A. SPECIFIC AIMS

Leishmania are obligate intracellular parasites of mammalian phagocytic cells. They are transmitted to humans and other mammals via the bite of sandflies. Upon inoculation into a mammalian host, parasites are engulfed by phagocytic cells, commonly macrophages. The parasite-containing phagosomes sequentially fuse with the early and late endosomes and finally the lysosomes. *Leishmania* organisms replicate within these acidified compartments, which are referred to as parasitophorous vacuoles (PVs), and are eventually released from infected cells (1,2). Surprisingly, the late events of this infectious cycle remain poorly understood. For many years, release of parasites was thought to occur upon host cell lysis. This model was based on early microscopic studies of *Leishmania* infection, which revealed the presence of disrupted host cell membranes and free parasites in late stages of infection. However, this paradigm was re-examined in a recent study showing that *L. major*-containing vesicles clustered at the plasma membrane and that parasite release occurred gradually, suggesting that parasites may utilize host exocytosis machinery. Host cell lysis was not apparent in this study, although the microscopic images could be consistent with either apoptotic or necrotic cell death (3). In fact, both apoptosis and necrosis may occur to some extent, but the balance may be altered by factors such as cell culture conditions, inoculum size, parasite virulence factors, and host susceptibility. Additional studies will be necessary to clarify the mechanism of *Leishmania* release. Interestingly, while resting macrophages provide a hospitable environment for *Leishmania* growth, activated macrophages produce nitric oxide and rapidly kill parasites. Therefore, in the course of a natural infection, parasite survival may be improved if host cell death occurs by apoptosis, thereby limiting the degree of immune system activation (2). Whether this occurs is not known. To answer this question and better understand the late events of *Leishmania* infection, we propose the following studies:

1. Test the hypothesis that *Leishmania* uses host vesicle-trafficking proteins to exit infected cells.

Leishmania parasites reside within PVs of infected cells. To escape from infected cells, they must cross two membrane barriers: the PV membrane and the host plasma membrane. Engaging host exocytosis machinery would provide an efficient mechanism to exit the cell. To test this, we will use immunofluorescence and confocal microscopy to identify vesicle-trafficking proteins that are associated with the PV. To test their functional requirement, we will express dominant negative constructs for important proteins involved in vesicle trafficking or, alternatively, employ siRNA-mediated knockdown of these proteins. We will also test whether overexpression of these proteins improves parasite release.

2. Test the hypothesis that host cells die by apoptosis during *Leishmania* infection

From previous studies it is unclear whether death of the host cell occurs prior to or following release of parasites. Both may occur in the course of *in vivo* infection. Moreover, it is not known whether cell death occurs primarily by an apoptotic or necrotic pathway. Since necrosis is characterized by an early loss of plasma membrane integrity, we will infect cells and monitor their integrity over the course of infection. We will simultaneously assess cells for markers of apoptosis, such as phosphatidylserine exposure, loss of mitochondrial membrane potential, DNA fragmentation, and caspase activation.

B. BACKGROUND AND SIGNIFICANCE

Leishmania lifecycle:

Leishmania parasites alternate between two morphologically distinct forms: the promastigote and the amastigote. While the flagellated promastigote form is primarily associated with survival in the sandfly gut, it is also the form initially transmitted to mammalian hosts. Promastigotes produce the surface-associated glycolipid lipophosphoglycan (LPG), which promotes uptake

into phagocytic cells (1). Once parasites enter a host cell, the LPG interacts with host factors to delay phagosome-lysosome fusion until the parasite has differentiated into the more acid-resistant amastigote form, demonstrating that parasites are capable of manipulating host vesicle trafficking for their own benefit (4). After fusion occurs, the amastigote forms replicate in the host cell and upon release can infect other target cells.

Interestingly, although LPG mediates promastigote uptake into host cells, amastigotes express little or none of this substance. Therefore, amastigotes must employ alternative strategies for inducing uptake. One such mechanism involves their opsonization by host IgG and uptake by an $F_c\gamma$-mediated interaction. In addition, recent studies with *L. amazonensis* suggest that parasites may express phosphatidylserine on their surface and thus infect cells by mimicking apoptotic bodies (5,6). Besides inducing engulfment of the parasite, this may have the added benefit of inducing TGFβ and IL-10 production, both of which have anti-inflammatory effects on immune cells (7).

Leishmania and disease:

Human infection with *Leishmania* parasites occurs throughout the world. However, different species are responsible for the disease burden in different geographic regions (primarily Old World vs. New World). In addition to their association with a particular geographic region, individual species are frequently associated with a particular type of clinical illness (8). As a whole, members of the *Leishmania* genus produce diverse disease manifestations, but for a given species, these manifestations tend to cluster into one of three main disease patterns. The most common pattern is cutaneous leishmaniasis, which is characterized by one or more persistent skin ulcers. Although in most cases, the host immune response will eventually control the infection, the patient may remain latently infected with the potential for relapse (9). A second pattern known as mucucutaneous leishmaniasis occurs predominantly in South America and produces an irreversible tissue destruction of the nose, mouth and throat. Further destruction can be prevented with drug therapy, but damage incurred prior to treatment is irreversible. The most severe pattern of disease is visceral leishmaniasis, in which the parasites disseminate to the liver, spleen and bone marrow. It occurs more frequently in immunocompromised individuals and is fatal without effective anti-parasitic therapy (10).

Although Leishmanial disease has a worldwide distribution, most of the estimated 1.5-2 million new infections that occur annually affect populations in developing countries (10). In these countries, the number of people affected by Leishmanial disease is increasing for several reasons. First, more people are exposed to the sandfly vectors as a result of development and expansion of populations into formerly rural areas. Secondly, the presence of a large population of immunosuppressed individuals due to the HIV epidemic has increased the likelihood of disease progression. Lastly, *Leishmania* parasites are showing increasing resistance to currently available treatments and consequently relapses are more likely.

Parasite growth in culture:

Parasites of multiple species have been isolated from infected patients and can be propagated in culture. Although these organisms are related and similar in many respects, their ability to cause different patterns of clinical disease is evidence that important differences do exist. Therefore, performing the current studies with multiple parasite species will be important. Established mouse macrophage cell lines such as RAW and J774 provide a relatively easy way to propagate parasites in culture and study large numbers of cells. For studies in a more physiologically relevant context, *Leishmania* parasites can be studied both *in vivo* in infected mice and *in vitro* in primary mouse macrophages. Early studies in the mouse model revealed that some strains of mice (BALB/c) are highly susceptible to persistent *L. major* infection and develop a T_h2-type immune response, while others (C57BL6) mount an efficient pro-

inflammatory T_h1-type immune response and eradicate the parasite. A recent study found that parasites produced in cells from two different strains of mice differed in their ability to express phosphatidylserine and induce anti-inflammatory cytokines (6). Differences in the mechanism of parasite release may also contribute to the differences observed in immune response so it will be important to study these events in both strains.

Exocytosis in macrophages:
In order for vesicles to traffic to their proper destination within the cells, specific interactions must occur between vesicle membrane-associated proteins (v-SNAREs) and target membrane-associated proteins (t-SNAREs). Other proteins, such as the Rab GTPases, facilitate this process. Many of the proteins specifically involved in trafficking of vesicles to the plasma membrane have been identified in macrophages because of their connection to cytokine secretion. For example, vesicle-associated membrane protein 2 (VAMP-2) and 3 (VAMP-3) are important v-SNAREs associated with exocytosis and syntaxins 2, 3, 4 and 7 are important t-SNAREs (11,12,13).

Significance.
Leishmania spp. cause a spectrum of disease, ranging from self-limited skin ulcers to a life-threatening disseminated infection. While leishmanial disease is common in much of the developing world, very few treatments exist. Those that do are frequently toxic to humans. Moreover, parasites are increasingly resistant to the currently available drugs (10). Therefore, a better understanding of parasite biology and the identification of new drug targets are critical goals. The recent availability of sequence from the *L. major* genome will greatly facilitate achievement of this goal, but targeted approaches for discovering critical pathways will still be necessary. A recent study in the organism *Trypanosoma cruzi*, a related intracellular parasite of macrophages, illustrates the potential for the present work to promote the identification of new drug targets. The study revealed that the presence of apoptotic cells enhanced the growth of the parasites. This was shown to be due to modulation of inflammatory signals delivered to macrophages. The growth enhancement observed was blocked by the administration of drugs that interfere with this signaling, such as aspirin. Based on these findings, studying the ability of *Leishmania* to affect cell death and modulate inflammatory signals may allow for similar novel treatment approaches (14).

C. RESEARCH PLAN

Aim 1: Test the hypothesis that *Leishmania* uses host vesicle-trafficking proteins to exit infected cells.

Although release of *Leishmania* parasites from infected cells has traditionally been thought to occur when the infected cells bursts, recent video-microscopy studies of *L. major*-infected macrophages suggest that release actually occurs in a controlled way reminiscent of exocytosis (3). Therefore, previously identified proteins involved in exocytosis will be tested for their ability to promote parasite release.

Experiment 1. Test whether host membrane trafficking proteins localize to PV.
If host vesicle-trafficking proteins play a role in parasite egress from the cell, some of these proteins should localize to the PV membrane. The vesicle-associated proteins VAMP-2 and VAMP-3 represent likely candidates since they facilitate exocytosis in macrophages. For example, VAMP-3 is critical for transporting vesicles containing TNFα to the plasma membrane (12). We will test whether these proteins are present in PV membranes by immunofluorescence and confocal microscopy of infected cells. To do this we will infect mouse macrophages with *L. major* parasites. At regular time points over the course of the infection, we will fix and permeabilize the infected macrophages and perform indirect immunofluorescence labeling of

vesicle-associated proteins VAMP-2 and VAMP-3 using commercially available antibodies against these proteins. Non-specific IgG will be used as a control for antibody specificity. To determine whether these proteins co-localize with the PV, we will simultaneously immunolabel known PV membrane components, such as the lysosomal markers LAMP1 and LAMP2 and the late endosomal marker CD68 (1,15) To identify the parasite, we will immunolabel with serum from infected human patients (9)

Experiment 2. Test whether inhibition of vesicle-trafficking proteins blocks parasite release.
If host proteins are important for release of *Leishmania* parasites, impairment of these factors should inhibit parasite release from infected cells. To test this, we will inhibit vesicle-trafficking proteins and monitor parasite release from infected cells. To monitor parasite release, we will remove extracellular parasites by washing the macrophage culture and then stain and count the number of parasites. To prevent re-infection of other cells in culture, we may need to treat with Annexin V and $F_c\gamma$ to block macrophage recognition of parasites or Aml hydrochloride to block macropinocytosis (6). In addition to monitoring parasite release, intracellular parasite localization will be monitored by phase contrast microscopy (3).

Several methods have been developed to study inhibition of vesicle trafficking proteins. In one approach, we will transfect macrophages with DNA expression constructs encoding dominant-negative SNARE proteins prior to infection with *Leishmania* parasites. These dominant-negative proteins possess intact cytoplasmic domains but lack transmembrane domains. We expect that SNAREs implicated in exocytosis will be associated with parasite release whereas SNAREs associated with other vesicle-trafficking events will not. However, while dominant-negative SNAREs associated with other types of vesicle transport (e.g. early to late endosomes) may not block release, they may impair *Leishmania* infection by blocking an earlier stage of vesicle transport. Therefore, it may be necessary to create constructs that can be induced at points during infection. As an alternative strategy to dominant negative proteins, we will transfect cells with siRNA specific for each membrane trafficking protein prior to infection. Successful protein knockdown will be confirmed by Western blotting. Use of mismatched siRNAs and complementation with siRNA-resistant expression constructs will be important controls for specificity of the knockdown. A third strategy will involve treating infected cells with tetanus or botulinum toxin, which specifically cleave SNAREs (16). With any of these approaches, loss of function in a protein critical for parasite release will result in fewer parasites in the media and more parasites localized to vesicles within the cell.

Experiment 3. Test whether overexpression of vesicle-trafficking proteins enhances release.
If host proteins are important for *Leishmania* release, overexpression of these proteins may increase parasite release. To test this we will transfect macrophages with DNA expression constructs encoding proteins identified as candidates for a role in exocytosis and then quantify parasite release as in the previous experiment. Overexpression of SNAREs not involved in exocytosis are not expected to alter parasite release.

Aim 2: Test the hypothesis that host cells die by apoptosis during *Leishmania* infection
Inducing apoptosis in host cells may be both advantageous and harmful to the parasite, depending on when it occurs in the course of infection. Early in infection, *Leishmania* depend on the host cell to provide nutrients and a safe environment in which to grow and divide. Consequently, several parasites including *Leishmania* spp. possess mechanisms for inhibiting apoptosis. For example, *L. major* blocks release of cytochrome c from the mitochondria by a TNFα- and NFκB-independent mechanism (17). In contrast, induction of apoptosis may be advantageous during the late stages of infection. Whereas *Leishmania* replicate unopposed in resting macrophages, they are rapidly killed by activated macrophages. Since debris from necrotic cells promotes inflammation and macrophage activation, induction of apoptosis may

provide a means to limit immune system activation. In a study of another intracellular macrophage parasite *T. cruzi*, exposure of macrophages to apoptotic cells enhanced parasite growth relative to what was seen in macrophages exposed to necrotic cells (14). The increased growth resulted from elaboration of TGFβ and IL-10 by macrophages in response to engulfment of apoptotic bodies. Similarly, *Leishmania* parasites showed greater infectivity in macrophages exposed to apoptotic cells (5). This finding together with the experiments showing increased infectivity upon *parasite* expression of phosphatidylserine provide two mechanisms by which *Leishmania* limits the immune response and is able to survive in hosts. In the present study, we will address the question of whether *Leishmania*-infected cells undergo apoptosis.

Experiment 1. Evaluate infected cells for markers of apoptosis by flow cytometry.
Two species of *Leishmania* parasites have been shown to inhibit apoptosis, at least early in infection. For this study, we will monitor cells throughout the course of infection and following parasite release. To do this we will harvest infected macrophages at regular time points throughout infection to test for markers of apoptosis. Mock-infected cells will serve as a control. At each time point, the percentage of necrotic cells will be measured as the fraction of cells that lose the ability to exclude propidium iodide dye. The cells will also be stained for markers of apoptosis, including TUNEL staining for DNA fragmentation, Annexin V staining for membrane exposure of phosphatidylserine, cleavage of fluorescently labeled substrates for activated caspases, and DiOC6 staining for loss of mitochondrial membrane potential. By screening a panel of different apoptotic markers, we will determine which, if any, will be most appropriate for future studies. We expect that early in infection, infected cells will express reduced levels of apoptotic markers and at later time points, apoptotic markers will become increasingly prevalent. On the other hand, if cells die primarily by a necrotic mechanism, cells will show increased membrane permeability over time but will not show staining for apoptotic markers.

Experiment 2. Evaluate infected cells for death markers and parasite content.
Using the data generated in the previous experiment, we will further characterize when the markers of cell death occur relative to parasite release from the infected cell. To do this we will fluorescently label parasites with a dye such as CMFDA (15) and isolate infected cells by fluorescence activated cell sorting (FACS). This will be important to ensure that all cells are infected at the beginning of the study. Alternatively, we could infect cells with a higher inoculum, which would increase the likelihood of infection but may also alter results because of the large number of parasites per infected cell. In either case, we will harvest cells at late time points during infection and perform immunolabeling for parasites. Based on the results of the previous experiment, we will also stain for markers of either apoptosis or necrosis. If cells undergo apoptosis, this pathway may be actively induced by the parasite late in infection or it may occur following the removal of anti-apoptotic factors upon parasite release. If the former is true, apoptotic markers and anti-parasite staining should co-exist in the same cell. If the latter is the case, staining for apoptotic markers should appear in cells lacking parasites. On the other hand, if cells were previously found to undergo necrosis, it is possible that loss of membrane integrity either precedes or follows release of the parasite. Analysis of cells dually labeled with parasite antibodies and propidium iodide will help to answer this question.

Experiment 3. Visualize individual cells by immunofluorescence and confocal microscopy.
Initial evidence suggesting that cells did not burst prior to parasite release came from microscopy studies. Here we will build on these earlier studies by staining for membrane integrity and apoptotic markers to more directly assess the status of the infected cell. In this experiment, we will perform confocal microscopy on infected macrophages harvested late in infection and immunolabeled with antibodies specific to parasites and apoptotic markers.

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