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The cytotoxic T cell: MHC restriction, strain specificity and role in immunity to *Theileria parva* infection.

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Introduction

In recent years evidence has emerged that major histocompatibility complex (MHC)-restricted T cell responses play an important role in protection of cattle against East Coast fever. This, usually fatal disease occurs in East and Central Africa and is caused by an intracellular tick-borne protozoan parasite, namely *Theileria parva*. As the disease has a major economic impact on cattle production, considerable effort is devoted to defining the protective immune responses which can control the disease. It has been known for many years that cattle can be immunised against the disease by infection with sporozoites (infective stage from the tick) and simultaneous treatment with long-acting tetracyclines (Radley *et al*, 1975). However the existence of different geographical strains which do not always cross-protect, has hampered the introduction of viable parasites as a vaccine for general application in the different countries concerned. Moreover, in cross-challenge experiments with two parasite stocks, not every animal immunised with one stock is protected against challenge with the other. This parasite heterogeneity (strains) and individual animal variation in protection has stimulated further research on the characterization of the parasite and the protective immune mechanisms in order to identify cross-protective antigens for use in vaccination.

The schizont-infected cell

Theileria parva sporozoites are injected into the mammalian host by the tick during its feeding process. The sporozoites then enter bovine lymphocytes by receptor-mediated endocytosis and during the subsequent 12-24 hours the host cell membrane surrounding the parasite is destroyed so that the parasite comes to lie free in the cytoplasm (Fawcett *et al*, 1984). The intracellular parasite starts to undergo nuclear division resulting after 1-2 days post-infection, in the appearance of a typical multinucleate body, named a schizont. At this time the host cell undergoes blast transformation and starts to proliferate in synchrony with the schizont so that there is a clonal expansion of the initially infected cell population (Hullinger *et al*, 1964). Later in infection schizonts undergo merogony and merozoites thus formed infect erythrocytes, giving rise to piroplasms which are infective for the tick. However, it is the schizont stage of the parasite which is responsible for most of the pathology of the disease (Irvin and Morrison, 1987). Large numbers of schizont-infected lymphocytes are found throughout the lymphoid system and are associated with extensive lymphocytolysis. Schizont-infected cells also invade non-lymphoid tissues such as the lung and the gastrointestinal tract and, in the lungs, commonly result in severe pulmonary oedema. Susceptible cattle usually die within 4 weeks of infection.

The unique relationship of the parasite with the host cell allows the establishment of continuously growing infected cell lines by infecting normal lymphocytes *in vitro* with sporozoites (Brown, 1983). This culture system has proved invaluable in defining the nature of the target cells for infection and in studying T cell re-

sponses against the parasite. By the use of monoclonal antibodies (MAb) specific for different populations of bovine lymphocytes and accessory cells, it has been shown that B cells, both major subpopulations of T cells ($BoCD4^+$ and $BoCD8^+$) and an additional population of lymphocytes lacking B cell and T cell markers (BoWC1) can be infected *in vitro* with the parasite (Morrison *et al,* 1989). However, the majority of infected lymphocytes detected *in vivo* are T cells.

Parasite strain heterogeneity

Immunization with one stock of the parasite does not provide protection against all other stocks of the parasite. However, the parasite strain heterogeneity with respect to protection is probably limited as the use of mixtures of 2 to 3 stocks for immunisation has provided protection against challenge with a number of stocks from different geographical locations (Irvin, 1985). Although the cross-protection experiment is the best method for identifying the immunologically important strains of the parasite, it is expensive and does not always give conclusive results. Indeed, when breakthrough infections occur, they often do so only in a proportion of animals. These findings may be due in part to the use of uncloned parasite stocks, some of which appear to be heterogeneous as evidenced by laboratory methods. Nonetheless, even when cloned parasite stocks or stocks wherein no diversity has been detected, are used, animals do not always react in the same way on cross-challenge.

Parasite heterogeneity between, as well as within stocks has also been demonstrated by other methods. These include the use of parasite-specific MAb and DNA probes. When fixed parasite preparations are screened with a panel of parasite-specific MAb, different reactivity patterns with the panel are observed for the parasite stocks (Minami *et al,* 1983). Parasite heterogeneity between stocks and within stocks of the parasite has also been observed in Western blotting with MAb specific for a parasite antigen which displays marked polymorphism among populations of *T parva* (Toye *et al,* 1991). Similar differences have also been detected with parasite specific DNA probes which code for DNA sequences present as multiple copies in the *Theileria parva* genome: when used on Southern blots of parasite DNA, polymorphism in the restriction fragment lengths of parasite DNA is detected between and within stocks of *Theileria parva* (Conrad *et al,* 1989). Also differences in the protein spot patterns of two-dimensional gel electrophoresis of schizonts has been detected between and within stocks of *Theileria parva* (Sugimoto *et al,* 1989). However, whether these differences detected in the laboratory directly reflect the parasite heterogeneity in cross-protection remains unanswered; nonetheless, a correlation has quite often been observed.

Mechanisms of immunity

There are a number of features of immunity to East Coast fever that strongly suggest that protective immunity is operative against the schizont-infected cell. First, all of the successful methods of immunisation involve the establishment and development of infection to the schizont stage. Second, immune cattle, challenged with sporozoites, may exhibit transient low levels of schizont parasitosis before the infection is eliminated. Third, cattle can be immunised with schizont-infected cells and these animals are solidly immune to homologous challenge with sporozoites. There is, however, no compelling evidence that the anti-schizont antibodies play a significant role in protective immunity. Indeed, neither passive transfer of antibodies from immune to susceptible cattle, nor active induction of anti-schizont antibodies to titers comparable with those accompanying immunisation with live parasites, could protect animals against East Coast fever. This is not surprising given that the schizont does not have to leave the infected cell for its propagation and that parasite antigens have never been detected by antibodies on the surface of parasitised cells.

By contrast, evidence that cell-mediated immune responses, in particular cytotoxic T lymphocytes (CTL), play a key role in protection against East Coast fever, has accumulated during the last decade. Immunisation or challenge of immune cattle with *T parva* results in the induction of CTL which are specific for parasitised lymphoblasts. In particular the fact that *Theileria*-specific CTL are detected transiently in the peripheral blood and the local draining lymph node at the time of remission of infection, strengthens the belief of their importance in protective immunity (Eugui and Emery, 1981). Moreover, such a mechanism of immunity is compatible with the finding that establishment of

infection in the animal is required for induction of protection because CTL are only induced after they recognise antigenic changes on the cell surface in the context of self major histocompatibility complex (MHC) molecules.

Theileria-*specific cytotoxic T lymphocytes*

To study the parasite specificity and MHC restriction of *Theileria*-specific cytotoxic cells, one has to activate their precursors or memory-cells (CTLp) by *in vivo* challenge with the parasite or *in vitro* stimulation with infected autologous infected cells. By *in vivo* as wells as *in vitro* stimulation it has been shown that the *Theileria parva*-specific cytotoxic cells kill autologous parasitised lymphoblasts but not uninfected lymphoblasts (Goddeeris *et al,* 1986a; Morrison *et al,* 1987). By selecting panels of target cells of defined class I MHC phenotypes and by testing the capacity of MAb specific for class I and class II MHC molecules to inhibit cytotoxicity, the cells were shown to be restricted by class I MHC determinants. With the development of MAb specific for lymphocyte populations, the effector cells were confirmed to belong to the BoCD8$^+$ T cell population. All these results have also been confirmed with *T parva*-specific CTL clones (Goddeeris *et al,* 1986b). Thus, in analogy with other systems, the *Theileria*-specific CTL seem to recognise parasite peptides in association with autologous class I MHC molecules on the surface of the infected cell. To date however, the parasite target peptides or peptide-containing proteins have not been identified.

In order to determine the frequency of the CTLp and analyse their individual characteristics, limiting dilution microassays were used (Taracha *et al,* 1992). In immune animals CTLp frequencies ranged from ±1/2 000 to ± 1/15 000 whereas no CTLp were detected in naive animals at a cell input of 20 000 per well.

Correlation between cross-protection and parasite strain specificity of the CTL

If CTL are indeed important in protective immunity, then one would expect that differences in the capacity of parasite stocks to cross-protect would be reflected in the strain specificities of the CTL. Analyses of the strain specificity of *T parva*-specific CTL have been focused on two parasite stocks, namely Muguga and Marikebuni. Cross-protection experiments with these two stocks have shown that immunisation with Marikebuni protects against challenge with Muguga whereas a proportion of animals immunised with Muguga remains susceptible to challenge with Marikebuni. However, correlating strain specificity of CTL with results of *in vivo* cross-protection has been difficult as the Marikebuni stock contains a heterogeneous mixture of parasites (Toye *et al,* 1991). Only recently have Marikebuni sporozoites been cloned and used for immunising cattle. Only results of cross-challenge experiments with the Muguga stock (homo-geneous) and a cloned parasite of the Marikebuni stock will be discussed (Taracha, 1991).

Seventeen cattle were immunised with *T parva* (Muguga) and 5 with cloned *T parva* (Marikebuni 3219). Frequencies of CTLp in each of the animals were determined in a limiting dilution microassay in which the diluted peripheral blood mononuclear leukocytes were reactivated with autologous cells infected with the parasite used for immunisation. Strain specificities of the CTLp were determined by testing cytotoxicity on Muguga-infected and Marikebuni-infected targets. All immunised animals had CTLp frequencies of higher than 1/14 200 (from 1/2 071 to 1/14 200). However, 6 of the 17 Muguga-immunised animals and 3 of the 5 Marikebuni-immunised animals had CTLp which recognised both parasite strains. Upon challenge of the animals with a lethal dose (all naive control animals died) of the heterologous parasite, all animals, except the 6 Muguga-immunised animals and the 3 Marikebuni-immunised animals with cross-reactive CTLp, had severe reactions (2 died) characterized by fever for 6-14 days and moderate (1-10%) to high parasitosis (> 10%) in the local and contralateral lymph nodes for 7-22 days (tables I and II). After cross-challenge, CTLp were reanalysed for strain specificity by reactivating the peripheral blood mononuclear cells with the initial parasite used for immunisation: all surviving animals had CTLp which were cross-reactive *ie* recognised Muguga and Marikebuni. This experiment clearly demonstrates firstly, that the strain specificity of the *Theileria*-specific response correlates with *in vivo* cross-protection, and secondly that in a proportion of the animals, cross-reactive CTL are not induced after immunisation with a single strain of the parasite, although this strain expresses cross-reactive epitopes on its host cell. The latter observation brings us to the individual variation among the

Table I. Correlation between strain specificity of the CTLp and cross-protection.

Immunising parasite	*No of animals*	*Parasite strain specificity of CTLp*		*Parasitosis on cross-challenge*	
		Muguga	*Marikebuni*	*Severity* [a]	*Duration (d)*
Muguga	11	+	–	++ / +++	7–21
	6	+	+	+	1–3
Marikebuni	2	–	+	+++	9-13
	3	+	+	– / +	0–1

[a] Parasitosis in the local draining lymph node: – = no infected cells detected, + = < 1% infected cells, ++ = 1–10% infected cells, +++ = > 10% infected cells.

Table II. Influence of the immunising parasite stock on the strain specificity of the CTL precursors of the peripheral blood (analysed in a limiting dilution assay on two sets of cotwins).

Cotwin [a]	*BoLA-phenotype*	*Immunising parasite*	*Strain specificity*	*BoLA restriction*	*Challenge with heterologous parasite* [b]
E81	w10,KN104/KN8	Muguga	Muguga	KN104	++
E82		Marikebuni	Marikebuni	w10	+++
E260	w10,KN104/w6	Muguga	Muguga	KN104	++
E259		Marikebuni	Marikebuni + Muguga	KN104	–

[a] Both sets of cotwins received the w10,KN104 haplotype from sire 1422. [b] Parasitosis in the local draining lymph node: – = no infected cells detected, ++ = 1–10% infected cells, +++ = > 10% infected cells.

animals and the role of the MHC molecules which restrict the *T parva*-specific CTL.

Influence of the restricting MHC molecule on the strain-specificity of the CTL

That the restricting MHC molecule can indeed have an influence on the parasite strain specificity has been demonstrated previously by comparing the strain specificities of CTL clones which originated from the same animal but had different MHC restrictions (Goddeeris, 1990; Goddeeris *et al*, 1990). Two animals had been immunised with *T parva* (Muguga) whereas one animal with *T parva* (Marikebuni 3219): for each animal 2 CTL clones with different MHC restrictions were analysed for their parasite strain specificity (table III). For each animal, one clone recognised the immunising strain while the other clone recognised the immunising and the heterologous strain of the parasite. These data clearly demonstrate that the restricting MHC molecule

Table III. Influence of the restricting MHC molecule on the strain specificity of the CTL clones (2 of each animal).

Donor animal (MHC phenotype)	*Immunising stock*	*CTL clone*	*Restricting MHC specificity*	*% MHC-restricted cytotoxicity* [a] *in cells infected with*	
				Muguga	*Marikebuni*
D232 (w6.2/w2)	Muguga	T23.14	w6.2	49	7
		T23.23	Undefined molecule of the w6.2 haplotype	97	67
C196 'w6/w7)	Muguga	T26.20	w7	37	23
		T26.44	Undefined molecule of the w6 haplotype	63	0
F104 (w10,KN104 /w10,KN104)	Marikebuni	T36.39	KN104	64	69
		T36.17	w10	1	85

[a] Results of cytotoxicity were obtained in a 4-h ^{51}Cr-release assay at an effector to target ratio of 2:1 (clones of D232 and C196) and 4:1 (clones of F104).

has an influence on the parasite strain specificity of the CTL. Different MHC molecules appear to select different parasite epitopes which can be strain-specific or cross-reactive. This may be explained by the determinant selection model which proposes that MHC molecules have different affinities for different peptides and that immune responses are preferentially induced by those MHC-peptide combinations which associate with highest avidity (Buus *et al,* 1987). Peptide binding studies have been performed with purified MHC class I molecules (Chen and Parham, 1989) and have demonstrated that the capacity of a particular MHC allele to bind peptide antigen parallels the responder status.

Influence of the immunising parasite on the strain-specificity of the CTL

As the CTL to be compared, had to originate from different animals, *ie* one immunised with the Muguga stock and another with a Marikebuni clone, identical twins were used to avoid any interference from factors inherent with a different genetic background of the animals. Two sets of twins were selected and in each set, one animal was immunised with *T parva* (Muguga) and the other with *T parva* (Marikebuni 3219). In the first set of twins (E81 en E82) CTLp were strain-specific: the Muguga-immunised animal had only Muguga-specific CTLp and the Marikebuni (3219)-immunised animal had only Marikebuni (3219)-specific CTLp (table II). It was of interest to observe that the restricting MHC specificities were different which indicates an influence of the immunising parasite on selection of the restricting MHC specificity. Results with the second set of twins were even more interesting: in both animals CTLp were restricted by the same MHC specificity (KN104) but had different strain-specificities (table II). Thus, the immunising parasite also has an influence on the strain-

specificity of the CTL which is independent of its effect on the restricting MHC specificity.

An important observation here is that one MHC specificity can present different parasite epitopes on the same infected cell: KN104 MHC molecules on the surface of the Muguga-infected targets were associated with a Muguga-specific epitope as well as a cross-reactive epitope. Why then are the cross-reactive KN104-restricted CTL only induced after a Marikebuni immunisation and not after a Muguga immunisation? On the one hand, it is likely that in the Muguga-immunised animal (E260), the Muguga-specific peptide/KN104 combinations were more immuno-inductive for their specific CTL than were the cross-reactive peptide/KN104 combinations for their CTL, or that the Muguga-specific peptide had a higher affinity for the KN104 MHC molecule than the cross-reactive peptide which could result in a higher concentration on the membrane and better induction of its CTL. The latter situation has been demonstrated in studies with murine class II MHC molecules, in which differences in binding between immunogenic peptides and Ia have been observed, and T cell responses were directed predominantly to those Ia-peptide combinations which showed strongest binding (Buus *et al,* 1987). On the other hand, in the Marikebuni-immunised animal (E259), cross-reactive peptides were able to induce KN104-restricted CTL because the cross-reactive peptide/KN104 combination was not inhibited or overshadowed by a more immunodominant Marikebuni-specific peptide: the cross-reactive peptide has a higher affinity for the KN104 molecule than the Marikebuni-specific peptide, and/or the cross-reactive peptide/KN104 associations were more immuno-inductive for their T cells than any other peptide/MHC associations.

Influence of other factors on the MHC restriction and parasite strain specificity of CTL

When the two Marikebuni (3219)-immunised animals E82 and E259, which inherited their "w10,KN104"-haplotype from the same sire, are compared, it is striking that their CTLp selected different restricting MHC molecules (w10 and KN104) of the identical BoLA haplotype (table II). Moreover, two Muguga-immunized animals, E81 and D580, which shared the BoLA-haplotype "w10,KN104" from sire 1422 and had the MHC specificity KN8 on the other BoLA-haplotype in common, produced CTLp with different strain specificities although they were restricted by the same MHC molecule, KN104: animal E81 produced Muguga-specific CTLp (table II) while animal D580 produced CTLp killing Marikebuni- as well as Muguga-infected targets. Thus, it appears that factors other than the immunising parasite strain influence the strain-specificity and/or MHC restriction of the *Theileria*-specific CTL.

First of all, it is possible that the second BoLA haplotype (KN8) was not identical although its BoLA specificity at one locus was the same (*eg* the two Muguga-immunised animals) and that differences in MHC molecules other than the restricting ones influence the T cell repertoire selection. Second, non-MHC differences may also account for differences in the T cell receptor repertoire. Evidence indicates that the generation of the T-cell repertoire involves two selective processes occurring in the thymus in which thymocytes with high avidity for self molecules (with self peptides) are deleted while those with low or intermediate affinity for self-MHC molecules are selected for expansion (Blackman *et al,* 1988). Thus MHC molecules other than the restricting ones can have an influence on the generation of the T cell repertoire. Moreover, it has been shown that autologous peptides constitutively occupy the antigen binding site on Ia (Buus *et al,* 1988). Thus, autologous polymorphic proteins can also have an influence on the T cell repertoire as, during T cell education, their autologous peptide/MHC combinations can differ between animals.

Conclusion

From these studies the following conclusions can be drawn. First, a correlation between strain-specificity of *T parva*-specific CTL and *in vivo* cross-protection has been confirmed. Thus, *T parva*-specific CTL seem to be an, if not the, important immune mechanism for protection against East Coast fever. Second, the parasite strain type as well as the BoLA phenotype of the animal have important influences on the generation of cross-reactive CTL. It is suggested that the influence of the MHC occurs at the level of the MHC/epitope combination as well as at the level of the T cell repertoire generation. These conclusions have important implications for the selection of parasite antigens as subunit vac-

cines: cross-reactive epitopes will have to be separated from more immunodominant strain-specific epitopes and if several cross-reactive epitopes are needed, they will have to be incorporated in separate vectors to avoid competition in the same target cell.

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Interactions virus-lymphocytes pour la production d'interféron-α.

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Les interférons (IFN) sont des protéines souvent glycosylées, synthétisées par des cellules de vertébrés en réponse à divers signaux inducteurs (virus, bactéries, stimulation antigénique) (Lefèvre, 1989) ou au cours de situations physiologiques particulières, notamment les phases précoces du développement embryonnaire (La Bonnardière et Martal, 1991). Les IFN sont capables d'induire, dans les cellules sensibles à leur effet, un état de résistance à l'infection par un large spectre de virus à ARN et à ADN, *via* le déclenchement de nouvelles synthèses d'ARN et de protéines. Au cours d'une infection virale, ce sont les IFN-α et β qui sont sécrétés, les IFN-α étant principalement produits par les leucocytes alors que l'IFN-β est synthétisé par des cellules d'origines tissulaires très diverses (cellules épithéliales, fibroblastes, leucocytes).

Si l'on considère la *production d'IFN viro-induit par les leucocytes*, 2 mécanismes distincts sont mis en œuvre, selon que l'induction d'IFN requiert ou non une multiplication du virus dans les cellules productrices : dans le cas d'une induction d'IFN résultant d'une multiplication virale (ex: virus Sendaï), ce sont essentiellement les monocytes qui synthétisent les IFN-α/β et la fréquence des cellules mononuclées du sang humain synthétisant les ARNm correspondants peut atteindre 1-3% (Gobl *et al*, 1988). Mais hormis ce mode «classique» d'induction d'IFN, des données récentes montrent que des préparations virales inactivées ou des tapis cellulaires infectés puis fixés par le glutaraldéhyde peuvent induire des leucocytes à produire l'IFN (Lebon, 1985; Dianzani et Capobianchi, 1987) : dans ce cas, qui suppose un contact membranaire entre une structure virale et la surface des leucocytes, c'est une population cellulaire très peu fréquente (1–10 pour 10^4 PBL chez l'homme) qui produit l'IFN. Il existe donc 2 populations leucocytaires productrices d'IFN-α/β : l'une, qui est induite par exemple par le virus Sendaï infectieux, est constituée de monocytes ($CD14^+$, phagocytaires, adhérentes), l'autre, induite par exemple par le virus herpès simplex (HSV), non infectieux, est constituée de cellules mononuclées peu fréquentes, non phagocytaires, non adhérentes, pour lesquelles le terme «NIP cells» *(Natural interferon producing cells)* a été proposé (Ito *et al*, 1981; Sandberg *et al*, 1990). L'analyse des interactions entre virus et «NIP cells» entraînant la production d'IFN-α en est à ses débuts et fait l'objet de cette courte synthèse bibliographique.

Nature de la structure virale inductrice d'IFN-α par contact membranaire

Des cellules infectées par divers virus (paramyxovirus, herpes virus, coronavirus, lentivirus...) puis fixées par le glutaraldéhyde dans des conditions qui suppriment tout virion infectieux sont capables, après un contact assez bref avec des lymphocytes sanguins, d'induire la synthèse d'IFN-α par ces derniers (Lebon *et al*, 1980; Capobianchi *et al*, 1985; Kurane *et al*, 1986; Charley et Laude, 1988). Ce protocole expérimental d'induction d'IFN-α ainsi que le fait que des virions non infectieux peuvent induire l'IFN indiquent que la réplication virale n'est pas indispensable à ce mode d'induction et que le mécanisme inducteur implique un contact membranaire. De fait, Lebon (1985) a suggéré l'existence d'un récepteur membranaire exprimé par les cellules productrices d'IFN-α et impliqué dans le phénomène d'induction : en effet, les substances lysosomotropes (chlorures d'ammonium) empêchent l'induction d'IFN-α par des cellules infectées par HSV et fixées. De plus, ce