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***Theileria parva* and the bovine CTL response: down but not out?**

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SUMMARY

Theileria parva is a tick-borne intracellular protozoan of cattle, with obligate sequential differentiation stages in lymphocytes and erythrocytes. Immunity is mediated by cytotoxic T lymphocytes (CTL) that target and clear parasitized lymphocytes but allow persistence of infected erythrocytes, which are required for transmission to the tick. The life cycle of *T. parva* is haploid with the exception of a brief diploid stage in the tick vector during which sexual recombination occurs. There is evidence for antigenic diversity in field parasite populations, although broad immunity can be acquired following exposure to a limited number of strains. The CTL response in individual animals is tightly focused and its specificity is strongly influenced by major histocompatibility complex (MHC) phenotype. This review discusses the issue of how CTL immunity is likely to impact on parasite population structure in the light of available information on diversity of the parasite and its ability to recombine.

Keywords

cattle; immune selection; population structure; *Theileria parva*

REVIEW

In host–parasite systems that involve the reciprocal application of evolutionary pressure, or co-evolution, it has been argued that an equilibrium establishes over time between host resistance and pathogen virulence (1). This equilibrium may be based on avirulent parasites and low investment in resistance by the host, or high levels of host resistance and highly virulent parasites. Where host resistance arises from adaptive immunity, co-evolution is driven by maintenance of immune recognition by the host on the one hand and avoidance of recognition by the parasite on the other (2).

Like other vector-borne pathogens, *Theileria* parasites face selective pressure in both ruminant host and tick vector. Much of this pressure derives from immune mechanisms involving polymorphic recognition and/or effector molecules (3,4). The genus incorporates a number of species with the capability of transforming nucleated cells to a state of uncontrolled proliferation – a trait unique among eukaryotes. One of these parasites, *T. parva*, causes a severe and often fatal lymphoproliferative disease of cattle known as East Coast fever (ECF) in eastern, central and southern Africa. Transmitted by *Rhipicephalus appendiculatus* ticks, the disease exerts substantial economic impact in the region. This arises not only from mortalities, but also from losses in production and the costs of acaricides for tick control, which is the principal means of prevention. In addition, the disease constrains dairy and beef sectors in endemic areas by limiting the introduction of

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more productive European breeds of cattle, which are more susceptible to infection. A 1989 analysis estimated annual costs associated with the disease in Africa at \$169 million (5).

Increasing prevalence of acaricide resistance in tick populations has intensified the search for alternative control measures. Available vaccination options rely on infection with live parasites and simultaneous treatment with oxytetracycline – the so-called infection and treatment method (6). Widespread uptake of this approach has been constrained by its reliance on a cold chain and, because it gives rise to a carrier state, concerns regarding the introduction of new strains into areas previously free of them. Current research efforts are therefore focused on development of improved vaccines based on subunit components of the parasite (3). The extent to which *T. parva* is subject to immune selection by the bovine host is of substantial relevance to the likely success of such vaccines.

The life cycle of *T. parva* is typical of apicomplexans and involves obligate differentiation steps in both the mammalian host and the tick vector (7). The cycle is predominantly haploid, with only a brief diploid phase occurring in the tick. Infective sporozoites are inoculated by the feeding tick and rapidly invade host lymphocytes by a process similar to receptor-mediated endocytosis (8). Following lysis of the enveloping host cell membrane, the parasite comes to lie free in the cytosol and undergoes schizogonous division to become a multinucleate schizont. This process is associated with transformation of the infected cell and, by associating with the cellular spindle apparatus, the parasite ensures that each daughter cell inherits the infection (9). Clonal expansion of infected cells within the lymph node draining the site of infection is followed by metastasis to other lymphoid and non-lymphoid tissues. Invasion of tissues is accompanied by substantial pathology and cell death, so that susceptible animals often die within 3 weeks of infection (10). In a proportion of infected cells proliferation ceases and the parasite undergoes further differentiation to form uninucleate merozoite forms. Upon release from the dying cells these invade erythrocytes, where they develop into piroplasms (7,11). Recovery from infection is associated with a persistent carrier state of this parasite form (12,13), and its ingestion by subsequently feeding ticks completes the mammalian phase of the life cycle. Because piroplasms of *T. parva* undergo only limited replication (14), it is believed that maintenance of the carrier state requires persistence of small numbers of schizont-infected lymphocytes.

Lysis of infected erythrocytes in the tick gut results in release of piroplasms into the gut lumen, where they differentiate into either micro- or macro-gametes (male and female respectively) (7). These undergo syngamy to form diploid zygotes, which invade gut epithelial cells and differentiate to motile kinete forms. This process appears to be synchronized with the moulting of the tick (15). Kinetes are released into the haemocoel of the tick and migrate to the redeveloping salivary gland, where they invade specialized cells of type III acini (4,7,16). As the tick feeds on its next host, the kinete undergoes further development within the infected cell to form a large syncytial structure known as the sporoblast (16,17). Each sporoblast has been estimated to give rise to 10^4 – 10^5 uninucleate haploid sporozoites, which are released into the animal from about the fourth day of feeding (16).

The precise stage at which meiotic reduction occurs in the process of differentiation from zygote to sporozoite is somewhat unclear, although studies with DNA-binding dyes suggest that the kinete is haploid (18). This observation is consistent with the situation for malaria parasites, where meiosis occurs shortly after zygote formation (19,20), and electron microscopic studies during the course of sporogony indicate that nuclear division in the sporoblast is mitotic (17). This issue has relevance to the diversity of sporozoites within a single acinar cell. If the kinete is indeed haploid, all sporozoites derived from a single sporoblast can be expected to be genotypically identical. In contrast, at least four distinct

genotypes might be expected if a meiotic reduction occurs in the sporoblast prior to the generation of sporozoites. Clarification of this issue will require analysis of single infected acinar cells with polymorphic molecular markers.

Cattle that recover from ECF, or those immunized by infection and treatment, are solidly protected against homologous challenge (6). However protection is not assured against heterologous strains and there is evidence for considerable genetic diversity among field populations of *T. parva* (6,21,22). Nonetheless, broad protection can be achieved by immunization with relatively few strains (21), which suggests that a limited number of antigenic determinants are targeted by the immune system. There is good evidence that protection is mediated by parasite-specific class I MHC-restricted CD8⁺ cytotoxic T lymphocytes (CTL) (23). These are present at moderate frequency in immune animals and increase dramatically in number following challenge, peaking around the time that parasitosis is controlled (24). In addition, adoptive transfer of responding CD8⁺ T cells between immune and naïve identical twin calves under challenge confers protection (25). Given that CD8⁺ T cells recognize antigens as processed peptides bound to polymorphic MHC class I molecules on the surface of infected cells (26), the specificity of CTL responses to *T. parva* would be expected to vary between individual cattle.

In contrast to the MHC region of humans, which contains three class I loci that are expressed in all individuals (27), that of cattle appears to incorporate at least four classical loci, of which only a proportion is expressed in individual animals (28,29). Furthermore, the number of expressed class I genes varies between haplotypes (28). For example, while the A18 haplotype was observed to express only one classical class I gene, three genes were found on the A14 haplotype (28). The significance of this haplotype variation for immune recognition remains to be determined, and it is unclear whether all alleles expressed by multilocus class I haplotypes have equal functional significance.

Most information on the immune response of cattle to *T. parva* has been obtained using the Muguga stock of the parasite. Originally isolated in the 1940s, this was maintained for many years by cattle–tick passage until the development of tissue culture isolation and cryopreservation methods in the early 1970s (30,31). It has limited diversity and is considered antigenically homogeneous. In a recent genotypic analysis of the Muguga stock in our laboratory with 64 satellite markers, we found only four genotypes in the stock and these differ by only 1–2 markers (Frank Katzer, unpublished). A feature of the CTL response in Muguga-immunized cattle is that it is almost invariably presented, or restricted, by a product of only one of the parental class I MHC haplotypes (32,33). Where markers are available that distinguish individual products within haplotypes, restriction can often be resolved to only a single product (32). This is consistent with a tightly focused response that targets only few antigenic determinants of the parasite. When examined at a population level, some haplotypes can be observed to have a greater propensity for restricting the Muguga-specific response than others. Hence, dominant haplotypes consistently out-rank other haplotypes when present in heterozygous animals (33). When all haplotypes in a population are compared on the basis of their tendency to restrict the response, a hierarchy is observed, so that less dominant haplotypes restrict the response only when shared with one lower in the hierarchy. In view of the apparent tight focus of the CTL response, these observations suggest that the MHC phenotype of the animal is a major determining factor in its specificity.

There is also good evidence that the specificity of the response is influenced by the parasite genotype. The Marikebuni stock of *T. parva* has undergone only a limited number of passages since its isolation in the early 1980s (34) and is known to be heterogeneous both at molecular and antigenic levels. Although cattle immunized with this stock are invariably

protected against Muguga, only a proportion of Muguga-immunized animals resist challenge with Marikebuni (35). Protection is associated with the specificity of the CTL response, so that those animals that resist Marikebuni challenge make a cross-reactive response, whereas those that remain susceptible generate only Muguga-specific CTL (36). The basis of this dichotomy is incompletely understood, but appears to relate to selection of immunological determinants in the context of individual MHC phenotypes (37). This is well illustrated by an experiment in which identical twin calves bearing the KN104 MHC class I specificity were immunized, one with the Muguga stock and one with a Marikebuni clone (32). When the specificity of the CTL response in these animals was determined, the Marikebuni-immunized calf generated CTL that recognized both parasites, while its Muguga-immunized twin made only a strain-specific response. In line with the tight focus of the response discussed earlier, the CTL response in both calves was restricted by KN104. Only the Marikebuni-immunized animal resisted challenge when the calves were subsequently inoculated with the reciprocal parasites. In a related experiment, calves bearing the KN104 specificity were immunized with Muguga and observed to generate a strain-specific CTL response restricted by KN104 (32). When subsequently challenged with Marikebuni, the animals underwent a patent infection, but recovered. Significantly, when evaluated for the specificity of their response upon recovery, both calves were observed to have developed cross-reactive CTL that were also restricted by KN104. These observations suggest that although the KN104 MHC specificity is capable of presenting epitopes that are shared between Muguga and Marikebuni, a strain-specific epitope is for some reason dominant when immunization occurs in the context of Muguga. Conversely, an epitope shared between Muguga and the Marikebuni clone appears dominant when the latter is used for immunization. Immunodominance almost certainly extends beyond these two stocks and, although cattle in the field are likely to be exposed to more complex parasite populations, it must have substantial influence on the susceptibility of immune cattle to heterologous challenge.

Given the nature of the *T. parva* life cycle, the CTL response can exert selective pressure on the parasite only if it compromises transmission. If not, the population structure of the parasite in the field would be expected to be determined principally by vigour, in the absence of additional specific immune mechanisms, with those parasites that are best adapted to transmission between cattle and tick hosts prevailing. A number of recent publications are at variance with the notion that population structure of *T. parva* in the field is determined solely by vigour. Oura *et al.* using mini- and microsatellite mapping of parasite isolates from geographically diverse areas, have observed substantial diversity consistent with some form of evolutionary selection (38,39). This is supported by previous observations of Bishop *et al.* using molecular markers based on ribosomal polymorphisms and repetitive sequences (40).

In the light of the evidence for immunodominance, it is worthwhile considering the extent to which meiotic recombination of the parasite in the tick might influence its recognition by the CTL response. Consider a scenario in which an animal bearing alleles A_1 and A_2 and B_1 and B_2 at MHC class I loci A and B respectively is infected with two strains of *T. parva* with four bi-allelic antigen loci each. Assuming a narrow focus of the CTL response, let us suppose that the product of only one antigenic locus is presented by each MHC allele, so that the animal responds to two parasite antigens and, upon recovery, becomes a carrier of the piroplasm stages of both parasites. A subsequently feeding tick would be expected to ingest both gamete species, allowing them to undergo sexual recombination. Meiotic recombination of four parasite loci with two alleles each has the potential to yield 2^4 or 16 combinations. An animal immune to allelic products of two parasite loci would still be susceptible to 2^2 or 4 of these recombinants on the basis that they lack the alleles targeted by the response. Indeed, the animal in question would, in theory, be susceptible to infection

with the progeny of the parasites to which it had become immune. Assuming that cattle with identical MHC phenotypes respond to the same antigenic specificities, this simple model suggests that recombination in the tick could provide the parasite with a means of avoiding the CTL response.

The extent to which this model reflects the situation in the field is, however, clouded by a number of uncertainties. Although it has been observed that immunization with a limited number of *T. parva* strains provides broad protective cover (21), the number of determinants or loci targeted by the CTL response in individual animals has not been determined. Further, only limited information is available on the extent of allelic diversity that exists in CTL determinants of *T. parva*; reassortment of antigenic loci with no allelic variation would be expected to be immunologically neutral. Finally, although the four chromosomes of *T. parva* are likely to reassort during meiosis, it is not clear whether significant recombination of antigenic loci occurs within chromosomes (although a degree of linkage disequilibrium has been inferred by statistical modelling (39)). At a more fundamental level, it remains to be established whether the CTL response prevents establishment of the piroplasm stage of the parasite.

Two recent developments have allowed progress in clarifying these issues. First, the recent sequencing of the *T. parva* genome (41) has facilitated the development of a panel of micro and mini-satellite markers (38), which has provided opportunities for evaluating genotypic diversity in field isolates of the parasite and for estimating frequencies of crossover in recombining populations. Second, scientists at the International Livestock Research Institute in Kenya have identified several CTL determinants of *T. parva* (42) and positioned them within the genome. Where polymorphisms exist in antigen genes co-located on a given chromosome, an estimate can be obtained of the level of reassortment of antigenic loci in recombining parasite populations. We have recently been applying the satellite panel in an analysis of genotypic diversity in a stablate of the Marikebuni isolate and have found evidence for reassortment of two CTL antigen-encoding loci interspersed by distance of ~1.36 Mb on chromosome 3. We have identified two alleles at each of these loci in 32 multilocus genotypes that occur in the stablate, on the basis of PCR restriction fragment length polymorphism. Three of four possible combinations of these alleles occur among the genotypes, suggesting that recombination does occur between the loci (Frank Katzer, unpublished). Whether such reassortment results in immune evasion is clearly dependent on the degree of allelic diversity among CTL antigens of the parasite. An estimate of the latter will await sequence analysis of known CTL antigen genes in a suitable number of parasite clones. However, it would be expected to reflect the degree to which the CTL response imposes a selective pressure on parasites carrying the determinant(s) for which it is specific. Selection against a given parasite clone would require significant reduction in its transmission and hence prevention of differentiation to the piroplasm stage.

It is unclear at present how *T. parva*-specific CTL influence the emergence of piroplasms. Observations of only limited replication of *T. parva* piroplasms (14) have been interpreted as evidence that the carrier state is actually maintained by persistence of small numbers of schizont-infected cells, perhaps at immunocompromised sites. Under these circumstances, the influence of the CTL response on the emergence of piroplasms will be greatest during the acute stages of infection. It is well known that immune cattle develop schizont parasitosis following challenge with homologous or cross-reacting isolates and, although a surge in piroplasm parasitosis is often observed (see (43)), it remains to be determined whether this arises from immunizing or challenge parasites. However, multiple carrier states are common in the field, suggesting that super-infection does give rise to piroplasms (39,44,45). At another level, observations in a related parasite, *T. annulata*, indicate that individual parasite clones vary in the efficiency with which they differentiate to piroplasms

(46,47). If a similar scenario applies to *T. parva* it would be expected that those clones that differentiate efficiently might more readily evade selection, as these would be more likely to produce piroplasms before clearance of schizonts, even if only for a limited period. In the light of these observations, it is safer to assume that, if the CTL response does affect the emergence of piroplasms, it is a partial rather than an absolute effect. Hence, in an immune animal challenged with a heterologous isolate incorporating cross-reacting parasite clones, the piroplasm parasitosis of those clones would be reduced in quantity and/or duration rather than abrogated. These piroplasms could still be ingested by subsequently feeding ticks and undergo recombination, albeit in reduced numbers. Half of the progeny of this recombination would inherit the antigenic determinant targeted by the CTL response.

Although representation of these progeny in the ensuing sporozoite population would depend heavily on gamete frequency, other factors are likely to play a role. For example, malaria parasites have been reported to vary in the proportions of male and female gametes that they produce, with consequent effects on the degree of out-crossing vs. selfing (48,49). Such variation in *T. parva* might be expected to influence the contribution of individual gamete populations to the sporozoite gene pool. Events subsequent to ingestion of the parasite by the tick are also likely to influence composition of the gamete pool. For example, it is possible that bovine serum antibody responses directed at piroplasm surface antigens give rise to some parasite attrition in the immediate period following ingestion. A perhaps more significant determinant of survival for parasite recombinant genotypes is the tick immune system. Infection with *T. parva* is detrimental to the survival of *R. appendiculatus* ticks (50) and, although little is known of tick immune mechanisms, insect vectors are known to deploy a number of defensive mechanisms capable of compromising parasite survival, including oxidative metabolites in the gut lumen and haemocyte activity (51-53). Preliminary evidence for selection against parasite populations in the tick has been reported for *T. annulata* in *Hyalomma* ticks (54). Selection against *T. parva* in *R. appendiculatus* is clearly effective, given the discrepancy between the numbers of gametes ingested during feeding and the numbers of infected acinar cells that result,¹ and must alter the proportions of recombinant parasite progeny that are retained in the sporozoite population. It may therefore provide an opportunity for some recovery of progeny carrying alleles targeted by the CTL response in the source animal. Adding an additional level of complexity, the over-dispersal of *T. parva* infections in field populations of *R. appendiculatus* (55,56), suggests that individual ticks vary in the efficiency of these immune mechanisms. Whether this variation relates to specificity in the context of different parasite populations is unclear.

Notwithstanding these considerations, if it is assumed that a proportion of recombinant parasite clones carrying antigens targeted by the CTL response do survive to the sporozoite population, the question arises of how these parasites might fare following transmission to another animal. This will clearly depend on the immune status of that animal. In the event that it is naïve, no further selection would be expected on the antigenic determinants targeted by the response and the frequency of parasite clones that bear them in the ensuing piroplasm population would reflect the vigour with which they expand in the infected animal. On the other hand, even if the animal is immune, further selective pressure on the antigenic determinants will be expected only if the specificity of the response is the same as that of the original animal. As discussed earlier, available evidence suggests that this would require that the animals share the same MHC phenotype.

¹A female *R. appendiculatus* nymph ingests approximately 80 µL of blood during the course of feeding. On the basis of a bovine erythrocyte count of $5 \times 10^6/\mu\text{L}$ and an average acute piroplasm parasitaemia of 5%, this equates to an uptake of 2×10^7 piroplasms. Even highly abundant tick infections are of the order of only 10^2 infected acini, which is consistent with parasite losses of at least five orders of magnitude during differentiation in the tick.

These arguments imply that the capacity of the CTL response to impose selective pressure on *T. parva* depends on the immune status of the herd (i.e. the probability that a tick which has fed on an immune animal will feed on another immune animal in the subsequent instar) and the degree of MHC heterozygosity in the herd. The first of these variables is itself dependent on the endemic status of ECF in the herd; for example, high transmission rates would be expected to promote high levels of herd immunity and therefore potentially favour selection. The extent of MHC heterozygosity in the herd is determined primarily by breeding practices and the number of sires in use. Hence, in the more intensive management systems such as those employed by progressive dairy farmers in endemic areas, more limited MHC heterozygosity would be expected as a result of the use of artificial insemination, with a consequently greater selective pressure exerted by the CTL response. This would presumably be reflected in reduced parasite diversity in the shorter term. Conversely, increased MHC heterozygosity resulting in less selection and greater parasite diversity would be expected in more extensively managed herds requiring greater sire numbers. In this regard, evidence for variation in levels of parasite diversity between geographically distinct cattle populations has been obtained using satellite marker analysis (39), although it is not clear whether this was associated with differences in herd management systems.

The identification of a number of *T. parva* CTL antigens by ILRI scientists has intensified efforts towards development of a subunit vaccine for ECF. In addition to the possibility that meiotic recombination in the parasite may permit immune evasion, the issue of whether CTL immunity compromises transmission and influences parasite population structure is an important one in respect of the utility of such a vaccine. In line with the considerations discussed above, a product with broad protective cover deployed in an intensively managed herd with relatively low MHC heterozygosity would be expected to impose significant selective pressure on the parasite. The resulting decline in parasite challenge would provide only limited boosting of immunity by natural challenge. This would necessitate frequent revaccination and give rise to a state of endemic instability. In the longer term, such a vaccine might be expected to act as a driver for selection of antigenic variants of the parasite. Ironically, failure to compromise parasite transmission may be a desirable feature for novel vaccines against *T. parva*. This would maintain the endemic status of the disease, allowing boosting by natural challenge and less intensive vaccination. The absence of selective pressure on the parasite would also ensure continuing efficacy.

As outlined above, the substantial diversity observed in field populations of *T. parva* and the development of multiple carrier states appear inconsistent with immunity in cattle resulting in prevention of transmission. It is arguably more probable that this diversity relates to other aspects of the parasite life-cycle. The major bottleneck in the cycle occurs in the tick, where very large numbers of gametes ultimately give rise to relatively few sporoblasts. The processes that lead to this high level of attrition may well impose a much greater evolutionary pressure on the parasite than the bovine immune system.

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