The Entry of *Theileria parva* Sporozoites into Bovine Lymphocytes: Evidence for MHC Class I Involvement

Michael K. Shaw, Lewis G. Tilney, and Anthony J. Musoke

International Laboratory for Research on Animal Diseases, Nairobi, Kenya

Abstract. We have examined the process of *Theileria parva* sporozoite entry into susceptible bovine lymphocytes and have begun to identify one of the possible molecular interactions involved in the process. The entry process involves a defined series of events and we have used a number of experimental procedures in combination with a method of quantitation to examine various aspects of this process. *T. parva* sporozoites are nonmotile organisms and the initial sporozoite–lymphocyte interaction is a chance event which can occur at 0–2°C. All subsequent stages in the process are temperature dependent, require the participation of live intact sporozoites and host cells, and involve some cytochalasin-inhibitable rearrangement of the host cell surface membrane or cytoskeleton. Sporozoite entry can be inhibited by antibodies (mAbs) reactive with major histocompatibility complex (MHC) class I mol-

ecules (IL-A 19, IL-A 88) and with beta 2 microglobulin (B1G6), whereas mAbs reactive with MHC class II molecules (IL-A 21, J 11), and a common panleucocyte surface antigen, (IL-A 87; a bovine equivalent of CD 11a) have no effect. These results indicate that MHC class I molecules play a role in the process of *T. parva* sporozoite entry into bovine lymphocytes although as yet the precise role has not been determined.

Once internalized within the lymphocyte, a process that takes <3 min at 37°C, the sporozoite rapidly escapes from the encapsulating host cell membrane; a process which occurs concurrently with the discharge of the contents of the sporozoite rhoptries and microspheres. The intracytoplasmic parasite is covered by a layer of sporozoite-derived fuzzy material to which host cell microtubules rapidly become associated.

I NTRACELLULAR parasites ranging from viruses and bacteria to protozoa have evolved an enormous range of intricate and elaborate mechanisms to allow them to enter, in many cases selectively, and become established within host cells. Not only have these organisms evolved a variety of methods of attachment to and entry into their host cells, but they also employ a wide range of mechanisms to survive within an often extremely hostile intracellular environment (e.g., *Leishmania* in macrophages).

In general, intracellular parasites can be separated into two groups, depending upon whether the parasites remain enclosed within a membrane of host origin throughout their intracellular existence (e.g., *Toxoplasma gondii*; *Plasmodium*; *Leishmania*) or escape from any encapsulating membrane(s) and exist free within the host cell cytoplasm (e.g., *Theileria*) (Moulder, 1985). In all cases obligate intracellular parasites depend upon the maintenance of the integrity of the host cell to survive, whilst at the same time using the host cell to obtain many, if not all, of their metabolic requirements. In many cases these intracellular organisms alter the metabolic and/or growth characteristics of their host cells (Trager, 1986; Sherman, 1988), changes which often lead

ultimately to the death of the host cell. Studies on the biology of such intracellular organisms are important not only for what they can tell us about the biology of the host cell, but also because such studies can lead to better methods of controlling those organisms which cause disease in both man and his domestic animals.

The *Theileria* are tick-borne, protozoan parasites that infect both wild and domestic animals throughout much of the world (see Dolan, 1989). Of particular interest to us is *Theileria parva*, which causes an acute and often fatal disease in cattle known as East Coast fever; a disease of great economic importance throughout East and Central Africa. The infective form of the parasite, the sporozoite, develops within the tick salivary gland and is transmitted to the bovine host when the tick, *Rhipicephalus appendiculatus*, feeds. The sporozoite enters a subpopulation of bovine T and B lymphocytes (Baldwin et al., 1988a; Morrison et al., 1989) where it differentiates into the multinucleate schizont stage, and in the process induces and subsequently maintains host cell blastogenesis leading to a rapid clonal expansion of parasitized cells. The fact that *T. parva* sporozoites invade and develop only within a subpopulation of T and B lymphocytes suggests that a specific interaction(s) between the sporozoite and host cell mediates invasion, although the molecular basis of this interaction(s) is not understood.

The processes involved in sporozoite recognition, attachment, and entry of susceptible host lymphocytes are not only intrinsically interesting but are of great practical importance from the standpoint of controlling the disease by vaccination against the sporozoite. Previous studies (Fawcett et al., 1982; Fawcett and Stagg, 1986) have reported that *T. parva* sporozoites are able to invade bovine lymphocytes in vitro in an energy-independent manner; a process they termed "passive endocytosis" (Fawcett et al., 1982). In contrast, Jura (1984) showed that invasion of bovine lymphocytes by the closely related parasite, *T. annulata*, was an active, temperature-dependent process. In view of these conflicting reports, the purpose of the present study was to reexamine the process of *T. parva* sporozoite entry as a basis for continuing work on the molecular mechanisms of recognition and entry of this intracytoplasmic parasite into its host cell. In particular, the present study has shown that *T. parva* sporozoite entry occurs in a sequential series of discrete stages; that the process is energy dependent and requires the participation of live intact sporozoites and host cells; that the process involves cytochalasin-inhibitable changes in the lymphocyte surface membrane and cytoskeleton; and that bovine major histocompatibility complex (MHC)[1] class I molecules are involved in the process.

Materials and Methods

Parasites and Cells

T. parva (Muguga stock) and the tick, *Rhipicephalus appendiculatus* maintained in a laboratory at ILRAD, were used in these studies. Ticks were infected as nymphs by feeding them on experimentally infected cattle when the erythrocyte piroplasm counts were high. Engorged nymphs were maintained at 23–25°C and 80% relative humidity and allowed to moult to the adult stage.

T. parva sporozoite suspensions were prepared by grinding up salivary glands dissected from *T. parva*–infected adult *R. appendiculatus* that had fed on a rabbit for 4 d (see Brown, 1987). Debris was removed by centrifugation (1,500 g for 2 min) and the supernatant containing the sporozoites saved.

Bovine peripheral blood lymphocytes (PBLs) were obtained from the blood of uninfected cattle; the blood was defibrinated with glass beads as described previously (Lalor et al., 1986) and PBLs isolated on Ficoll-Hypaque (Pharmacia Fine Chemicals, Sweden) gradients. The PBLs were washed three times in Alsever's solution and resuspended in RPMI 1640 culture medium with 10 mM Hepes buffer supplemented with 10% heat-inactivated FBS. (Gibco Laboratories, Paisley, Scotland).

Infection of Cells with T. parva Sporozoites

Bovine PBLs were infected in vitro with *T. parva* by incubation with sporozoites derived from salivary glands of infected adult ticks (Brown, 1987).

In an attempt to standardize the infection procedure, 250–300 μl of sporozoite suspension (2,000 infected salivary gland acini/ml) were added to 200 μl of PBL suspension (5–7.5 × 10⁶ cells/ml) in a 1.5-ml Eppendorf tube (Brinkman Instruments, Inc., Westbury, NY) and the mixture incubated at 37°C for either 30 min or for various times as described in the text. To maximize sporozoite-PBL contact during the incubation period, the samples were mixed regularly every 2–3 min by inverting the tubes several times.

Effect of Temperature

In an initial series of experiments, sporozoites and PBLs were incubated at 37°C for 3, 15, and 30 min before fixing in suspension and processing for

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1. *Abbreviations used in this paper*: MHC, major histocompatibility complex; PBLs, peripheral blood lymphocytes.

EM. In a second set of experiments we attempted to verify the observation of Fawcett et al. (1982) that *T. parva* sporozoites could invade PBLs at 0–2°C. Sporozoites and PBLs, precooled on ice [0–2°C], were mixed together and incubated on ice for 60 or 90 min. After incubation the sporozoite-PBL mixtures were either fixed in suspension at 0–2°C with ice-cold fixative, or as Fawcett et al. (1982) had done, centrifuged for 3 min in an Eppendorf centrifuge (model 5414; Brinkman Instruments Inc.) before fixing on ice with ice-cold fixative.

Protease Inhibitors

To investigate the effects of protease inhibitors on sporozoite entry, either a mixture containing antipain, leupeptin, pepstatin A and chymostatin (Sigma Chemical Co., Poole, England) each at a final concentration of 50 μg/ml (from a stock mixture of 25 mg of each in 5 ml DMSO), or PMSF (Sigma Chemical Co.) at a final concentration of 5 or 10 mM (from a stock of 100 mg/ml in DMSO) was added to the sporozoite suspension immediately before the addition of PBLs. The sporozoite-PBL mixtures were incubated with the protease inhibitors for 30 min at 37°C and the samples fixed in suspension and processed for EM.

Experiments to Investigate the Requirement for Live, Intact Sporozoites and PBLs for Successful Parasite Invasion

Sporozoite and PBL Ghosts. Sporozoite ghosts were prepared by incubating sporozoites in an equal volume of distilled water for 5 or 10 min at room temperature (22°C). These ghosts were then added to PBLs in culture medium and the combination incubated for 30 min at 37°C.

PBL ghosts were prepared by incubating PBLs with 0.5 mg/ml saponin (Sigma Chemical Co.) for 5–10 min at room temperature (22°C). The PBL ghosts were gently pelleted, washed twice in culture medium to remove the saponin, resuspended in sporozoite suspension, and then incubated for 30 min at 37°C.

Irradiated Sporozoites. Infected tick salivary glands were irradiated at 100 krads with a cesium source and sporozoite suspensions prepared as described above, incubated with PBLs for 30 min at 37°C and processed for EM. Sporozoites irradiated at 64 krad or greater consistently fail to infect and transform bovine PBLs in vitro (M. Butera, personal communication) and can, therefore be considered "dead" or nonviable.

Sodium Azide. Initially sporozoites and PBLs were incubated for 30 min at 37°C in the presence of 65 mM (0.1%) sodium azide. Subsequently, to determine whether sodium azide was preferentially affecting either the sporozoite or PBLs, sporozoites or PBLs were preincubated with 10 mM sodium azide for 5 min at 37°C, pelleted, washed rapidly twice in culture medium, and then resuspended in the presence of either PBLs or sporozoites, respectively. The mixtures were incubated for 30 min at 37°C before fixing and processing for EM.

Cytochalasin

Cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) and cytochalasin D (Calbiochem-Behring Corp., San Diego, CA) were dissolved in DMSO. PBLs were incubated in either, cytochalasin B at 10 μg/ml for 15 min at 37°C, or in cytochalasin D at 0.5, 5, 10, or 20 μg/ml for 60 min at 37°C after which sporozoite suspension was added to the cells still in the presence of cytochalasin, and the mixtures incubated for 30 min at 37°C before fixation and processing for EM. As controls, PBLs were preincubated with DMSO for 60 min at 37°C before incubation with sporozoites for 30 min at 37°C.

To examine the effect of cytochalasin D on sporozoites, sporozoites were incubated with 5 μg/ml cytochalasin D for 15 min at 37°C, pelleted, washed twice in culture medium, and resuspended in the presence of PBLs. The mixture was incubated at 37°C for 30 min before fixing and processing for EM.

Experiments to Investigate the Regulation of Rhoptry and Microsphere Discharge

Ionophore A 23187. Free sporozoites isolated in RPMI-HEPES culture medium containing 2 mM CaCl₂ were incubated with 20 mM A 23187 (Calbiochem-Behring Corp., San Diego, CA) (from a stock solution of 1 mg/ml in DMSO) for 5 min at 37°C before fixing and processing for EM.

mAbs

mAbs IL-A 19 (isotype IgG2a) and IL-A 88 (isotype IgG2a) both react with

nonpolymorphic determinants of bovine MHC class I molecules (Bensaid et al., 1989; Toye et al., 1990). mAbs W6/32 (isotype IgG2a) and BIG6 (isotype IgG2b) which recognize epitopes on all HLA-A, B, and C heavy chains, and human beta-2-microglobulin, respectively, are also reactive with different monomorphic determinants on bovine MHC class I molecules (Bensaid et al., 1988, 1989).

mAbs IL-A 21 (isotype IgG2a) and J 11 (isotype IgG1) both react with nonpolymorphic determinants of bovine MHC class II molecules (Baldwin et al., 1988b).

mAb IL-A 87 (isotype IgG2a) is a bovine equivalent of CD 11a and reacts with a pan-leukocyte cell surface antigen that occurs on the cell surface in similar numbers to MHC class I molecules (Splitter and Morrison, 1991).

In each case, the mAbs were used either as ascites fluid or as tissue culture supernatant.

In all experiments the individual mAb was added, at a 1/20 or 1/50 dilution, to 200 μl of PBLs and the mixture incubated at room temperature (22°C) for 5 min before the addition of 300 μl of sporozoite suspension. The samples were incubated for 30 min at 37°C and then fixed and processed for EM. For each mAb or set of mAbs tested a control mixture of sporozoites and PBLs was incubated and processed at the same time.

EM

After incubation under the various conditions, the sporozoite-PBL mixtures were fixed in suspension by the addition of an equal volume of either a freshly prepared solution containing 1% glutaraldehyde (from an 8% stock supplied by Electron Microscope Sciences, Fort Washington, PA.), 1% OsO_4 and 0.05 M phosphate buffer (pH 6.3), or in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 6.3). In the case of the first fixative, fixation was carried out on ice (0–2°C) for 40–45 min after which the samples were pelleted, washed several times in distilled water to remove excess phosphate and then en bloc stained with aqueous 0.5% uranyl acetate for 6–16 h. Glutaraldehyde-fixed samples were fixed at room temperature (22°C) for 30–60 min, pelleted, post-fixed in 1% OsO_4 in 0.05 M phosphate buffer (pH 6.3) for 30 min, washed, and en bloc stained with uranyl acetate as described above.

To examine the contents of the rhoptries samples of sporozoites and PBLs incubated for either 15 or 30 min at 37°C were also fixed for 2 h in freshly made 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2.0% tannic acid (Mallinckrodt Science Products Inc., St. Louis, MO) and 0.5 mg/ml saponin. The fixed pellets were washed in several changes of 0.2 M cacodylate buffer and then post-fixed in cacodylate buffered, 1.0% OsO_4, washed in distilled water, and en bloc stained with aqueous 0.5% uranyl acetate for 6–16 h.

The samples were dehydrated with either ethanol or acetone and embedded in an Epon–Araldite mixture.

Ultrathin sections (50–70-nm thickness) were collected on uncoated copper grids, double stained with aqueous uranyl acetate and lead citrate, and examined in an electron microscope (model EM IOA; Zeiss, Oberkochen, Germany).

Quantitation

In all our experiments a large excess of sporozoites was added to the PBLs and thus in a 30-min incubation period many sporozoites would bind to and enter susceptible lymphocytes. In fact, in a single thin section through an infected lymphocyte between 1–6, and occasionally 10 or more, sporozoites were commonly seen either attached to or internalized within the cell.

To quantify the effects of the various experimental treatments on the process of sporozoite entry we calculated the infection rates or infection index for each individual experiment. The infection index is defined as the percentage of PBLs showing either surface-bound or fully internalized sporozoite profiles in one section. To determine the infection index, we cut a thin section through each pellet of infected cells and counted the number of infected PBLs (that is those cells with sporozoites either bound to or fully internalized within the cell) per 300–500 cells included in that section. To confirm that this method gave reliable and reproducible results, we cut sections from the top, through the center and from the bottom of a single pellet. The infection index for the three regions of pellet were 31.8%, 34.3%, and 32.3%, respectively, (average 32.8% ± 1.3%). However, whilst the infection index was similar throughout the pellet, it is probable that the values obtained represent an underestimation of the actual or true infection levels as cells in one particular section showing no evidence of infection may actually be infected on adjacent ones.

Since our experiments were carried out using sporozoites from different batches of infected ticks, and preparations of PBLs from several cattle col-

lected over a 6-mo period we also compared the percentage infection from different tick batches and different cattle. The infection rates ranged between 26.9–39.0% (average 32.6 ± 3.9%), which is very similar to the range of percentage susceptible cells reported from numerous other studies using different techniques. From all our control samples, we therefore feel confident that our method of counting can be taken as a reasonable measure of the effect of the various treatments on the process of sporozoite invasion. Nevertheless we always ran control incubations with each experimental group.

For all the various experimental treatments used both the percentage infection index and the proportion of sporozoites fully internalized as a percentage of the total number of cell-associated sporozoites (i.e., bound and entered) was calculated for each individual experiment.

Results

Structure of T. parva Sporozoites

The ultrastructure of the sporozoites of T. parva has been described previously by Fawcett et al. (1982) and, with one major difference, conforms to the general descriptions of zoites for this and other genera of the Apicomplexa (Scholtyseck, 1979; Mitchell and Bannister, 1988). However, in the context of the present study, one difference from the description of Fawcett et al. (1982) in relation to the nomenclature and, by connotation, function, of one of the secretory organelles is worth emphasizing.

In the Apicomplexa at least three distinct types of putative secretory organelles have been described from the various invasive stages; namely rhoptries, micronemes, and electron-dense granules or microspheres. In general, the rhoptries and micronemes occur closely associated within the apical region of the zoite and appear, collectively, to play a major role(s) in the actual process of host cell invasion. In contrast, the electron dense granules or microspheres are not normally associated with the apical region but are found throughout the cytoplasm of the invasive stage. These microspheres discharge their contents independently at various points around the cell periphery and are, thus, quite distinct from the rhoptry–microneme system (see Bannister et al., 1975; Entzeroth, 1984, 1985).

T. parva sporozoites contain only two types of secretory organelles, namely the rhoptries and the numerous membrane-bounded electron-dense bodies scattered throughout the cytoplasm and termed "micronemes" by Fawcett et al. (1982). However, specifically in the case of T. parva sporozoites, while the rhoptries appear to resemble the rhoptries in the zoites of other Apicomplexans, the second type of secretory organelle which is not associated with, and functions separately from the rhoptries, more closely resembles the electron dense granules/microspheres of, in particular, the Plasmodium sporozoites and merozoites. Thus in the present paper we will use the term "microspheres" for these structures rather than the term "micronemes" used by Fawcett et al. (1982).

Invasion of Bovine Lymphocytes by T. parva Sporozoites Involves a Defined Series of Events

The sequential process of sporozoite recognition and entry into bovine lymphocytes is illustrated in Fig. 1. Some of these stages have been described by earlier investigators (e.g., Fawcett et al., 1982, 1984; Webster et al., 1985); other stages and/or details of these stages are described here for the first time. We have included an overall view of the pro-
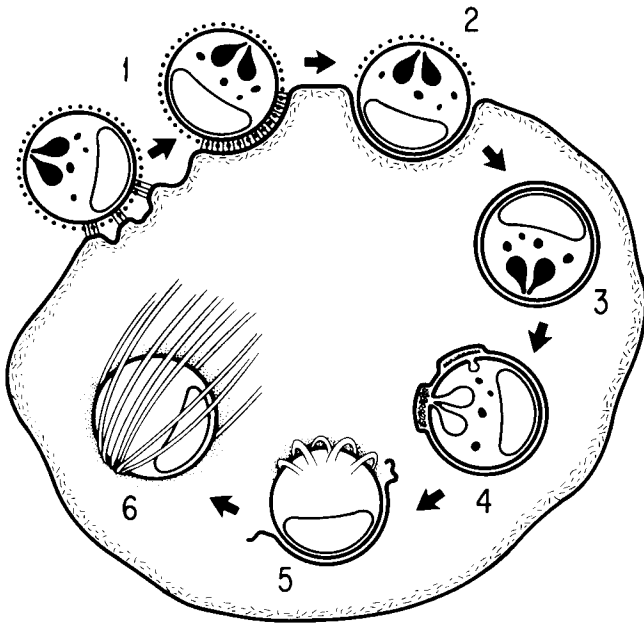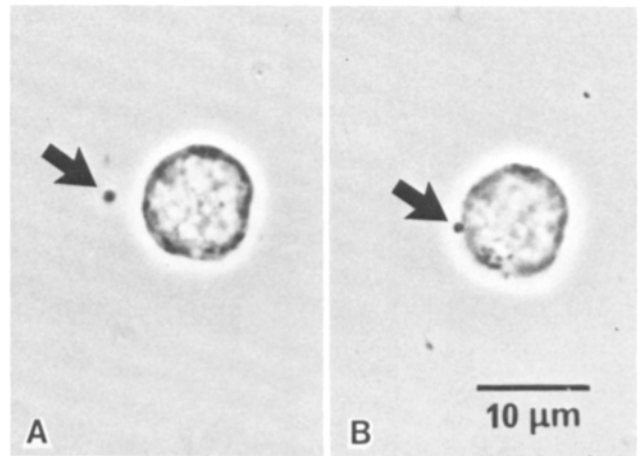
Figure 1. Drawing illustrating the sequence of events during the entry and establishment of *T. parva* into bovine lymphocytes. For details see text.

Figure 2. Sequential phase contrast light micrographs showing (A) a live *T. parva* sporozoite (*arrow*) close to a bovine lymphocyte to which it subsequently (B) comes into contact and to which it attaches. Once attached to the host cell surface the parasite very quickly enters the lymphocyte.

cess of sporozoite entry here whilst the details of the individual stages will be described in the subsequent sections. The process involves a defined series of events (Fig. 1) which include (*1*) the initial recognition and binding of the sporozoites to one or more sites on the lymphocyte plasma membrane (see Fig. 3, *a* and *b*); (*2*) the formation of a very close, continual junction between the sporozoite and lymphocyte membranes; the two closely apposed membranes are separated by a thin (∼6-nm thick) dense layer of material (Fig. 4, *a* and *b*; see Fawcett et al., 1982); (*3*) the progressive circumferential "zippering" of the closely apposed sporozoite and lymphocyte membranes (Fawcett et al., 1982), concomitant with the loss of the sporozoite surface coat (Webster et al., 1985) and movement of the sporozoite into the lymphocyte resulting in the parasite becoming fully internalized within the host cell whilst still surrounded by the closely apposed lymphocyte plasma membrane; (*4*) the separation of the enclosing host cell membrane from the sporozoite; a process which involves the discharge of the contents of the rhoptries and microspheres and the appearance of a 10–15-nm thick layer of fuzzy material on the surface of the sporozoite; (*5*) the dissolution of the host cell membrane and the escape of the parasite into the host cell cytoplasm; (*6*) the formation of an orderly array of microtubules surrounding the sporozoite and closely associated with the fuzzy material on the parasite surface.

Sporozoite Binding Occurs at 0–2°C

T. parva sporozoites are nonmotile organisms and, therefore, the initial contact between the sporozoite and a susceptible host cell is a chance event. By examining living sporozoites and PBLs by light microscopy we can show that once contact between a susceptible host cell and a sporozoite has occurred recognition and binding to the host cell surface appears to be instantaneous (Fig. 2). Once bound the sporozoite invariably enters the lymphocyte and rapidly becomes internal-

ized. We must emphasize, however, that only ∼30–35% of the PBLs are susceptible to infection (Table I).

To discount the possibility that sporozoite binding was because of chance contact with the host cells upon glutaraldehyde fixation, sporozoites were incubated under identical conditions, with either bovine macrophages, fibroblasts, or granulocytes. In all cases no sporozoites were ever observed bound to the cell surface. Similarly, when sporozoites were incubated with mixtures of bovine PBLs and granulocytes, sporozoites were found bound only to or internalized within the PBLs (Shaw and Tilney, unpublished observations). Furthermore, Stagg et al. (1983) found that, in vitro, *Theileria* sporozoites infected only a limited range of bovid cells and that sporozoite attachment and infection were not observed with nonsusceptible bovid cells, nor were PBLs from a range of nonbovid mammalian species infected by the parasite. Thus, we feel confident that the presence of sporozoites bound to susceptible host cells is the result of a specific parasite–host interaction and not the result of chance contact upon glutaraldehyde fixation.

The first step in sporozoite binding seems to be an association of the sporozoite with one or more small areas on the lymphocyte plasma membrane (Fig. 3 *a*), followed by the formation of a more extensive area of contact characterized by the parallel apposition of the sporozoite and host cell membranes with slender strands of low density material bridging the 20–40-nm gap (Fig. 3 *b*). Thus the initial interaction between the sporozoite and host cell surface involves some change or rearrangement in the host cell membrane. Whether this change is an active process or because of a "mass action" effect caused by the formation of increasing numbers of strands of material bridging the gap between the two membranes is unclear. Some increase in the density and/or organization of the host cell actin cytoskeleton was occasionally seen in the region of sporozoite binding indicating that the host cell cytoskeleton may play some role in, at least, sporozoite binding (see below).

Sporozoite binding to the surface of susceptible host cells can occur at 0–2°C, although the numbers binding were slightly lower than at 37°C (Table I). However, whilst the

Table I. T. parva Sporozoite Invasion of Bovine PBLs Is Temperature Dependent and Requires Live, Intact Sporozoites: Addition of Irradiated Sporozoites, Sporozoite Ghosts, and the Effects of Azide on Sporozoite Invasion of Bovine Lymphocytes

	Infection index	Sporozoite internalization
	%	%
Controls	32.6 ± 3.9 (range 26.9–39.0%)	33.5 ± 6.2 (range 19.9–45.8%)
Sporozoites and PBLs incubated on ice (0–2°C) for 60 min	21.9	0
Pretreated sporozoites		
Irradiated	28.3	1.3
Ghosts	24.4	8.6
Sodium azide		
65 mM (0.1%)	1.1	0
Pretreated sporozoites		
10 mM	2.1	0
Pretreated PBLs		
10 mM	33.1	16.4

Bovine PBLs were incubated in vitro with *T. parva* sporozoites for 30 min at 37°C and processed for electron microscopical examination as described in Materials and Methods. The infection index is defined as the percentage of PBLs showing either surface-bound or fully internalized sporozoites in one section. Sporozoite internalization is defined as the percentage of cell-associated sporozoites that are fully internalized within an infected PBL.

Values for the control incubations represent the mean (± SD) from 11 experiments. Values for the experimental incubations are the mean of two experiments.

sporozoites could bind to the host cells at 0–2°C, no sporozoites were ever found either zippered to the surface or internalized within the lymphocytes.

Sporozoite "Zippering" and Entry Is Temperature Dependent and Requires Live, Intact PBLs and Sporozoites

While the initial recognition and binding process is not temperature dependent, the subsequent zippering (Fig. 4) (and internalization) stage is, indicating that it is an energy-dependent process (cf., Jura, 1984). This finding is at variance with the previous reports of Fawcett and colleagues (Fawcett et al., 1982; Fawcett and Stagg, 1986) who claimed that *Theileria* sporozoites could invade lymphocytes and macrophages at 0–2°C as well as at 37°C. The discrepancy between the present results and those of Fawcett et al. (1982)
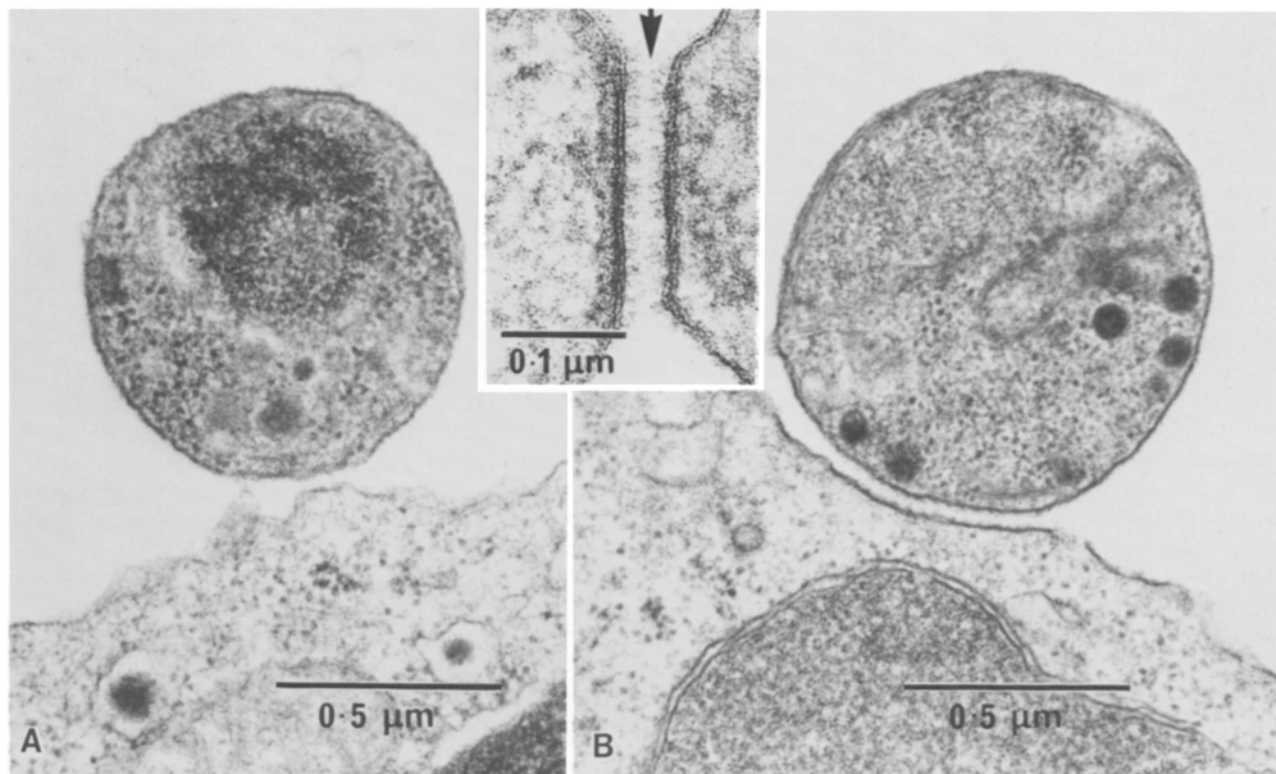
Figure 3. Thin sections of *T. parva* sporozoites attached to the surface of lymphocytes. (*A*) The initial attachment occurs at one or more small areas on the lymphocyte membrane, followed (*B*) by the formation of a more extensive area of attachment. (*Inset*) Slender strands of parasite coat material can be seen bridging the gap between the parasite and the host surface (*arrow*).
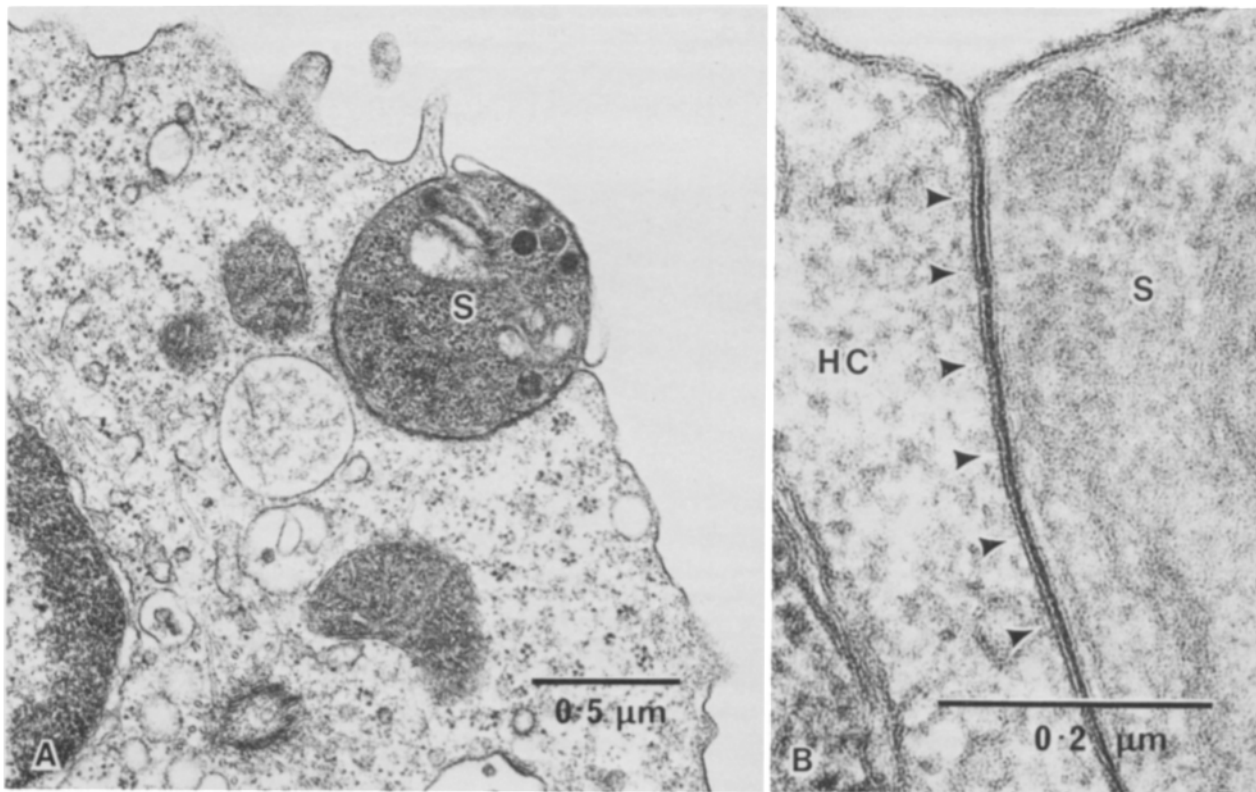
Figure 4. (*A*) Sporozoite (*S*) in the process of entering a lymphocyte: the sporozoite and lymphocyte membranes establish intimate contact and there is a progressive circumferential "zippering" of the two membranes resulting in the sporozoite occupying a deepening recess in the host cell surface. (*B*) The interface between the sporozoite (*S*) and host cell (*HC*) consists of a pentalaminar junction formed by the apposition of the two unit membranes with no discernable intercellular gap present. Note that there is no apparent localization of material on the cytoplasmic face of the lymphocyte membrane (*arrows*).

and Fawcett and Stagg (1986) appears to be because of the inevitable warming of the samples during centrifugation before fixation in the latter studies. This was confirmed in a subsequent experiment when sporozoites and PBLs were incubated on ice for 90 min and then centrifuged for 3 min before fixation. Whilst no sporozoites were found zippered to or internalized in samples incubated and fixed on ice without centrifugation, numerous sporozoites were found zippered to and fully internalized in cells centrifuged before fixation.

The presence of a temperature block between the initial binding and subsequent zippering stage reinforces the idea that the binding and zippering stages of sporozoite entry are two separate and independent events. This conclusion can be independently verified and extended using sporozoite ghosts or irradiated sporozoites. Both will bind to intact lymphocytes (Fig. 5, *a* and *b*) but will only very rarely zipper or enter the host cell (Table I). Similarly, live, intact sporozoites will also bind to lymphocyte ghosts (Fig. 5 *c*) but cannot zipper with the host cell surface membrane. The failure of zippering to occur when either sporozoite ghosts, irradiated sporozoites, or lymphocyte ghosts were used in in vitro infection experiments indicates that the process requires the participation of both live, intact sporozoites, and lymphocytes.

Pretreatment of the sporozoites with 10 mM sodium azide significantly inhibits sporozoite binding and entry, whereas pretreatment of the PBLs did not affect the binding and entry of the sporozoites (Table I). This result would suggest that sporozoite entry requires the active participation of the para-

site. However, the differences in the binding capacity of irradiated sporozoites, sporozoite ghosts, and azide-treated sporozoites is difficult to explain. One possible explanation could be that azide pretreatment results either in the direct loss of, or in some structural change(s) to the sporozoite surface coat. In contrast, while irradiation or pretreatment of the sporozoites with distilled water may render the sporozoites nonviable, these pretreatments may not be sufficient to remove or structurally damage the sporozoite surface coat to the extent of preventing the initial binding process.

Once the live sporozoite has made initial contact with a susceptible host cell the process of binding, zippering, and internalization is rapid, with large numbers of parasites being found fully internalized within 3 min at 37°C. However, at this point the vast majority of these internalized sporozoites still contained rhoptries and microspheres and remained enclosed within the tightly apposed sporozoite and host cell membranes.

Although some activation of the host cell cytoskeleton was noted during the initial binding process, no localization of material on the cytoplasmic face of the host cell membrane was seen (Fig. 4 *b*). This would suggest that the zippering and internalization process does not involve anything analogous to clathrin-coated pit formation.

Sporozoite Escape from the Encapsulating Host Cell Membrane

Once the *T. parva* sporozoite has become internalized within the host cell the next step is the escape of the parasite from

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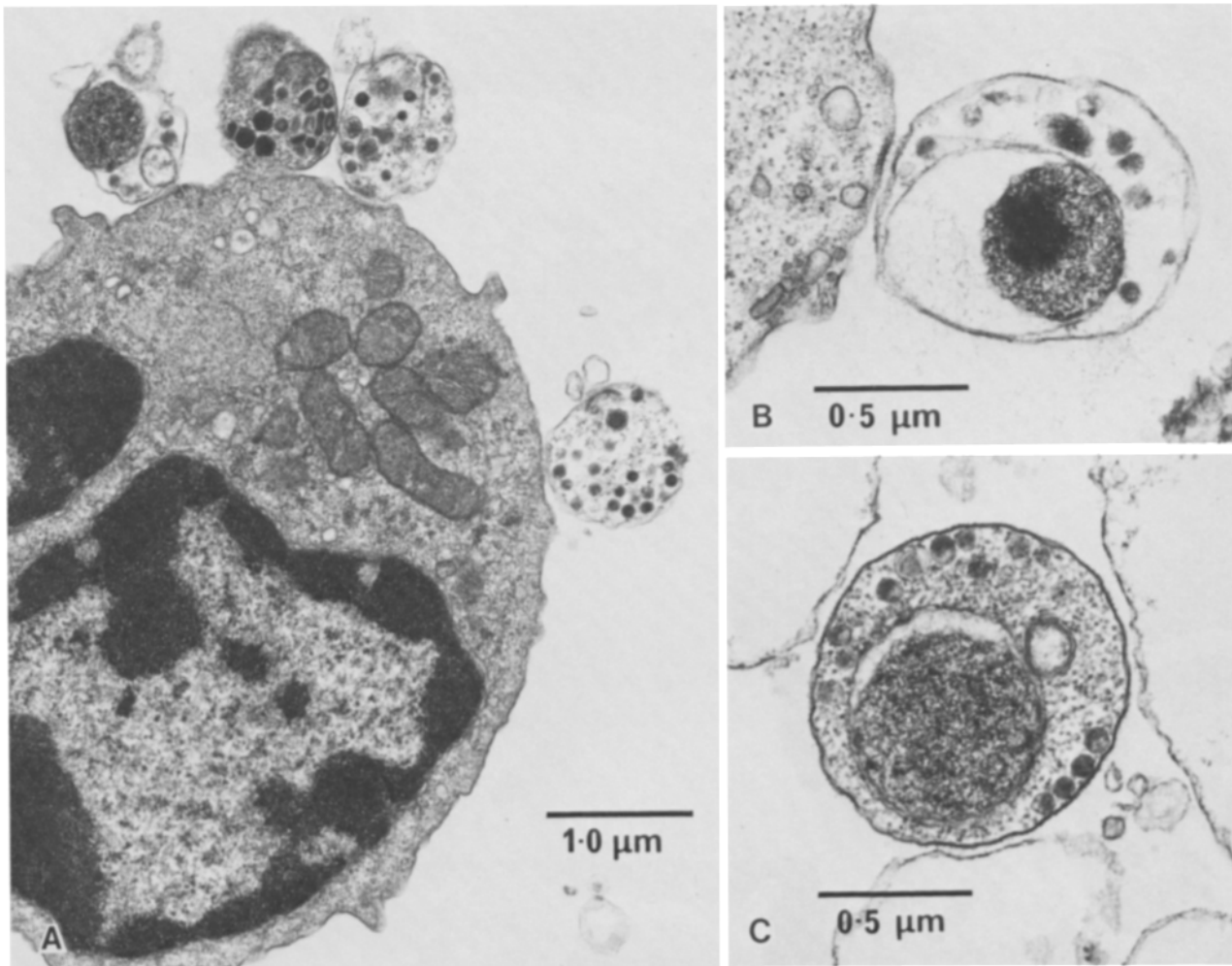
Figure 5. Both irradiated (*A*) or ghost (*B*) sporozoites will bind to intact lymphocytes but cannot enter the host cell. (*C*) Similarly, live intact sporozoites can also bind to the surface membrane of lymphocyte ghosts but cannot enter the cells.
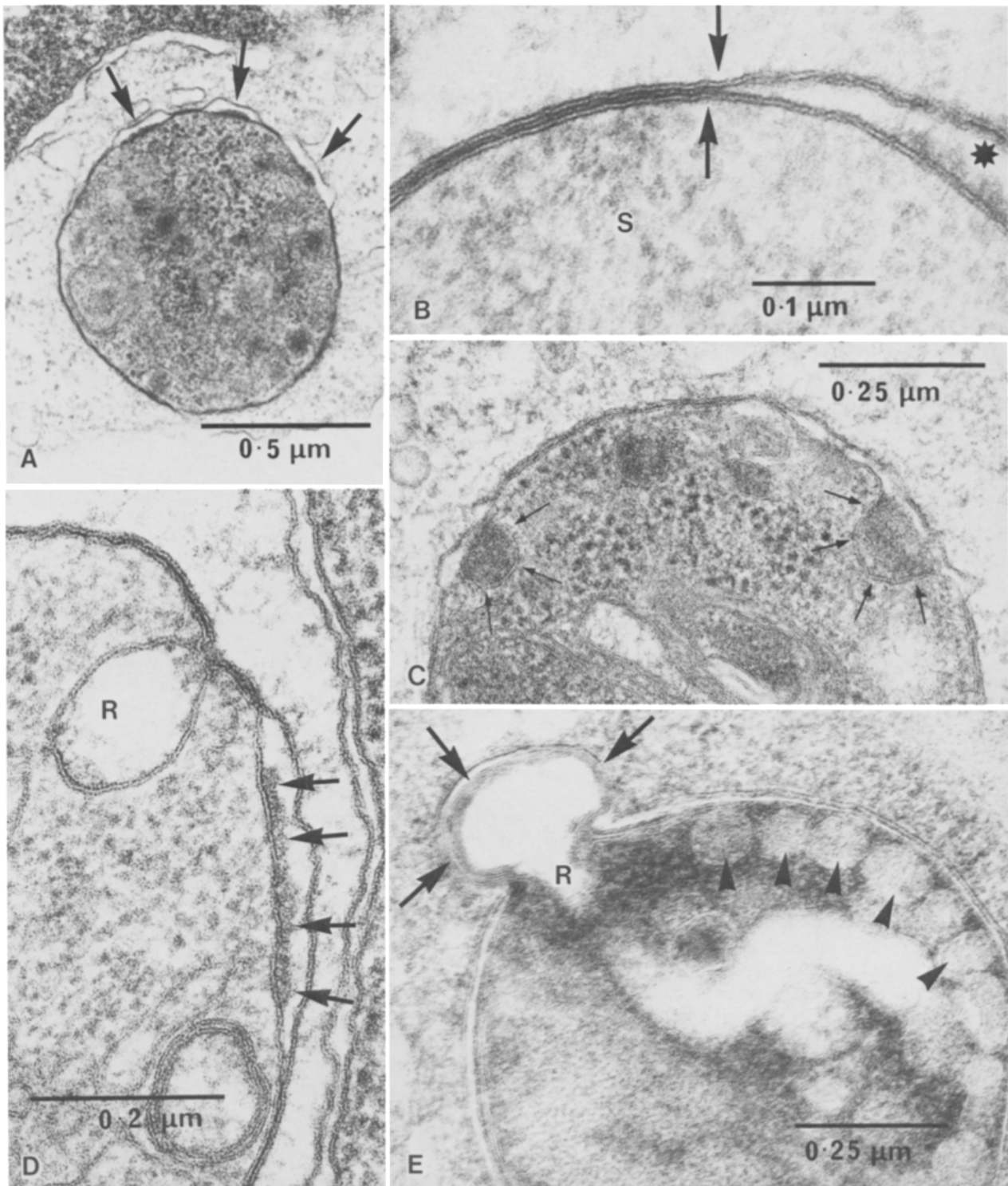
the encapsulating host cell membrane. The loss of the enclosing host cell membrane was invariably preceded by the separation of the tightly apposed host and parasite membranes (Fig. 6, *a* and *b*) and the appearance of an electron lucent vacuolar space. This separation of the two membranes coincided with the discharge of both the microspheres (Fig. 6 *c*) and rhoptries (Fig. 6 *d*) with the denser contents of the microspheres forming a layer of dense fuzzy material on the surface of the sporozoite (Fig. 6, *b* and *d*). Additional indirect evidence that the rhoptries and microspheres are responsible for the separation of the two membranes is that the initial separation of the closely apposed host cell and parasite membranes invariably occurred in the regions of the sporozoite which contained these organelles and not over those regions of the sporozoite where the nucleus was located.

In a number of sections of totally internalized sporozoites, profiles of rhoptries in the process of releasing their contents were observed. Frequently the discharging rhoptries contained numerous rounded or tubular vesicles and in a few cases the released material also had a loose membranous appearance. In contrast, the microspheres contained and discharged a dense, finely granular material. This difference in the contents of the rhoptries and microspheres was even more apparent in sporozoite–lymphocyte samples fixed in a

mixture of glutaraldehyde–tannic acid. In these samples, multilamellar membraneous whorls of dense material were frequently observed being extruded from the rhoptries (Fig. 6 *e*), and loose membraneous material partially filled the expanded space between the host cell and sporozoite membranes. The microspheres, by contrast, never contained or discharged lamellar-like material and were invariably composed of a homogeneous, finely granular material (Fig. 6 *e*).
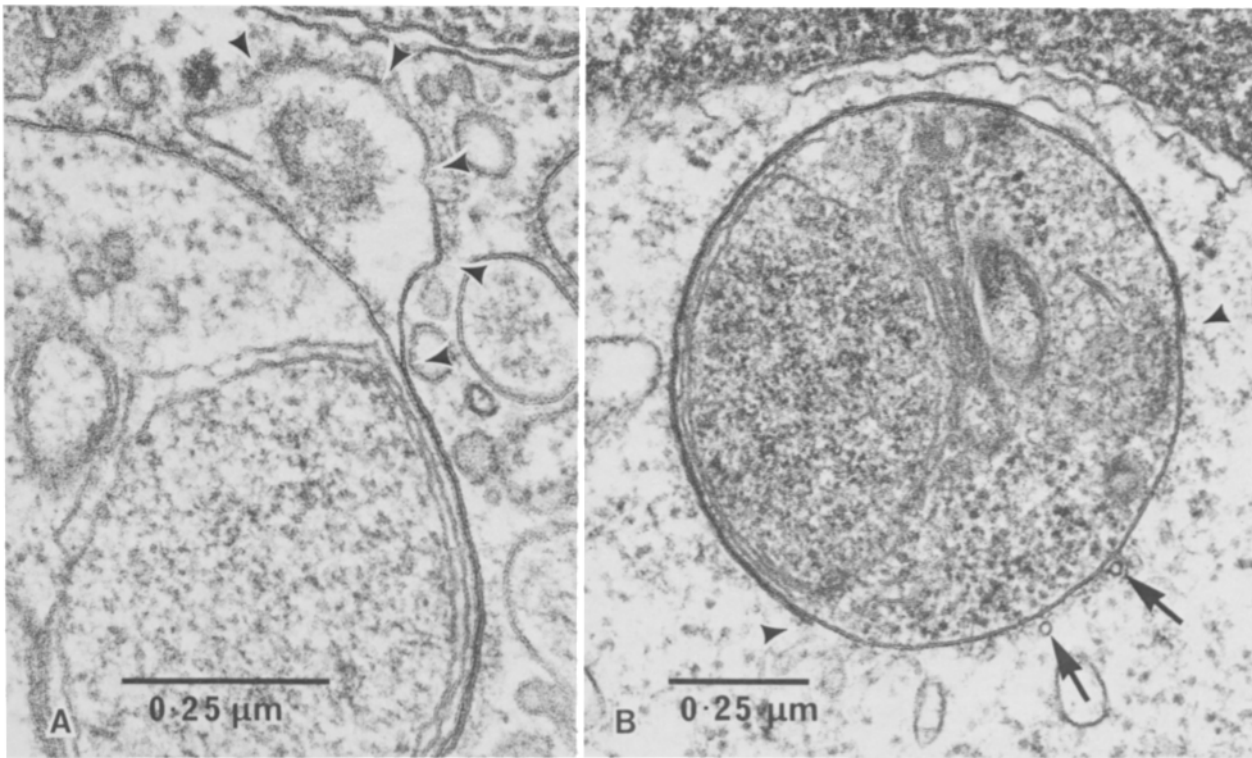
Once the enclosing host cell membrane has been separated from the sporozoite the loss of host membrane occurs by the breakdown or dissolution of the membrane (Fig. 7, *a* and *b*) beginning at one or more points but eventually including the whole membrane. The enclosing host cell membrane appears to be dissolved progressively from the broken ends of the membrane which may lie at some distance from the sporozoite (Fig. 7 *a*), and is not lost by fragmentation (cf., Fawcett et al., 1982).

After the gradual dissolution of the enclosing host cell membrane, microtubules begin to form around the parasite (Fig. 7 *b* and 8) in close association with the 15–25-nm thick layer of moderately dense material on the surface of the sporozoite (Figs. 7 *b* and 8 *a*). Contrary to Fawcett's suggestion (Fawcett et al., 1984), this layer of fuzzy material does not represent the remains or residues of the dispersed host

Figure 6. (*A*) Initially the fully internalized sporozoite is completely enclosed in a tightly apposed, enveloping host cell membrane. The loss of this enclosing host membrane is preceded by the separation of the two apposed membranes often at several places around the circumference of the sporozoite (*arrows*). (*B*) High magnification view of part of the fully internalized sporozoite (*S*) showing the separation of the host cell and parasite membranes (*arrows*). Note the presence of a layer of dense fuzzy material on the surface of the parasite in the region where the two membranes have separated (*asterisk*). (*C*) Concomitant with the separation of the apposed host and parasite membranes, the microspheres open at the zoite surface suggesting the exocytosis of their contents (*arrows*), which appears to form a layer of dense fuzzy material on the surface of the parasite. (*D*) Section through a fully internalized sporozoite showing a rhoptry (*R*) opening to the exterior and presumably in the process of discharging its contents. Note also the presence of fuzzy material (*arrows*) on the surface of the sporozoite in the region where the host and parasite membranes have separated, thus clearly demonstrating that this layer is not derived from the disintegration of the enclosing host cell membrane. (*E*) Section through a fully internalized sporozoite fixed in the presence of tannic acid, showing the extrusion of multilamellar membranous dense material (*arrows*) from a rhoptry (*R*). Note that the microspheres (*arrowheads*) are composed of a finely granular material and do not contain lamellar-like material.

Figure 7. (*A*) Once separated from the sporozoite the enclosing host cell membrane (*arrows*) dissolves progressively from the broken ends of the membrane which as in this micrograph may lie at some distance from the sporozoite. (*B*) Partially escaped sporozoite: in this micrograph the host cell membrane has been lysed from part of the parasite (between *arrowheads*) and microtubules are becoming associated with the dense fuzzy material on the exposed parasite surface (*arrows*).
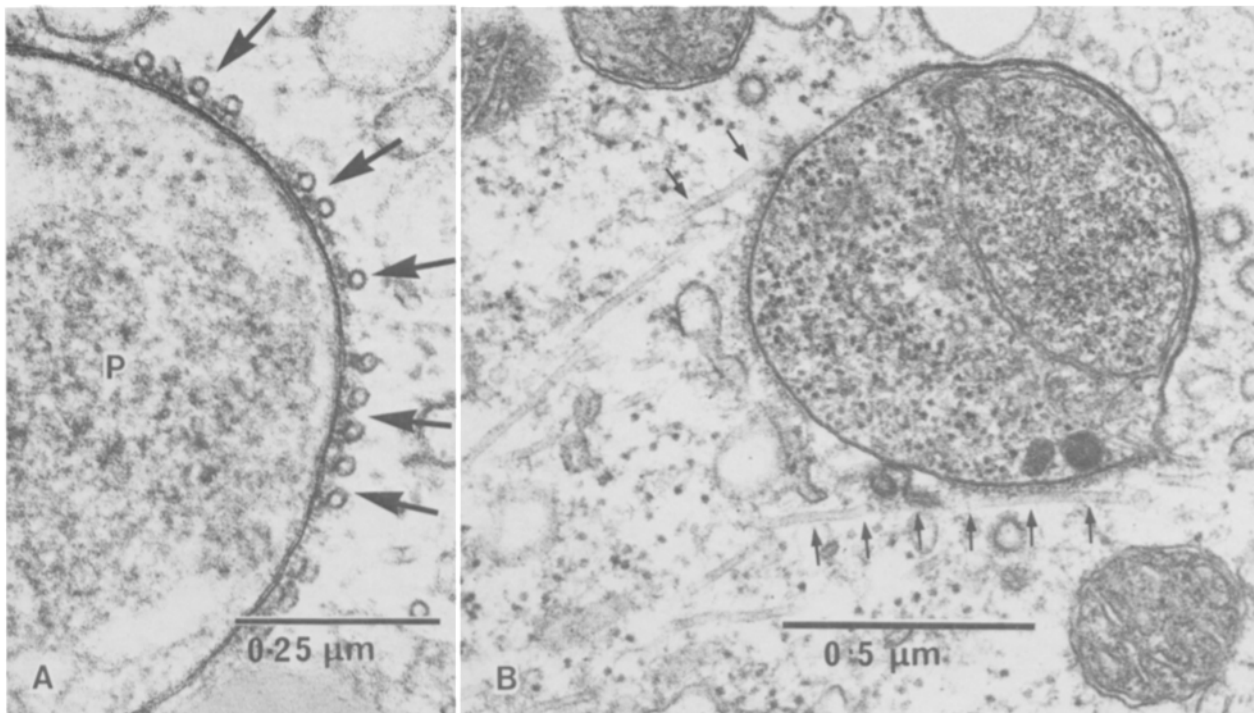
Figure 8. (*A*) Within 30 min of invasion the escaped, intracytoplasmic parasite (*P*) is surrounded by an orderly array of host cell–derived microtubules (*arrows*) which (*B*) pass tangentially to the parasite (*small arrows*) and converge towards the centriole–Golgi region of the host cell.

Table II. The Effect of Protease Inhibitors PMSF and a Mixture Containing Antipain, Leupeptin, Pepstatin A, and Chymostatin on T. parva Sporozoite Invasion of Bovine Lymphocytes

	Infection index	Sporozoite internalization
	%	%
Controls	32.2 ± 2.8 (range 28.3–34.9%)	28.6 ± 6.2 (range 19.9–33.6%)
PMSF		
5 mM	20.4	22.8
10 mM	5.9	7.1
Sporozoites preincubated for 15 min at 37°C in PMSF		
5 mM	2.5	12.5
Mixture of protease inhibitors		
50 µg/ml	23.8	38.1

Values for the control experiments are the mean (± SD) from three experiments.
Values for the experimental incubations are the mean of two experiments.

cell membrane, but arises from the secreted contents of the sporozoite microspheres and in many sections covered the sporozoite surface despite the fact that the host cell membrane was still present (Fig. 6, *b* and *d*).

The whole process of sporozoite binding, entry, and escape from the encapsulating host cell membrane with the ensuing formation of the surrounding basket of microtubules occurs rapidly at 37°C, microtubules being present around the parasite within 15 min (Fig. 7 *b*).

Effect of Protease Inhibitors

Sporozoite entry was inhibited by 5.0 and 10.0 mM PMSF (Table II), although at 10.0 mM PMSF the lymphocytes were adversely affected by the inhibitor with gross cytopathic effects evident. However, whilst PMSF significantly inhibited sporozoite entry, where infection did occur the percentage of sporozoites becoming fully internalized within the host lymphocytes was almost identical to the controls (Table II). This would strongly suggest that PMSF was affecting the initial binding process; sporozoites that could bind to the host cell surface could subsequently zipper and become internalized at frequencies identical to control situations. To

Table III. The Effect of Cytochalasins B and D on T. parva Sporozoite Invasion of Bovine Lymphocytes

	Infection index	Sporozoite internalization
	%	%
Controls	32.4 ± 5.0 (range 26.9–39.0%)	35.5 ± 2.7 (range 31.3–38.8%)
Pretreated PBLs		
Cytochalasin B		
10.0 µg/ml	11.2	6.5
Cytochalasin D		
0.5 µg/ml	14.1	51.1
5.0 µg/ml	11.1	42.1
10.0 µg/ml	10.5	12.2
20.0 µg/ml	0	0
Pretreated sporozoites		
Cytochalasin D		
5.0 µg/ml	45.8	33.3

Values for the control incubations are the mean (± SD) from five experiments. Values for the experimental incubations are the mean of two experiments.

test this idea sporozoites were pretreated with 5 mM PMSF (15 min at 37°C) before being incubated with PBLs. This pretreatment significantly increased the level of inhibition (Table II), although in this case the sporozoites appeared abnormal suggesting that pretreatment may have adversely affected the parasite.

By comparison, the mixture of antipain, leupeptin, pepstatin A, and chymostatin at 50 µg/ml caused only moderate inhibition of sporozoite entry (Table II).

Effect of Cytochalasin

The effects of cytochalasins B and D on sporozoite binding and internalization are shown in Table III.

Whilst both cytochalasins B and D inhibit sporozoite binding to cytochalasin-treated PBLs, cytochalasin D does not change the percentage of fully internalized sporozoites (Table III). This would suggest that cytochalasin D was affecting, possibly by slowing down, the initial binding process but once the sporozoites bind to the host cell surface they zipper and enter at frequencies similar to control situations. In contrast, when sporozoites rather than PBLs were initially treated with 5 µg/ml cytochalasin D, washed, and subsequently incubated with PBLs, no inhibition of invasion was observed (Table III) indicating that the cytochalasin-induced inhibition of invasion was due solely to changes in the lymphocyte actin cytoskeleton. Significantly, examination of thin sections of cytochalasin-treated sporozoite–PBL mixtures revealed that the lymphocytes tended to become rounded up and contained aggregates of short filamentous material which probably represented actin microfilaments.

In the case of cytochalasin B, whilst inhibition of sporozoite invasion was less than observed with cytochalasin D, there was very strong inhibition of parasite zippering and internalization (Table III). However, cytochalasin B is known to have other secondary inhibitory effects on monosaccharide transport across the plasma membrane (see Cooper, 1987) and therefore the inhibition of sporozoite internalization may be because of other effects unrelated to the disruption of the lymphocyte actin cytoskeleton.
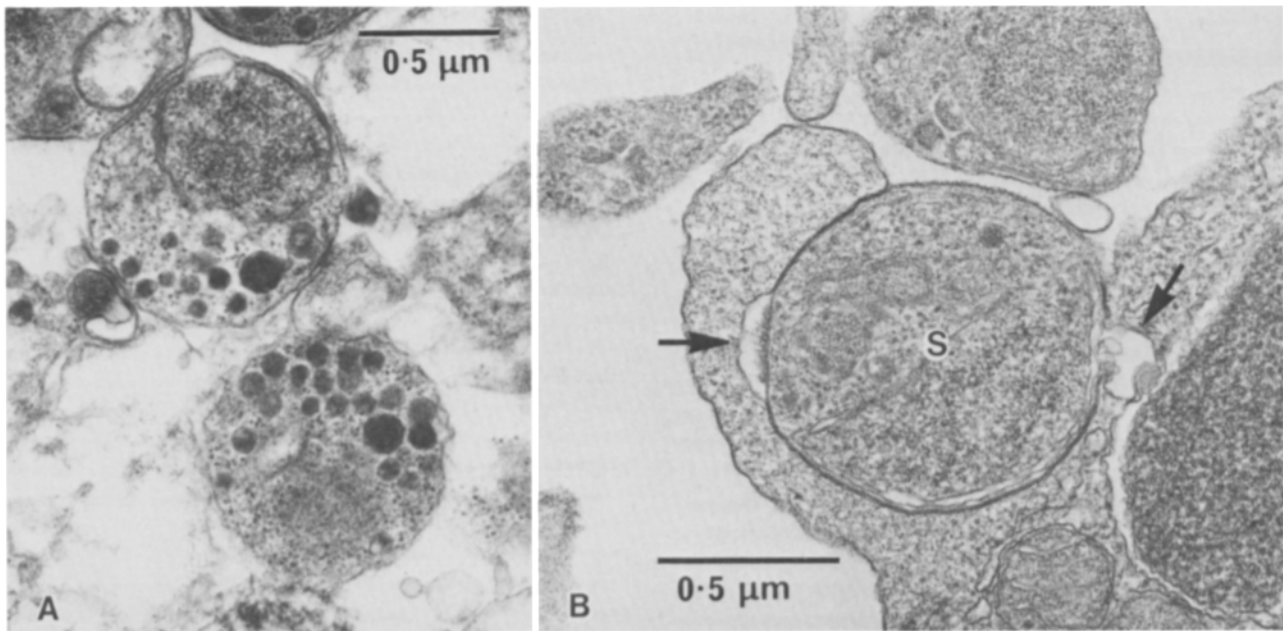
How Is Rhoptry and Microsphere Discharge Regulated?

In control experiments, rhoptry, and microsphere discharge occurs only after the sporozoite has become fully internal-

Figure 9. (*A*) Sporozoites treated in vitro with ionophore A 23187 still contain both rhoptries and microspheres indicating that the process of discharge is not induced by calcium ions. (*B*) Cytochalasin D-treated sample (5.0 µg/ml) showing a partially entered sporozoite (*S*) in which the initial separation of the zippered sporozoite and host cell membranes has started (*arrows*).

ized within the host cell. The discharge of these organelles is related to the process(es) involved in the parasite escaping from the encapsulating host cell membrane.

Incubation of free sporozoites with the ionophore A 23187 in vitro did not cause the discharge of either the rhoptries or microspheres (Fig. 9 *a*) suggesting that the process is not induced by calcium in a manner similar to exocytosis as documented for cortical granules, mast cell secretion, etc. (for a discussion of signal transduction events associated with exocytosis in ciliates, see Satir, 1989).

In cytochalasin D–treated samples in which the rate of sporozoite binding and entry appears to be slowed down, we observed a number of cases in which the discharge of the microspheres (and rhoptries) and the separation of the zippered membranes had started before entry was complete

(Fig. 9 *b*). This suggests that the discharge of these organelles is on some type of clock mechanism whereby the process of zippering initiates a sequence of, as yet undefined, events which in time leads to the discharge of the microspheres and rhoptries and the escape of the parasite from the encapsulating host cell membrane. This interpretation is also supported by the observation that in sporozoite–PBL samples incubated for 3 min at 37°C, the majority of fully internalized sporozoites still contained their microspheres and rhoptries, and were completely enclosed by the tightly apposed lymphocyte membrane. In a very few cases the initial stages of microsphere discharge and membrane separation, whereas by 5-min microsphere and rhoptry discharge was commonly found (data not shown).

Table IV. The Effect of mAbs against Bovine MHC Class I and MHC Class II Molecules on T. parva Sporozoite Invasion of Bovine Lymphocytes

	Infection index	Sporozoite internalization
	%	%
Controls	31.2 ± 3.2 (range 27.1–35.1%)	38.1 ± 5.2 (range 31.9–45.8%)
mAbs against MHC class I		
IL-A 19	2.8	0
IL-A 88	1.6	0
W6/32	0.4	0
B1G6	0.7	0
mAbs against MHC class II		
IL-A 21	33.6	41.7
J 11	35.5	30.2
mAbs against a common panleucocyte marker (bovine equivalent of CD11a)		
IL-A 87	34.4	45.7

Values for the control incubations are the mean (± SD) from five experiments. Values for the experimental incubations represent the mean of two experiments.

The Role of Bovine MHC Class I Molecules in Sporozoite Invasion

Our present results have shown clearly that sporozoite binding and zippering to the lymphocyte surface membrane are two separate although sequential processes which have different metabolic requirements. Whilst it has been shown previously that mAbs against the immunodominant part(s) of the sporozoite surface coat can abolish or greatly reduce parasite invasion of susceptible host cells (Dobbelaere et al., 1984; Musoke et al., 1984), little is known about the host cell surface molecules involved in the processes of parasite recognition, binding, and entry into the host cell.

In an initial attempt to identify the host cell surface molecule(s) involved in the process of parasite invasion we have investigated the effect of a number of mAbs raised against lymphocyte surface molecules (Table IV). The most striking result from these initial studies was the almost total inhibition of invasion by monomorphic mAbs reactive with bovine MHC class I molecules and with beta-2-microglobulin (Table IV) with >90% inhibition and no sporozoites becoming internalized within the host cell. Interestingly, these mAbs appeared to inhibit sporozoite entry by preventing the initial recognition/binding event. Whilst this result can be interpreted as meaning that class I molecules are involved in the process of sporozoite invasion, secondary effects such as a general coating of the surface membrane with antibodies and/or a general disorganization of other membrane proteins cannot be totally ruled out. For example, invasion of erythrocytes by *Plasmodium knowlesi* merozoites is entirely independent of alpha sialoglycoprotein and yet is inhibited by antibodies to alpha SGP (Miller et al., 1977; Hadley et al., 1986). To test these possibilities we used an mAb-reactive with a pan-leucocyte-surface molecule (IL-A 87), which occurs in similar numbers to MHC class I (Splitter and Morrison, 1990). This mAb and other mAbs directed against other surface molecules did not inhibit infection (Table IV).

Discussion

The primary purpose of this paper is to define in greater detail the stages involved in the process of sporozoite entry into susceptible bovine lymphocytes as a prelude to studies aimed at trying to understand the molecular basis of host cell specificity and the invasion process.

Sporozoites, although Nonmotile, Are not Passive Participants in Host Cell Entry

Previously Fawcett et al. (1982) had reported that *T. parva* sporozoites could enter lymphocytes at 0–2°C by an energy-independent process they termed "passive endocytosis." However, our results clearly show that this is not the case. Sporozoite entry involves a defined series of sequential stages and, while the initial sporozoite–lymphocyte recognition event can occur at 0–2°C, all the subsequent stages are temperature dependent, require the participation of live, intact sporozoites and host cells, and involve some rearrangement in the host cell surface membrane or submembrane cytoskeleton. Furthermore, the entry process occurs rapidly with sporozoites becoming fully internalized within 3 min at 37°C, and established free in the cytoplasm within 15–30 min.

We have confirmed the previous observation of Fawcett et al. (1982, 1984) that *T. parva* sporozoite entry into bovine lymphocytes occurs via a "zippering" of the host cell and parasite membranes presumably involving a sequential and circumferential interaction of molecules on the parasite surface with those on the host cell surface (see Silverstein, 1977). However, in the case of parasite entry into nonphagocytic cells (the situation in the present case), Silverstein (1977) suggested that the force necessary to move the host cell membrane around the parasite was probably generated by the contractile elements of the parasite. In the present case, the free sporozoites are nonmotile and pretreatment of them with cytochalasin D does not prevent entry, although sporozoite "ghosts" and irradiation-killed sporozoites, whilst they will bind to the host cell surface, do not become internalized. Similarly, sporozoites pretreated with azide whilst they will bind to the surface of susceptible host cells, albeit in greatly reduced numbers, do not become internalized. Thus, while we have no direct evidence for any locomotory activity on the part of the sporozoite (cf., the situation in other Apicomplexan parasites where inhibitors of parasite locomotion will also inhibit invasion; see Russell, 1983; Sinden, 1985; Mitchell and Bannister, 1988), the requirement for live, intact sporozoites for successful invasion would suggest that the parasite is not a "passive participant," but plays some essential role in the zippering and/or internalization process. Further work is in progress to determine more precisely the role of the sporozoite in the host–parasite interactions occurring during the zippering and internalization phase of the entry process.

The Use of Protease Inhibitors Indicates That Proteases May Be Involved in the Entry Process

The selective inhibition of sporozoite binding by the protease inhibitor PMSF suggests that binding requires enzymatic activity. The binding of sporozoites at 0–2°C does not preclude the possibility of enzymatic activity being required/involved in the initial recognition and binding process particularly as binding occurs at a slower rate than in the controls. This is in keeping with observations on host cell entry by other related Apicomplexan parasites which indicate that a parasite surface protease(s) is involved in the process of host cell entry by *Leishmania* (Etges et al., 1986, 1989; Bordier, 1987), whilst other studies on, in particular malaria merozoites, have shown that the invasion process requires enzymatic activities which can be inhibited in the presence of protease inhibitors (Hadley et al., 1983; Dluzewski et al., 1986; Adams and Bushell, 1988; Schrevel et al., 1988; Braun-Breton and Pereira da Silva, 1988). Since the initial binding of the sporozoite to the host cell surface is rapidly followed by the tighter juxtaposition of parasite and host membranes (zippering), which occurs concomitantly with the loss of, at least part of, the sporozoite surface coat (Webster et al., 1985), this process may involve enzymatic activity.

The Actin Cytoskeleton of the Host Cell Is Necessary for Sporozoite Entry

The inhibition of binding by cytochalasins B and D pretreatment of the PBLs indicates that the host cell actin cytoskeleton plays some essential role in the sporozoite–lymphocyte membrane interaction(s) involved in the invasion process. In all these experiments although the binding process was often

significantly inhibited, once bound, parasites were establishing themselves within the lymphocyte at frequencies similar to control cultures (i.e., between 30–40% of invading sporozoites were inside the lymphocyte even though the total number of organisms was greatly reduced). One explanation for these observations is that the cytochalasins, by binding to actin, cause the disruption of the lymphocyte cytoskeleton. Significantly, the levels of cytochalasin-induced inhibition of sporozoite binding (between 60–100%) are similar to the degree of inhibition of the capping of lymphocyte surface antigens which has been found to correlate with the ability of cytochalasins to inhibit actin elongation in vitro (Yahara et al., 1982; Cooper, 1987). In the present case the cytochalasin-induced disruption of the lymphocyte cytoskeleton which would lead to a general perturbation of the lymphocyte surface may, in particular, induce a redistribution of membrane molecules thereby indirectly inhibiting the recognition and binding process.

The Role of The Rhoptries in Sporozoite Escape into the Host Cytoplasm

Before escaping into the cytoplasm of the host cell the tightly apposed membranes of the host and parasite separate. At the same time the rhoptries discharge their contents which in tannic acid–fixed preparations are seen to contain membranous materials which in other systems have been identified as phospholipids (Kalina and Pease, 1977). Similar tannic acid–preserved multilamellar, extrudable material has also been observed in the rhoptry–microneme system of the sporozoite and merozoite stages of *Plasmodium* species (Stewart et al., 1985, 1986; Bannister et al., 1986; Bannister and Mitchell, 1989) and recent reports have provided direct experimental evidence that, in the merozoite, rhoptry-derived lipidic, and proteinaceous materials are rapidly incorporated into the host cell membrane and also play a role in the formation of the parasitophorous vacuole (Mikkelson et al., 1988; Sam-Yellowe et al., 1988; Lustigman et al., 1988). By analogy with the situation in *Plasmodium* the extrusion of tannic acid–preserved membranous material from the *T. parva* sporozoite rhoptries may be responsible for the observed expansion and thereby separation of the encapsulating host cell membrane. This is consistent with the fact that during the initial separation of these membranes the parasite shows no noticeable signs of becoming smaller and, in fact, with the concomitant exocytosis of the large numbers of microspheres around the periphery of the parasite without any obvious evidence of sporozoite membrane recycling, probably increases rather than decreases in diameter. Whether the rhoptries, or possibly the microspheres, also secrete a lipase or similar substance(s) to facilitate the subsequent dissolution of the host cell membrane is not known.

Possible Function of the Microspheres in the Establishment of the Parasite within the Host Cell

Fawcett et al. (1984) previously noted the rapid association of host cell–derived microtubules around the newly established intracytoplasmic parasite. This orderly array of microtubules stays associated with the developing parasite and is consistently present around the schizont (Shaw, M. K., unpublished observations; Stagg et al., 1980). In the present study we found that the microtubule basket is closely associated with the fuzzy material secreted by the microspheres. Such a rapid and close association may be coincidental or may be because of the secretion, presumably via its microspheres, of microtubule-associating or microtubule-nucleating material. Irrespective of the correctness of our interpretation of the role of the fuzzy material in the establishment of this microtubule array, the *Theileria*-lymphocyte association is another, intriguing, example of an intracytoplasmic parasite subverting the host cell cytoskeletal system to its own purposes (cf., *Listeria* in macrophages; Tilney and Portnoy, 1989).

The Molecular Basis of Sporozoite–Host Interactions

We have started, in a preliminary way, to identify the molecular basis for the specific interaction(s) involved in *T. parva* sporozoite invasion of susceptible bovine lymphoid cells. We can now determine this as we have developed a quantitative assay with which to identify which steps in the invasion process are inhibited by certain substances and by how much. The finding that antibodies reactive with nonpolymorphic determinants of bovine MHC class I molecules significantly inhibit parasite invasion is an exciting although, at first sight, a somewhat perplexing and unexpected result. MHC class I molecules are ubiquitous to all nucleated cells and would, therefore appear unlikely to be involved in the initial, specific parasite–host cell recognition process given that *T. parva* sporozoites selectively invade only a subpopulation of T and B lymphocytes. However, whilst preliminary quantitation studies have not revealed any obvious or significant population (i.e., 30–40%) of bovine PBLs exhibiting high levels of class I expression, differences in the type and/or degree of surface expression of class I molecules have been reported (Bensaid et al., 1989) and, in particular, increased expression is known to occur during and as a result of cell activation (for example, McCune et al. [1975] reported a 10- to 30-fold increase in surface MHC molecules on virus transformed human lymphoblastoid cells as compared to nonactivated cells). Thus, it is possible that *T. parva* sporozoites are able to use differences in the level of MHC class I expression between different populations of lymphocytes to selectively infect only a specific group of lymphoid cells. As a precedent for the involvement of MHC molecules in "parasite" entry, it should be noted that Semliki Forest virus has been shown to use MHC molecules as specific host cell surface receptors during the initial interaction between the virus and host surface membrane (Helenius et al., 1978), although Oldstone et al. (1980) subsequently presented evidence to show that the MHC molecules were unlikely to be the major specific receptor required by Semliki Forest virus to penetrate and infect cells. Recently, in a host–parasite association more analogous to the present *Theileria*-bovine PBL situation, Velge et al. (1989) showed that mAbs reactive with HLA-DR and CD3 antigens significantly inhibited *Trypanosoma cruzi* infection of human T lymphocytes.

Alternatively, as is the case in the entry of other intracellular parasites and viruses (Tait and Sacks, 1988), two or more different molecules might be sequentially involved in, firstly, the recognition and binding process and secondly, in zippering and entry. In this case, given the physical nature of the zippering process and assuming that it is formed by a ligand–receptor interaction, it seems reasonable to suspect the involvement of a host-cell surface molecule(s) that occurs

both in relatively high numbers and is evenly distributed over the cell surface. Therefore, with these assumptions in mind, the possible involvement of MHC class I molecules, which occur over the lymphocyte cell surface in relatively high numbers, in, at least, the zippering process seems reasonable.

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