Example of a Successful student Application; Yours may vary - this is only to be used as a guideline to submitting an application!

Antibody Dependent Enhancement of Visceral Leishmaniasis

Your Name Here

Your Committee Chair Name

Department Name

Abstract

Leishmaniasis is an infectious disease caused by parasitic microbes belonging to the family *Leishmania*. The disease is spread by biting sandflies and can be found in the warmer regions of South America, Africa, Europe, and Asia. It is estimated that worldwide, 1.7 billion people are at risk of exposure. There are two ways most *Leishmania* infections will manifest: either as a cutaneous infection of the skin or as a visceral infection that attacks the internal organs. The latter is often fatal when untreated. Earlier research in animals has shown previous exposure to the cutaneous infection causes increased severity of disease in individuals that are later exposed to the visceral infection. It may seem counter-intuitive for an infection to be worse the second time an individual contracts it, but I hypothesize a phenomenon called antibody dependent enhancement (ADE) may be to blame. The aim of my research is to test this hypothesis through the use of purified antibody treatments in animal infection experiments as well as "test tube" studies on cultured cells. The goal is to determine if antibody generated during a cutaneous infection is responsible for increased disease severity associated with re-exposure to the visceral infection.

Antibody Dependent Enhancement of Visceral Leishmaniasis

Background

Leishmaniasis is a disease caused by protozoan intracellular parasites belonging to the genus *Leishmania*. The genus is comprised of 53 species, around 20 of which are known to infect humans[1]. The parasite is transmitted by biting phlebotomine sandflies and can be found in the warmer regions of South America, Africa, Europe, and Asia. There are two ways by which *Leishmania* infection will usually manifest; either as cutaneous leishmaniasis (CL) or visceral leishmaniasis (VL). CL is the most common form of infection and is characterized by open, weeping sores. These sores, though grisly in appearance, are often reported to be painless by patients[2]. Generally this type of infection will self-heal in 1 to 8 months[3, 4]. VL is caused by a more diffuse infection that attacks the internal organs, particularly the spleen and liver. VL is characterized by fever, abdominal swelling, and a dark ashen coloration of the skin[5]. This form of leishmaniasis is usually fatal when left untreated[2, 5]. The manner in which the disease presents is dependent on the strain of *Leishmania* causing the infection.

The global impact of leishmaniasis is far-reaching. It is estimated that there are up to 3 million new cases each year, leading to 50,000 fatalities annually[5, 6, 7, 8]. The World Health Organization has identified leishmaniasis as a neglected tropical disease in need of further study. Leishmaniasis is also ranked second to malaria as the deadliest parasitic disease in terms of death toll[2]. The primary risk factor for people living in endemic areas is poverty and the associated living conditions. To be more specific, risk of exposure is higher for people who spend many hours outdoors, people who are not adequately clothed, and people living in rural areas. Increased rates of exposure have also been linked to urbanization, population mobility, environmental change, and animal husbandry[5].

Effective treatment options for this disease are available, though steep cost and demanding treatment regimens can be prohibitive for many. There are also prevention strategies for both individuals and communities. On the individual level, it is advised that one should use bed nets, dress adequately and use insect repellents if possible[9]. Community prevention generally comes in the form of municipal programs aimed at preventing malaria. Fortuitously, many of the techniques used to control malaria-carrying mosquitoes are also effective against the sandflies that carry *Leishmania*[10, 11]. Municipal prevention techniques include vector control, environmental management, disease surveillance, and control of animal hosts[9, 11]. Another, more antiquated method of prevention is leishmanization. This is the practice of collecting exudate from wounds of those afflicted with CL and administering them to a skin abrasion of another individual in the hopes of conferring some degree of immunity[12]. This method, though prone to side effects, has been shown to be relatively effective at preventing future cases of CL[3]. One issue that hasn't been addressed is the fact that strains of *Leishmania* known to cause CL and those that cause VL tend to co-occur geographically[8]. This means that someone who has been leishmanized or had CL may very well contract VL in their lifetime. This may seem to be a non-issue as one might assume that previous exposure to CL would confer some protection against VL. However, recent research done at CWU suggests this is not the case. In fact, it has been shown that mice who have recovered from CL and later contract VL exhibit greater parasite burden and increased severity of disease when compared to individuals that were naïve to *Leishmania*[13, 14]. It may seem contrary to conventional wisdom for an infection to be worse the second time an individual is exposed but this is not inexplicable.

This brings me to the basis of my proposed study. I hypothesize that the increased parasite burden observed in CL recovered individuals who are later infected by VL can be

attributed to antibody dependent enhancement (ADE). ADE is a phenomenon wherein an individual that has had previous exposure to a particular disease experiences a more severe infection when exposed to a closely related disease. The mechanism behind this is dependent on the presence of antibody generated during the first infection. During the initial infection, the host's immune system is naïve to the disease, allowing it to take hold. As the infection progresses and recovery begins, antibody is produced by the immune system. The antibody is highly specific to the pathogen and helps to neutralize it as well as direct the body's cells of immunity to locate and sometimes envelope and destroy the pathogen. If, however, the pathogen in question targets the cells of immunity for infection the presence of antibody can be counterproductive. When a second infection occurs the antibody will mark the pathogen, attracting cells of immunity called macrophages. These macrophages will envelope the pathogen, immediately becoming infected. This process exacerbates the infection by allowing the pathogen more opportunities to take hold while depriving the host of valuable macrophages.

Literature Review

ADE has been well documented in members of *Flaviviridae*: the family of mosquito-borne viruses that cause Dengue fever, chikungunya, and Zika, among others[15, 16, 17, 18, 19, 25]. Flaviviridae viruses behave exactly as previously described: they enter the host's body and commence the process of infection by infiltrating a macrophage[15]. Once inside the macrophage the virus replicates, eventually killing the cell, allowing the virus to escape, infect more cells, and proliferate. If the host has had one of these viruses in the past, circulating antibody will bind the new invading virus, attract high numbers of macrophages and accelerate the process of infection[15]. This is apparent in observations of people stricken by Dengue fever. Generally the disease will cause fever, rash, and flu-like symptoms[24]. A much smaller subset of patients, ~5%,

show far worse symptoms including joint pain, hemorrhaging, and shock[25, 26]. It was widely speculated that ADE was the cause of the harsher symptoms but a recent study has definitively shown that ADE is largely to blame [25]. Of course this is of concern to people living in affected areas and the doctors who care for them. ADE also poses an obstacle to the development of vaccines for flaviviruses.

Recent attempts at developing an HIV vaccine have also been hampered by ADE. In the case of HIV, the immune cells targeted for infection are CD4 T cells as well macrophages and others[21, 22]. HIV exhibits a somewhat different form of ADE known as antibody dependent complement-mediated enhancement[23]. Complement is a component of innate immunity that, through a series of cascading chemical reactions, can pierce the membrane of host cells that have been infected. After losing membrane integrity the cell will die, along with most of the viral particles replicating inside. In this case, antibody against HIV binds the virus and initiates a complement cascade through the classical pathway. This causes the death of infected cells and can reduce the number of circulating virions. Unfortunately, a compliment cascade will attract cells of immunity which carry compliment receptors[23]. By no coincidence, the immune cells that are drawn to the compliment-laden infected cell are targeted by HIV. When coming into close contact with infected cells that are in the process of dying, these immune cells can become infected, accelerating the infection[23]. It should be noted that this is an ongoing process that occurs during the course of HIV infection or as a result of attempted immunization. Obviously one cannot recover from HIV only to be reinfected, as is the case with *Leishmania* or Dengue.

There are few pieces of literature that explore the possibility of ADE in leishmaniasis. The pieces that are most relevant to my proposed project would be theses of two CWU master's graduates, C. Nation and H. Anderson. C. Nation started down this path of inquiry with a project

meant to determine whether previous exposure to *Leishmania major*, a causative agent of CL, would confer protection against infection by *Leishmania infantum*, a causative agent of VL. She in fact found the opposite. Mice that had recovered from *L. major* and were later infected with *L. infantum* showed a larger number of parasites in their tissues when compared to *L. infantum* infected mice that had no previous exposure[13]. H. Anderson then built off of this study to further explore this observed increase in parasitemia in recovered individuals. A key part of her study involved passive immunity transfer; that is, collecting blood serum from an individual who's had previous exposure and administering it to a naïve individual. Her results supported the findings of C. Nation by not only showing increased parasitemia in recovered individuals that were challenged by *L. infantum* inoculation, but also by showing higher parasite burden in passively immunized mice[14].

Two studies done by a team of Brazilian scientists showed a similar effect. They began by culturing *Leishmania amazonensis* parasites, a causative agent of CL, and homogenizing the parasites. They then produced an extract from the homogenate and injected mice with it. They then challenged the mice with *Leishmania braziliensis*, a causative agent of VL. The mice that had received the extract showed increased susceptibility to *L. braziliensis* [27]. In a subsequent experiment, these researchers identified a particular protein in the parasite extract that seems to be responsible for this effect.

Though the aforementioned studies all strongly point to ADE, the evidence isn't definitive. This is because none have specifically investigated the effect of purified antibody generated during active CL infection on the outcome of later infection with VL. The experiment carried out by H. Anderson involved serum transfer and though serum contains antibodies, it is a complex mixture. For more definitive evidence of ADE, the antibodies must be separated from

the serum to show that they are in fact behind the observed effects and not some other serum component. The Brazilian studies relied on parasite extract, not active infection. Purified antibody from an active infection is the key to demonstrating ADE in this system. It is my intention to do this for the proposed study.

Methods

My proposed study will utilize coupled animal infection experiments and *in vitro* experiments. The animal study will require six groups of mice; four test groups and two antibody donor groups. It will begin by infecting the two antibody generating groups and one test group with a small inoculum *L. major* (CL). Twelve weeks will be given for the infection to resolve and antibody to be generated[29]. Antibody will be collected from the two donor groups and purified using a protein G affinity column. One test group will then receive *L. major* specific antibody, the next group will receive inactivated antibody, and the final two groups will not receive antibody treatment. Immediately after this, all groups will be challenged with a large inoculum of *L. infantum* (VL). The infection will be allowed to progress for four weeks at which point the mice will be euthanized and spleens, livers, and lymph nodes will be removed for determination of parasite burden.

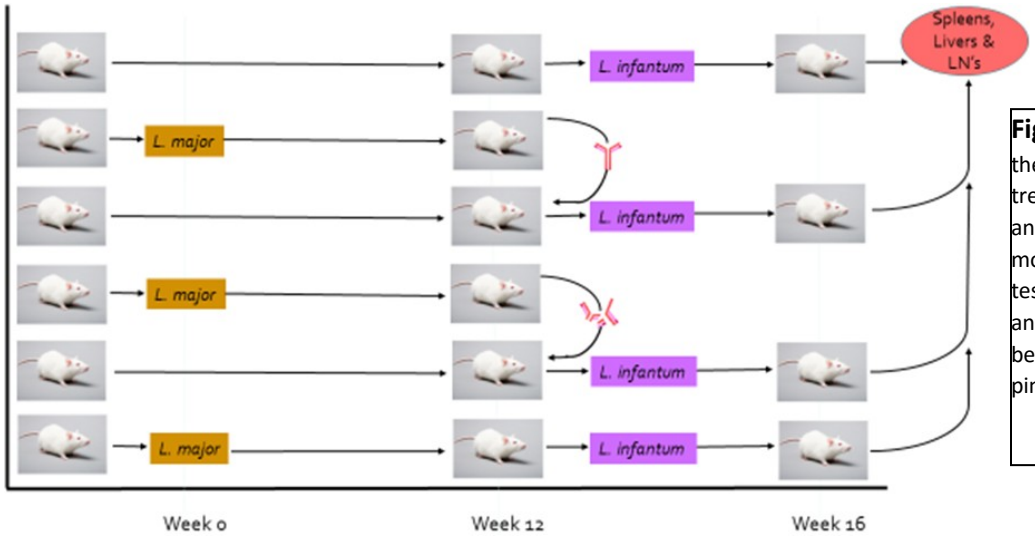

Figure 1: Schematic showing the experimental groups, treatments, and timeline of animal experiment. Each mouse image represents one test group. Intact & inactivated antibody being transferred between groups is shown in pink.

The amount of parasites in these tissues will be quantified using a limiting dilution. This involves homogenizing the tissue and suspending the homogenate in *Leishmania* culture medium. The homogenate/medium mixture will then undergo a series of dilutions. Each will dilute the sample by a factor of 1:5. From here each dilution will be plated on 96-well plates and allowed to incubate for one to two weeks. Each well on the plates will then be examined under a microscope to determine whether it is positive or negative for the presence of parasites. The proportion of positive to negative wells will then be used to construct a mathematical curve, from which the original number of parasites in the tissue can be estimated[20].

The in vitro study will be used to observe and quantify the effect of different antibody treatments on the uptake of *Leishmania* parasites by macrophages. A flow cytometer will be used; a machine capable of counting, characterizing, and sorting cells. Three test treatments will be used. Cultured macrophages will be combined with either: *L. major* specific antibody, inactivated antibody, or no antibody. The treated macrophages will then be combined will parasites and allowed to incubate. The mixtures will be run through the machine to determine what proportion of macrophages have become infected. A higher proportion of parasite uptake can then be correlated to disease exacerbation *in vivo.*

Timeline

The animal experiment will begin in January 2018 and terminate in April 2018. The *in vitro* study is currently underway and will continue until Spring 2018 at the latest.

Goals/Objectives

The aims of this study can be summarized as follows:

1. To replicate the findings of C. Nation and H. Anderson. That is, to show increased severity of infection in *L. major*- recovered or passively immunized animals after challenge with *L.*

infantum.

2. To use purified antibody to determine whether antibody dependent enhancement is a factor in the observed disease exacerbation through the use of coupled *in vivo* and *in vitro* experiments.

Anticipated Results

I predict that the previous findings of C. Nation and H. Anderson will be supported. I also predict that my results will indicate ADE has a role in causing increased disease severity observed in individuals that are re-exposed to *Leishmania*. Though, I cannot predict to what extent ADE may be at play.

Significance

Should my results be consistent with my predictions, it would be a significant discovery in this field. If antibody dependent enhancement does augment Leishmaniasis, having that knowledge would allow doctors to make more informed decisions with regard to treatment. These potential findings would also be of use for scientists working to develop a *Leishmania* vaccine. Knowledge of ADE would inform vaccine strategists to help determine how and where a potential vaccine should be deployed.

Dissemination

If I am fortunate enough to produce promising results I intend to submit my thesis to a journal for publication. A few journals I have in mind are *The American Journal of Tropical Medicine and Hygeine*, *Journal of Parasitology*, *Parasite*, or perhaps *Infection and Immunity*. Of course there are others but these are top journals and would be among my first choices. Currently, there does not appear to be any external funding for which this project would be eligible. Of course I am and will remain vigilant for funding opportunities.

Reference

1. Centers for Disease Control: Leishmaniasis: Biology. (2013, January 10). Retrieved April 14, 2017, from https://www.cdc.gov/parasites/leishmaniasis/biology.html

2. Centers for Disease Control: Leishmaniasis: Resources for Health Professionals. (2016, December 9). Retrieved April 14, 2017, from https://www.cdc.gov/parasites/leishmaniasis/health_professionals/

3. Nadim, A., Javadian, E., & Mohebali, M. (1997). The experience of leishmanization in the Islamic Republic of Iran. Eastern Mediterranean Health Journal, 3(2).

4. Centers for Disease Control: Leishmaniasis: FAQs. (2013, January 10). Retrieved April 14, 2017, from https://www.cdc.gov/parasites/leishmaniasis/gen_info/faqs.html

5. World Health Organization. Leishmaniasis. (2017). Retrieved February 23, 2017, from http://www.who.int/leishmaniasis/en

6. Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., … Murray, C. J. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. The Lancet, 380(9859), 2095–2128. https://doi.org/10.1016/S0140-6736(12)61728-0

7. Dorlo, T. P. C., Huitema, A. D. R., Beijnen, J. H., & de Vries, P. J. (2012). Optimal Dosing of Miltefosine in Children and Adults with Visceral Leishmaniasis. Antimicrobial Agents and Chemotherapy, 56(7), 3864–3872. http://doi.org/10.1128/AAC.00292-12

8. Pigott, D. M., Bhatt, S., Golding, N., Duda, K. A., Battle, K. E., Brady, O. J., … Hay, S. I. (2014). Global Distribution Maps of the Leishmaniases. eLife, 3, e02851. https://doi.org/10.7554/eLife.02851

9. Centers for Disease Control: Leishmaniasis: Prevention and Control. (2013, January 10). Retrieved April 14, 2017, from https://www.cdc.gov/parasites/leishmaniasis/gen_info/faqs.html

10. Zofou, D., Nyasa, R. B., Nsagha, D. S., Ntie-Kang, F., Meriki, H. D., Assob, J. C. N., & Kuete, V. (2014). Control of malaria and other vector-borne protozoan diseases in the tropics: enduring challenges despite considerable progress and achievements. Infectious Diseases of Poverty, 3, 1. https://doi.org/10.1186/2049-9957-3-1

11. Reyburn, H., Ashford, R., Mohsen, M., Hewitt, S., & Rowland, M. (2000). A randomized controlled trial of insecticide-treated bednets and chaddars or top sheets, and residual spraying of interior rooms for the prevention of cutaneous leishmaniasis in Kabul, Afghanistan. Transactions of the Royal Society of Tropical Medicine and Hygiene, 94(4), 361–366. https://doi.org/10.1016/S0035-9203(00)90104-4

12. Dunning, N. (2009). Leishmania vaccines: from leishmanization to the era of DNA technology. Bioscience Horizons: The International Journal of Student Research, 2(1), 73–82.

https://doi.org/10.1093/biohorizons/hzp004

13. Nation, C. S., Dondji, B., & Stryker, G. A. (2012). Previous exposure to a low infectious dose of Leishmania major exacerbates infection with Leishmania infantum in the susceptible BALB/c mouse. Parasitology Research, 111(3), 1407–1415. https://doi.org/10.1007/s00436-012-2899-5
14. CWU Master's thesis. Heidi Anderson, Dr. Blaise Dodji, Dr. Gabrielle Stryker, Dr. Alison Scoville, 2015

15. Boonnak, K., Dambach, K. M., Donofrio, G. C., Tassaneetrithep, B., & Marovich, M. A. (2011). Cell Type Specificity and Host Genetic Polymorphisms Influence Antibody-Dependent Enhancement of Dengue Virus Infection. Journal of Virology, 85(4), 1671–1683. https://doi.org/10.1128/JVI.00220-10

16. Chaichana, P., Okabayashi, T., Puiprom, O., Sasayama, M., Sasaki, T., Yamashita, A., … Ikuta, K. (2014). Low Levels of Antibody-Dependent Enhancement in Vitro Using Viruses and Plasma from Dengue Patients. PLoS ONE, 9(3). https://doi.org/10.1371/journal.pone.0092173

17. Moi, M. L., Takasaki, T., Saijo, M., & Kurane, I. (2014). Determination of antibody concentration as the main parameter in a dengue virus antibody-dependent enhancement assay using FcγR-expressing BHK cells. Archives of Virology, 159(1), 103–116. https://doi.org/10.1007/s00705-013-1787-3

18. Wang, Y., Si, L., Luo, Y., Guo, X., Zhou, J., Fang, D., … Jiang, L. (2015). Replacement of pr gene with Japanese encephalitis virus pr using reverse genetics reduces antibody-dependent enhancement of dengue virus 2 infection. Applied Microbiology and Biotechnology, 99(22), 9685–9698. https://doi.org/10.1007/s00253-015-6819-3

19. Zellweger, R. M., Eddy, W. E., Tang, W. W., Miller, R., & Shresta, S. (2014). CD8+ T cells prevent antigen-induced antibody-dependent enhancement of dengue disease in mice. Journal of Immunology (Baltimore, Md. : 1950), 193(8), 4117–4124. https://doi.org/10.4049/jimmunol.1401597

20. Titus, R. G., Marchand, M., Boon, T., & Louis, J. A. (1985). A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. *Parasite Immunology*, *7*(5), 545–555.

21. Jensen, M. A., & Van 't Wout, A. B. (2003). Predicting HIV-1 coreceptor usage with sequence analysis. AIDS, 5(2), 104-112. Retrieved April 14, 2017.

22. Laura Waters, Sundhiya Mandalia, Paul Randell, Adrian Wildfire, Brian Gazzard, Graeme Moyle; The Impact of HIV Tropism on Decreases in CD4 Cell Count, Clinical Progression, and Subsequent Response to a First Antiretroviral Therapy Regimen. Clin Infect Dis 2008; 46 (10): 1617-1623. doi: 10.1086/587660

23. Yu, Q., Yu, R., & Qin, X. (2010). The good and evil of complement activation in HIV-1 infection. Cellular & Molecular Immunology, 7(5), 334–340. https://doi.org/10.1038/cmi.2010.8

24. James Whitehorn, Jeremy Farrar; Dengue. Br Med Bull 2010; 95 (1): 161-173. doi: 10.1093/bmb/ldq019

25. Katzelnick, L. C., Gresh, L., Halloran, M. E., Mercado, J. C., Kuan, G., Gordon, A., Harris, E. (2017). Antibody-dependent enhancement of severe dengue disease in humans. *Science*, eaan6836. https://doi.org/10.1126/science.aan6836

26. Dengue: Guidelines for Diagnosis, Treatment, Prevention and Control. (2009). Retrieved April 14, 2017, from http://apps.who.int/iris/bitstream/10665/44188/1/9789241547871_eng.pdf

27. Silva, V. M. G., Larangeira, D. F., Oliveira, P. R. S., Sampaio, R. B., Suzart, P., Biointervention Student Group Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brazil, Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Brazil, Escola Bahiana de Medicina e Saúde Pública, Salvador, Brazil, … Pontes-de-Carvalho, L. (2011). Enhancement of Experimental Cutaneous Leishmaniasis by Leishmania Molecules Is Dependent on Interleukin-4, Serine Protease/Esterase Activity, and Parasite and Host Genetic Backgrounds . Infection and Immunity, 79(3), 1236–1243. http://doi.org/10.1128/IAI.00309-10

28. Silva, V. M. G., de-Araújo, C. F., Navarro, I. C., Oliveira, P. R. S., & Pontes–de-Carvalho, L. (2015). Enhancement of experimental cutaneous leishmaniasis by Leishmania extract: identification of a disease-associated antibody specificity. BMC Research Notes, 8, 197. http://doi.org/10.1186/s13104-015-1158-0

29. Dondji, B., Deak, E., Goldsmith-Pestana, K., Perez-Jimenez, E., Esteban, M., Miyake, S., … McMahon-Pratt, D. (2008). Intradermal NKT cell activation during DNA priming in heterologous prime-boost vaccination enhances T cell responses and protection against Leishmania. European Journal of Immunology, 38(3), 706–719. https://doi.org/10.1002/eji.200737660

MASTER'S STUDENT RESEARCH & CREATIVE ACTIVITIES FELLOWSHIP: SUPPLIES BUDGET TEMPLATE

Applicant's Name: Your Name Here

Project Title: Title of your Research

Budget Justification Describe essential items in detail. If you are requesting equipment, indicate the estimated unit cost for each item to be purchased, and briefly justify the need for each item. If you require materials and supplies, itemize them by nature of expense. Provide the basis for cost estimates or computations (e.g., vendor quotes, photocopies of catalog pages, internet URL of catalog, prior purchase of similar or like items, etc.). The chart below can be used as a template.

Note; See highlighted area below if you are receiving funds from other sources, be sure to list them. The total amount of request from the FDRC cannot exceed $1,000.00

Budget Itemization

Priority	Website	Description	Amount	Actual Cost	Amount Pending/ Received from other source(s)	Amount requested from the FDRC Committee
1	https://www.thermofisher.com/order/catalog/product/89961	Protein G spin colum, 5ml Cat # 89961	1 column	$484.00	$0	$484.00
2	https://geneseesci.com/shop-online/product-details/?product=25-104	96-well non treated plates Cat #: 25-104	Case of 100	$293.40	$0	$293.40
5	https://geneseesci.com/shop-online/product-details/?product=28-101	15ml conical tubes Cat# 28-101	Case of 500	$119.00	$0	$119.00
4	https://geneseesci.com/shop-online/product-details/?product=12-104	10ml serological pipets Cat# 12-104	Case of 200	$403.60	$300.00	$103.60
	• Other Source, Department of Biology contributing $300.00					
		Totals		$1300.00	* - $300.00	$1000.00

Budget justification

Item #

1. A column packed with recombinant protein G agarose. This is used to isolate antibody from blood serum. The serum is inserted into the column and allowed to percolate through it. The protein G binds the antibody while the other serum components pass through. A low pH buffer solution is then used to rinse the antibody from the column, allowing it to be collected. This is a conventional method of antibody collection and is indispensable to my study.

2. 96 well plates will be used in the limiting dilution I will carry out to measure parasite burden. Diluted samples of tissue homogenate are put in the small indentations of this plate. The plate is then examined for parasites and the ratio of indentations that contain parasite versus the ones that do not is used to estimate the number of parasites in the original animal tissues.

3. 15ml conical tubes will be used as containers to prepare the tissue homogenate solutions used for the limiting dilution.

4. 10ml serological pipets are used to measure and dispense liquid aseptically. I will use them to prepare the culture media used to grow the *Leishmania* parasites and the macrophage cells as well as handling infected tissue homogenates.

Attach Your Resume!

Example:

Objective

To obtain a Graduate Student Research Support Award through the CWU School of Graduate Studies.

Education

September 2016 - present Central Washington University Ellensburg, WA

- **Master of Science- Biology/Immunology**

September 2009 - June 2012 Central Washington University Ellensburg, WA

- **Bachelor of Science- Cell and Molecular Biology**
- **Chemistry Minor**
- **3.595 cumulative GPA**
  - Graduated Cum laude

Experience