^7$	$6.578 * 10^7$
Trial 2 Infected	$1.237 * 10^7$	$3.281 * 10^7$	$1.779 * 10^7$	$5.06 * 10^7$
Trial 3 Infected	$1.803 * 10^7$	$5.824 * 10^7$	$1.737 * 10^7$	$7.561 * 10^7$
Trial 1 Control	$1.047 * 10^7$	N/A	N/A	N/A
Trial 2 Control	$1.146 * 10^7$	N/A	N/A	N/A
Trial 3 Control	$1.767 * 10^7$	N/A	N/A	N/A

CHAPTER IV

Discussion

Resulting protein profiles of infected vs. control macrophage cells do indeed indicate the presence of different proteins. This was seen even in very early gels, which suggested the initial methods of protein separation and collection were working. Coomassie Brilliant Blue (the stain used on early gels) is not as sensitive as Sypro Ruby Red. Because of this, it was only used early in the experimental process (as seen in Figures 3.1 and 3.2) as it did not allow as many protein spots to be seen. Once Sypro was used to stain the gels, many more protein spots were able to be detected.

Similarly, 7 centimeter gels were first run to ensure the tentative methods actually allowed for proteins to be visualized. These can be seen in Figures 3.1 – 3.4. Once suitable results were obtained from these gels, protein samples were then run on 17 centimeter gels, as they provided a clearer and more accurate protein map, as seen in Figures 3.5 and 3.6.

Multiple trials of cell infections were performed. The proteins were isolated and 17 centimeter gels were run using these protein samples. The best three of these gels were then compared using the PDQuest software package. Gels were manipulated using the software in such ways that they allowed for better visualization of spots. They were cropped so that irrelevant areas on the outer edges were removed. They were also filtered, which made it easier for significant spots to be identified. Because of variations in gels, not all spots matched. If it was thought that the spots did indeed match, they were manually matched using features of the software. Manual matching allowed for

quite a number of additional spots to be matched, which were initially missed by the software. Spot matching among trials allowed for a composite to be created, as seen in Figures 3.7 and 3.9. The composite provides a more confident image of the proteome, as it was created from multiple trials of the same experiment. Trials and gels of control macrophage cells were also run in triplicate and compared in this way. Control composites were created and are presented in Figures 3.8 and 3.10.

The next step in protein analysis was the creation of higher order matchsets. This involved comparing and contrasting composites of the infected vs. control gels. Just as in lower order matchsets, spots were first automatically detected by the software, then spots that did not match were manually matched if appropriate. Analysis using PDQuest revealed 71 unique proteins in the control group. PDQuest also revealed 220 unique proteins in the infected group and 152 proteins that matched on both the control and infected gel images. As seen in Figures 3.11 and 3.12, unmatched (unique) spots are identified and encircled in red.

Expression of a protein in an infected sample, but not the control may indicate a protein involved in macrophages' response to *P. marneffei*. Conversely, expression of a protein in a control sample, but not an infected one may indicate a protein that gets shut off when the resting macrophage becomes activated to carry out phagocytosis.

An image of a gel overlay was created using composites of the infected and control cells (Figure 3.13). Because proteins from the infected gel appear green and those from the control gel are red, similarities and differences are easily observed between the two. Most proteins appear to line up to some degree, suggesting they are present with or without infection by the fungus. Imperfect alignment of spots may be

because of variances in or incomplete isoelectric focusing or streaking, so slight misalignment should be disregarded. There are some spots however, that do not seem to have a matching spot of the other color. These are proteins of interest that can be excised and sequenced in order to determine if they are in fact involved in the macrophage response to *P. marneffei*.

In order to achieve a better resolution of proteins, one possible change to make would be to reduce the protein load. The 17 centimeter gels used in these trials were all loaded using 255 μg of protein. Even though this is less than what is recommended in the BioRad instruction manual, it still may be too much for this particular experiment. Reducing this amount may reduce streaking and allow for the appearance of more well-defined protein spots.

The three-dimensional images presented in Figures 3.15 and 3.17 are interesting graphic representations that could not be seen by the eye alone. Portions containing numerous protein spots of both infected and control sample gels were selected and a 3-D image was created of that section of the proteome. This is a useful tool when studying proteins to determine which ones should be further examined, as the larger and more expressed proteins were not only consistently present, but would also likely be the ones to identify when hoping to uncover differences between infected and non-infected cells. Large protein spots were in fact seen in both gels. However they are not necessarily in the same location. Future identification of these unique spots may indeed uncover a protein unique to infection by *P. marneffei*.

The plating seen in Figures 3.18 – 3.20 was done in order to double check whether or not the conidia were viable and the macrophage cells phagocytized them

during infection. The plate in Figure 3.18 is that of only macrophage cells; they were not infected with conidia, thus no conidia are seen on the plate. Figure 3.19 represents the growth of conidia alone, without macrophage cells. The growth of colonies indicates the conidia were in fact viable at the time of infection. A sample of macrophage cells infected with conidia was plated and is shown in Figure 3.20. The appearance of reddish fungal colonies within the macrophage cell reveals the experiment was successful and cells did carry out phagocytosis of the *P. marneffei*.

Furthermore, cells were counted once infection had taken place. This was done using a fluorescent stain and microscope so that conidia inside macrophage cells could be seen. Macrophage cells were counted, as were the number of conidia within the macrophage and those outside of them. A significant number of conidia were seen inside the macrophages, indicating successful phagocytosis of the fungus by the macrophage.

Some of the goals of this project were accomplished. Proteins present in macrophages infected with *P. marneffei* and those in uninfected macrophages were indeed isolated and visualized, but no specific spots were excised from any gels for identification.

In the future, further procedures studying the macrophage response to *P. marneffei* may be carried out. In a continuation of this project alone, certain changes could be made to ensure better results. An example of this is determining an ideal protein load. This may reduce streaking and allow for more distinguished protein spots in gels. Another change could involve using macrophages that are all approximately the same "age" – that have all been passaged about the same number of times – for the different trials. This may not matter, but it would just be one more element that could be

controlled. One last change, using a narrower pH range when carrying out isoelectric focusing may be helpful because it will allow the proteins to appear more spread out. If specific spots are then identified and are to be removed for sequencing, it will be easier to physically remove them from the gel without disrupting other proteins.

This project can also branch off in many directions. For instance, gels showing proteins of infected and control macrophages can be compared with those of conidia alone. By doing this, proteins that may be present because of any conidia that might not have been washed away could be detected and disregarded. Also, macrophages could be infected with a different pathogenic fungus and gels could be run. These could be compared against gels of macrophages infected with *P. marneffei* in order to identify proteins specific to the cells' response to *P. marneffei*. This would all be advantageous in uncovering proteins involved in the unique macrophage response to *P. marneffei*.

CHAPTER V

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