Induction of cell-mediated immune responses with effector functions by antigens of Tritrichomonas foetus
by Jovanka Marija (Vujadinovich) Voyich

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Molecular Biology
Montana State University

Abstract:
Bovine trichomoniasis is an abortifacient sexually transmitted disease caused by the parasitic protozoa Tritrichomonas foetus (T. foetus). In this dissertation project, the immune responses of cattle to purified and crude antigens of T. foetus were examined including examination of possible effector mechanisms and mechanisms of pathogenesis to address the following hypothesis. Acquired immunity can be induced in cattle in response to infection and certain antigens of T. foetus. Examination of cell-mediated immune responses induced by parasite antigens should reveal mechanisms of pathogenesis and/or host effector mechanisms of protection.

Immunoaffmity chromatography was used to purify the adhesin Tfl90. Silver stained SDS-PAGE gels showed the presence of two immunogenic subunits of Tfl90 at 140kDa and 60kDa. The presence of carbohydrates and lipids previously shown to be in the LPG of T. foetus were demonstrated. Subcutaneous injections of antigen preparations primed bovine peripheral blood mononuclear cells (PBMC) as demonstrated by antigen specific proliferation and cytokine production upon ex vivo antigen exposure ofPBMC. Antigen-specific T cells derived from PBMC responded by production of IFNγ message and protein. Reactions of antibody from cattle parenterally immunized with Tfl90 revealed antigen specificity and Tf190-sensitization and significant increases in IgG1 and IgG2. Immune sera also significantly inhibited parasite adhesion to mammalian cell lines as compared to pre-immune sera. Intranasal immunization with Tf190 resulted in significant increases in parasite specific IgA in cervical mucus secretions from immunized animals that were more resistant to intravaginal challenge with T. foetus compared to controls.

Mouse infection models revealed innate mechanisms of immunity (PMN and macrophage influx) upon intravaginal exposure to T. foetus and the human trichomonad Trichomonas vaginalis (T vaginalis). Examination of the effects of parasites on macrophages revealed the ability of T foetus and T vaginalis to directly induce TNFa message and protein demonstrating the presence of a known mechanism of pathogenesis that is detrimental to pregnancy outcome. Finally, the ability of activated macrophages to suppress the growth, and in some instances destroy, Tfoetus was demonstrated.

The studies presented here show for the first time antigen specific T cell responses resulting in the production of protective antibody responses. Furthermore, anamnestic IFNy responses in T foetus antigen primed animals and subsequent in vitro evidence of direct TNFa activation support a mechanism for activating macrophages for production of NO, possibly resulting in the observed parasite suppression, and or destruction of parasites,elucidating potential effector pathways of protection and/or pathogenesis.

INDUCTION OF CELL-MEDIATED IMMUNE RESPONSES WITH EFFECTOR FUNCTIONS BY ANTIGENS OF *TRITRICHOMONAS FOETUS*

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This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Bovine trichomoniasis is an abortifacient sexually transmitted disease caused by the parasitic protozoa *Tritrichomonas foetus* (*T. foetus*). In this dissertation project, the immune responses of cattle to purified and crude antigens of *T. foetus* were examined including examination of possible effector mechanisms and mechanisms of pathogenesis to address the following hypothesis. **Acquired immunity can be induced in cattle in response to infection and certain antigens of *T. foetus*. Examination of cell-mediated immune responses induced by parasite antigens should reveal mechanisms of pathogenesis and/or host effector mechanisms of protection.**

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The studies presented here show for the first time antigen specific T cell responses resulting in the production of protective antibody responses. Furthermore, anamnestic IFNγ responses in *T. foetus* antigen primed animals and subsequent *in vitro* evidence of direct TNFα activation support a mechanism for activating macrophages for production of NO, possibly resulting in the observed parasite suppression, and or destruction of parasites, elucidating potential effector pathways of protection and/or pathogenesis.

CHAPTER 1

IMMUNITY AND *TRITRICHOMONAS FOETUS*

Introduction

Parasitic protozoa are a true success from an evolution standpoint. Collectively, they are complex in form having large genomes in comparison to other microbial pathogens like viruses and bacteria. They are very well adapted to their particular hosts, being clever at evading host immune responses with unique physiology and biochemical abilities (1). As a result, protozoa have been difficult to eliminate in humans and in animals.

There are many protozoan pathogens of humans, resulting in such serious diseases as malaria, amoebic dysentery, Chagas disease, African sleeping sickness, and leishmaniasis. Malaria is still the largest killer of mankind. The World Heath Organization estimates 300-500 million new cases/year will surface worldwide (2). Another protozoan *Trichomonas vaginalis* is responsible for trichomoniasis and is one of the most prevalent sexually transmitted diseases in the world with an annual incidence of ~ 170 million cases and an estimated 8 million new cases in the U. S. alone (2,3). More striking, however, is the estimated rate of asymptomatic cases being approximately 50% (3).

Wild and domestic animals also have detrimental protozoan pathogens causing both death and economic loss. Major parasitic protozoa infections in domestic animals include, coccidiosis caused by *Eimeria bovis* and *Eimeria zuernii*, sarcocystosis (*Sarcocystis sp.*) babesiosis (*Babesia bigemina* described by Smith and Kilborne 1893),

and trichomoniasis (*Tritrichomonas foetus*) (4). All of these diseases are capable of causing morbidity and mortality of fetuses. Babesiosis and coccidiosis can also cause death in adult animals.

The first individual to see protozoa was the Dutch microscopist Antony van Leeuwenhoek. In 1674 he discovered *Eimeria stiedai*. Soon after he discovered *Giardia lambia* by examination of his own stools (1). Now, over 300 years later we are only in the beginning stages of understanding immunity to the protozoan species. It is, therefore, very important to pursue research in these areas. The elimination of all microbial pathogens (whether bacteria, viruses, or parasites) requires a general understanding of immune responses induced by antigens of the pathogen. This knowledge can be acquired a number of different ways. It can be studied *in vitro* with cell lines or *in vivo* with animal models of immunization and infection. Regardless of the approach, in order to make advancements in chemotherapy or in vaccine development the immune response must be defined.

Immunity:

The immune system in higher vertebrates is a spectacular display of cells and signaling molecules that harmonize to protect the body from a myriad of foreign invaders (e.g. pathogenic microbes such as parasites, bacteria, and viruses). It is characterized by a highly specific ability to recognize an enormous repertoire of foreign antigens, while maintaining self-tolerance and is subsequently capable of eliminating these foreign

invaders with a diverse collection of potent effector mechanisms. Like the neurological system the acquired immune response also has memory.

It is divided into two basic systems, the innate and the adaptive. Innate immune responses are characterized by their ability to non-specifically recognize pathogens, whereas specificity and memory characterizes the adaptive response (5). Both systems include humoral and cellular components. An important difference, however, is the ability of the adaptive immune response to remember antigens. This ability allows the adaptive immune response to decrease its response time to previously seen pathogens by increased numbers of responding cells capable of increased response kinetics (6). The adaptive immune response is, thus, a delayed response that requires a previous exposure to the antigen. The adaptive immune response has different effector cells, mainly T and B cells (that recognize different forms of the antigen) and the numerous cytokines they secrete. It is the adaptive response which made the concept of vaccines a reality. An individual could be immunized, thus, increasing the kinetics of the response upon re-exposure to the same antigen (7, 8).

The innate immune response lacks the ability to remember antigens and, therefore, has consistent response kinetics with a constant number of precursor cells. Thus, in the past, the innate immune response was defined as the more primitive, less sophisticated branch of the immune system (9). It is true that the adaptive immune response evolved after the innate (~ 400 million years ago), and that the adaptive immune response is found only in cartilaginous and bony fish, amphibians, reptiles, birds, and mammals (10). However, it is now becoming evident that the innate immune system

instructs the adaptive immune response by communicating whether or not the adaptive system is to respond (9). The delivered messages are still somewhat difficult to decode in that the innate immune response sends the initiation signal to the adaptive system but does not always clearly identify the antigen. As a result, it sometimes fails to distinguish self from non-self, infectious from non-infectious, or dangerous from non-dangerous (9, 11, 12). Nevertheless it is now becoming evident that the innate immune response is an integral part of adaptive immunity.

Innate Immunity:

The first line of defense against microbial invasion is the innate immune system characterized by its ability to recognize antigens rapidly and without prior exposure (13). Effectors of the innate immune system include epithelial cells, serum proteins, cytokines, and leukocytes. Host resistance to pathogen invasion begins with physical barriers such as skin and mucosal membranes. Often the pathogen is initially denied access to the host making colonization impossible. If the physical barriers are penetrated the pathogen is then confronted with other elements of the innate immune system that include macrophages, natural killer cells, granulocytes, and complement.

Complement, first described by Jules Bordet in 1900, is a surveillance system made up of more than 30 membrane or plasma proteins capable of directly lysing microbes. It is the primary anti-microbial effector (9). It is also intimately involved in recruiting the cellular effectors of the innate immune system. For example, the coating of

microbes with the opsonnic complement component C3b allows for efficient uptake by phagocytic cells such as macrophages and polymorphonuculear cells (PMNs).

Elie Metchnikoff was the first to identify the process of phagocytosis (14). Metchnikoff correctly proposed that phagocytes were involved in the first line of defense. This observation made in 1884 was elemental in launching the field of immunology in particular the notion of cell-mediated immunity, and establishing the importance of phagocytosis in immunity.

One of the primary phagocytic cells is the neutrophil, the most abundant leukocyte in the blood of most mammals. Neutrophils are often referred to as professional phagocytes designed to quickly and efficiently respond to microbial antigens and are very much a part of the inflammatory process (15). They are highly influenced by chemoattractants and are equipped with adhesion molecules that allow for surveillance, adherence and transmigration through vascular endothelium into tissue sites of infection and inflammation (13, 16). Neutrophils are activated by a number of agents including endotoxin, interleukin-1 f-Met-Leu-Phe, and inflammatory cytokines such as TNFα (13, 17). Activation of neutrophils results in degranulation generating such effectors as lysozyme, cathepsin G, defensins, lactoferrin, hydrochloric acid and the production of toxic oxygen metabolites such as hydrogen peroxide. Unfortunately, the products of effectors such as oxygen radicals can easily go beyond what is necessary to contain an infection and the end result can be chronic inflammation and severe tissue damage.

Macrophages are key members of the innate immune system, having both microbicidal abilities and regulatory functions, thus, establishing homeostasis within the tissues (13). They are instrumental in growth, differentiation, and death of other cell types. Macrophages are potent effectors of the innate immune system, but are also key to T cell- mediated inflammatory responses (18). Some of their roles in immunity include the production of potent anti-microbial agents, functioning as antigen presenting cells (APC), presenting antigen to T cells, and destroying pathogens by phagocytosis. One of the most potent anti-microbial agents produced by an activated macrophage is nitric oxide (NO) (19). However, macrophages also produce other cytostatic/cytotoxic agents such as hydrogen peroxide and superoxide anion (19). Microbial, cellular, or soluble signaling proteins (broadly termed cytokines) can activate macrophages (13, 20). Macrophages secrete cytokines and are under the influence of several cytokines. An activated macrophage can secrete interleukin (IL) IL-12, IL-18, IL-6, IL-10, IL-1β, tumor necrosis factor α (TNFα), TNFβ, and transforming growth factor β (TGFβ). Collectively, the ability to produce and respond to cytokines places macrophages in a key position to interact and instruct the adaptive immune response. Thus, although macrophages are equipped to eliminate pathogens they can also damage tissues when present chronically or when they respond in excess of that necessary for pathogen elimination (19).

Adaptive Immunity:

Although the concept of immunology was described during the plague of Athens in 430 B. C., theories of acquired immunity appeared only in the last millennium (13). Tomio Tada defined the immune system as a super-system capable of self-regulation through self-organization (21). It begins a complex self-regulation from a single progenitor (22) giving rise to a complex super-system through a stochastic process of selection and adaptation. In the process the system generates individualism and independence to interpret external and internal stimuli *via* its own established behaviors. In short it is a fabulous illustration of a highly integrated life system, which we are only beginning to understand.

The adaptive immune response can be divided into two major categories of immunity. Humoral immunity is defined by secreted antibodies, immunoglobulins (Ig), produced by B-lymphocyte (B cells) that differentiate into plasma cells. Cell mediated immunity is defined by cell-cell interactions, led by T-lymphocytes (T cells) (23).

B cells originate in the bone marrow in adults and in blood islands, placenta, and liver in the fetus. B cells have an antigen-independent stage and an antigen-dependent stage of development. The antigen-independent stage of development generates a single IgM as its B cell receptor (BCR) for antigen (24). Ig gene rearrangement generates an enormous repertoire of B cells with unique BCR (25). It is estimated that the average individual generates approximately 10^9 different BCR by gene recombination. Encounter with antigen, coupled with secondary signals, results in division and isotype switching of the B cell. There are 5 major classes of immunoglobulins IgD, IgM, IgG, IgA, and IgE. The different classes offer different effector functions. For example, IgE causes mast cell

degranulation, while subclasses of IgG (in human IgG1, IgG2, and IgG3) along with IgM are most efficient at binding complement (23, 13). Clonal expansion of the terminally differentiated B cell derived plasma cell insures that the antigen specificity of the original B cell is maintained and produced in abundance (26). Plasma cells can secrete immunoglobulin at a phenomenal rate (10,000-20,000 molecules/minute).

T cells activate B cells most efficiently, however, they can be activated independent of T cells by direct receptor crosslinking (27). Many of the T cell-independent antigens contain repetitious structures, such as polysaccharides. Lipopolysaccharide (LPS) is an excellent example of a T cell-independent antigen. The resulting anti-LPS antibodies are usually IgM and are of limited specificity. Cooperation with T cells via contact and cytokine secretion results in effective isotype class switching and affinity maturation. Antigens often require cooperation between B and T cells in order to produce the type of antigen-specific responses that have effector functions. For example T cells produce interleukin-4 (IL-4) which is the most potent signaling cytokine in B cell switching to IgE and in signaling to non-complement fixing antibodies of the IgG subclass (IgG1 in mice and cattle, IgG4 in humans).

T cells get their name from the thymus, the organ where they undergo differentiation. T cells belong to two basic lineages based on the cell surface receptors they express either $\alpha\beta$ or $\gamma\delta$. The different subsets of T cells correspond to different effector functions. The $\alpha\beta$ T cells can be further divided into $CD8^+$ cytotoxic T-cells, $CD4^+$ inflammatory cells, and $CD4^+$ helper T cells (13). A small percentage of $\gamma\delta$ T cells can be $CD8^+$, however, the overwhelming majority in cattle are $CD4^-$, and $CD8^-$ (28). T

cells do not recognize antigen in its native form and instead recognize peptide fragments presented by antigen presenting cells in association with major histocompatability complexes I and II (MHC I and MHC II). There is evidence that γδ T cells can recognize antigen without MHC I or MHC II presentation and may use non-classical means of antigen presentation such as CD1 molecules (29). Additionally, there is evidence suggesting that γδ T cells can recognize non-peptide antigens such as lipids and glycoproteins (29).

Upon exiting the thymus, naïve $CD8^+$ T cells are destined to become cytotoxic T cells that destroy infected cells (often virus infected cells) by mechanisms involving perforin and granzymes or Fas/FasL mediated apoptosis (23). However, the effector functions of $CD4^+$ T cells are not predetermined upon emergence from the thymus; rather these functions are influenced by several different factors during downstream development. Mature $CD4^+$ T cells are usually classified into two major subsets termed T-helper 1 (Th1) or T-helper 2 (Th2) and, although not often identified, a third subset T-helper 3 has been defined (30, 31, 32). The initial classification of Th1 and Th2 $CD4^+$ cells was defined by Mosman et al (30) in 1986 using murine $CD4^+$ T cell clones. The $CD4^+$ T cells were subdivided into populations based on their particular cytokine profiles and were termed Th1 or Th2. Initially, Th1 cells were classified by their production of interleukin-2 (IL-2), and the inflammatory cytokines interferon γ (IFNγ) and lymphotoxin (TNFβ). The hallmark Th1 cytokine, IFNγ, regulates cell-mediated immunity, primes macrophages for activation, and aids in isotype switching of B cells to opsonic and

complement fixing IgG antibodies (such as IgG2a in mice and cattle) (31, 33, 34, 35). The defining effector function of a Th1 response is delayed type hypersensitivity (30).

Likewise, Th2 cells are also defined by the production of specific interleukins (IL-4, 5, 6, 10, 13). IL-4 is identified as the hallmark Th2 cytokine (30, 31, 35). The Th2 subset is defined by its influence on humoral immunity. It is the Th2 subset that greatly increases the switching of B lymphocytes to IgE, and non-complement fixing IgG isotypes (IgG1 in mice). The more recently described Th3 subset is characterized by the production of transforming growth factor β (TGFβ). TGFβ regulates control of cell growth and differentiation along with regulation of cell functions and target gene activities (32). The Th1/Th2 paradigm created a unique way of classifying immune responses by measurable factors (cytokines), thus, opening up the means of predicting the effector functions of a particular immune response. However, it soon became evident that the dichotomy between the two systems was not as clear as originally predicted. Furthermore, since the subsets were originally defined in mice, it became evident that although a similar pattern existed in humans and in other species like cattle, they were not so restrictive. For example, it is now evident that IL-10, originally a Th2 classified cytokine, is also produced by Th1 populations (36, 37, 38). It is also important to recall that the polar responses, i.e. Th1 *versus* Th2, were observed in chronically infected or chronically/hyper-immunized animals (31, 39, 40). Therefore, under natural conditions of infection or immunization the classification of Th responses may be more heterogeneous.

A controversial subset termed Th0 has been defined as T helper cells (clones of human and mouse cells) that produce both Th1 and Th2 cytokines (34, 39, 40, 41). The Th0 population may be precursors to Th1 and Th2 populations or may in fact represent a different subset of helper T cells that balance cell mediated immunity with humoral immunity. In addition, it was also discovered that the decision by $CD4^+$ T cells to become Th1 *versus* Th2 cells is not solely dependent on cytokine environment (although it seems to have the largest influence). It is also influenced by: (1) nature of antigen, (2) mode of antigen entry into host, (3) tissue distribution of antigen once inside host, (4) number of epitopes or ligand density, and (5) uptake by APC (42).

Nonetheless, the Th1/Th2 paradigm does offer a means of predicting effector responses by correlating expression of cytokines with immune responses. For example, IFNγ has strong regulatory effects on the production of IL-4, and since the reverse is true, the strong Th1 or Th2 responses do exist. Thus, identification of the canonical helper cytokine, i. e. IFNγ or IL-4, can predict rather distinct effector response pathways. For example, IFNγ can regulate macrophage activation, therefore, increasing the likelihood of TNFα and NO production. It can increase expression of MHC II, increase production of opsonic IgG antibodies, influence the activation of $CD8^+$ T cells and down regulate production of IL-4. In contrast, IL-4 is a potent regulator of antibody production, as it promotes B-cell switching to IgE and assists B cells in the production of IgM and non-complement fixing antibodies. IL-4 has regulatory influence on IFNγ (34, 43, 44). For example, an increase in IL-4 corresponds to a decrease in IFNγ production (34, 37).

The regulation of the T-helper subsets is not simply controlled by just the presence of the seminal cytokines. Instead, it is now evident that there are cytokines that strongly influence the production of IFNγ and IL-4, respectively. It is now becoming evident that there is a rich milieu of signaling and transcription that control the T cell developmental switch. In the last few years much progress has been made on the level of understanding of transcriptional control of cytokine expression. It seems that many of the cytokine receptors are associated with the Janus kinase family of tyrosine kinases (Jaks) (35). When the Jaks are activated via phosphorylation they in turn phosphorylate specific tyrosine residues on the cytokine receptor. The tyrosine residues are reservoirs for latent transcription factors called signal transducers and activators of transcription (STATs). After further phosphorylation and dimerization, STAT eventually is translocated to the nucleus. Proximal promoters of cytokine-inducible genes are then bound by STAT, thus, activating gene transcription. The specificity of STATs has to do with the order of kinase phosphorylation (45, 46). There are, thus, unique regulations of gene transcriptions. For example, IFNγ is under the transcriptional regulation of STAT 1, and STAT 1 and STAT 4 control production of a potent Th1 inducing cytokine IL-12. IL-4 is under the transcriptional regulation of STAT 6, and mice with STAT 6 gene knockouts do not make a Th2 response (35). Obviously, there are still some mysteries as to how certain cytokines are turned on or off or simply maintained. For example, IL-10 blocks the production of IL-12 but how is not yet known. No gene promotors from IL-10 can be found that inhibit IL-12 production. It is, therefore, hypothesized that IL-10 activates a process that accelerates the degradation of IL-12 (35).

The innate and adaptive immune responses are inherently linked. It is the two systems working together that most effectively eradicates a pathogen from its host. For example, *Listeria monocytogenes* is a bacterium that infects macrophages. Macrophages in turn produce cytokines, such as, IL-1, IL-12, TNF, and IL-15. In response to these cytokines natural killer (NK) cells are recruited. NK cells can then produce IFNγ that primes macrophages for activation, killing *Listeria* and allowing *Listeria* peptides to be presented to T cells. T cells under the influence of the cytokine environment generate the long-lived memory T cells that ultimately provide the protection against reinfection.

Tritrichomonas foetus:

Tritrichomonas foetus (*T. foetus*) is a sexually transmitted parasitic protozoan of cattle that is cosmopolitan in its distribution (47). It is a primitive eukaryote and a member of the family Trichomonadidae (described by Wenyon, 1926), whose members are characterized by 3-5 free flagella, a cytosome, an undulating membrane (with one flagella on the margin), and an axostyle extending through the posterior of the cell. The first described species of trichomonad was *T. tenax* (Levine). It was isolated from a human mouth by O.F. Muller in 1773 (he called it *Cercaria tenax*). In 1837, Donne isolated *Trichomonas vaginalis* (*T. vaginalis*) from the human vagina, and in 1854 Davaine found *T. vaginalis* in the stools of cholera patients. In 1928, Riedmuller discovered *T. foetus* (1, 4).

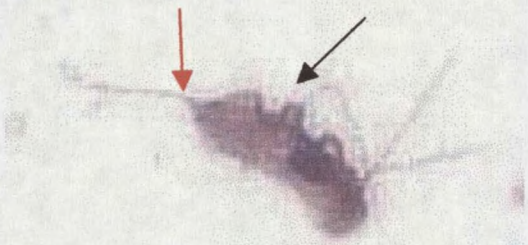
Morphologic studies of *T. foetus* were first done in 1933 by Wenrich and Emmerson, and again by Kirby in 1951 (4, 48). *T. foetus* is typically pyriform in shape and has 3 anterior flagella and one recurrent flagellum (Fig. 1). The flagella along with the undulating membrane and the supporting costa originate at the anterior portion of the parasite in the kinetosomal complex. *T. foetus* has a simple life-cycle with one morphologic form, the trophozoite, which is transmitted only through breeding.

T. foetus is an aerotolerant anaerobe that prefers anaerobic conditions, but can survive aerobic conditions for a limited time. (4) Therefore, it can be cultured axenically without elimination of oxygen, provided the media contains peptones, yeast extracts, serum, maltose and/or dextrose (47). Such media include modified Diamond's (49), CPLM (described by Johnson and Trussel 1943, (4), CTLM (50), and NIH and Brewer thyioglycolate broths (51).

T. foetus is quite interesting biochemically. It cannot synthesize its own purines or pyrimidines and, therefore, relies on salvage enzymes in particular hypoxanthine – guanine phosphoribosyltransferase (described by Wang, (4). *T. foetus* poorly metabolizes lipids and seems to be virtually dependent on environmental sources (52). Protein metabolism is poorly understood, but the organism has an abundance of proteases, (primarily cysteine proteases) that may actually play a role in pathogenesis. It also lacks mitochondria, mitochondria cytochromes, or a functioning tricarboxylic acid (Krebs) cycle. Respiration in the presence of oxygen instead occurs by ferredoxin-mediated electron transport. Meanwhile, oxidative degradation of carbohydrates (21 different kinds are utilized (53), occurs primarily in organelles called hydrogenosomes.

Hydrogenosomes contain several enzymes that metabolize carbohydrates, including malate dehydrogenase, hydrogenase succinate thiokinase, adenylate kinase and pyruvate:ferredoxin 2-oxidoreductase. These enzymes operate to produce acetate from pyruvate, although the Embden-Meyerhoff pathway generates the pyruvate initially.

A.



B.

Fig 1: Micrographs of *Tritrichomonas foetus* stained with Giemsa's stain. Note the 3 anterior flagella and pyriform shape of the parasite. (A.) Arrows are showing the undulating membrane and recurrent flagellum (black arrow), and the axostyle (red arrow). (B). A parasite has attached to a murine vaginal epithelial cell.