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Research paper

Dissection of the clonal composition of bovine αβ T cell responses using T cell receptor Vβ subfamily-specific PCR and heteroduplex analysis

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ABSTRACT

Although techniques that permit analysis of the clonal composition of T cell populations have been used extensively to provide a better understanding of the mechanisms that influence efficacy of T cell responses in humans and mice, such methods are lacking for other animal species. In this paper we report the establishment and validation of a panel of Vβ subfamily-specific semi-nested PCR assays, and a CDR3β heteroduplex technique for analysing the clonal diversity of bovine αβ T cell responses. Development of these methods was based on available sequence data for 48 functional Vβ genes classified within 17 subfamilies. These techniques were used to determine the clonal composition of parasite-reactive $CD8^+$ T cells obtained from two animals immunised with the protozoan parasite *Theileria parva*. Analyses of uncloned T cell lines as well as large panels of cloned T cells derived from each of these lines confirmed the specificity and sensitivity of the assays. Specific PCR products were obtained from 96% of the T cell clones examined, indicating that the currently identified Vβ genes represent most of the functional Vβ subfamilies in cattle. Heteroduplex analyses, coupled with sequencing of PCR products, identified over 20 clonal expansions within each of the T cell lines, distributed over a large number of Vβ subfamilies, although a limited number of clonotypes numerically dominated the response in both animals. The development and validation of these methods provides for the first time a generic set of molecular tools that can be used to perform detailed analysis of the TCR diversity and clonal composition of bovine T cell responses.

1. Introduction

The peripheral αβ T cell population expresses a highly diverse repertoire of clonally distributed T cell receptors (TCR). Estimates based on direct measurement indicate that in humans and mice individuals have repertoires of approximately 2×10^7 (Arstila et al., 1999) and 2×10^6 (Casrouge et al., 2000) unique T cell clonotypes respectively. This diversity is generated during thymocyte development by a process of somatic recombination whereby different permutations of discontiguous variable (V), diversity (D — β chains only) and joining (J) gene segments are joined to form the constituent α and β chains of the TCRs expressed by individual thymocytes. Nucleotide editing at the V(D)J junction by exonuclease and terminal deoxynucleotide transferase activity adds substantially to the diversity achieved during recombination (Davis and Bjorkman, 1988). Consequently, much of the variability is focused in the third complementarity determining region (CDR3), which is encoded by the V(D)J junction and forms the most intimate association with the antigenic peptide component of the peptide-MHC (pMHC) ligand (Garboczi et al., 1996; Garcia et al., 1996).

Individual pMHC are recognised by a small subset of the peripheral T cell population, which upon interaction with the pMHC are induced to undergo rapid proliferation and differentiation to form clonally expanded effector cell populations that subsequently contract to form a 'memory pool' (Sprent and Surh, 2002). Antigen (Ag)-specific populations responding to different pMHC can vary markedly in terms of both diversity of TCR gene usage and clonal

Abbreviations: Ag, antigen; CDR3, third complementarity determining region; FR, framework; pMHC, peptide-major histocompatability complex; TCR, T cell receptor; Vβ, T cell receptor β chain variable gene segment.

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composition. The Ag-specific T cells induced by some pMHC (e.g. the HLA-B57-restricted HIV-1 epitope GAG_{30-40} and the HLA-A11-restricted EBV epitope $EBNA4_{399-408}$), exhibit a severely restricted repertoire, with conserved expression of some or all of Vα, Vβ, CDR3α and CDR3β (Campos-Lima et al., 1997; Gillespie et al., 2006). At the other end of the spectrum, some pMHC (e.g. the HLA-A11 restricted EBV epitope $EBNA4_{416-424}$ and the HLA-A2 restricted HTLV-1 epitope Tax_{11-19}), induce a T cell response characterised by a highly diverse TCR repertoire (Utz et al., 1996; Campos-Lima et al., 1997). Detailed analyses of Ag-specific $CD8^+$ T cell populations indicate that they are generally polyclonal with some being composed of over 1000 different clonotypes (Pewe et al., 2004). In some cases, there is marked 'clonal dominance', with a single or limited number of clonotypes numerically dominating the response that also includes a large number of low frequency clonotypes (Maryanski et al., 1996; Naumov et al., 1998; Chen et al., 2001; Peggs et al., 2002), whilst in other responses the contribution of clonotypes is more uniform (Turner et al., 2003).

The $CD8^+$ T cell responses to many pathogens exhibit immunodominance, whereby despite the presence of enormous numbers of potentially immunogenic epitopes, only a limited number elicit a response (Yewdell and Bennink, 1999). In the case of pathogens that exhibit antigenic variability, the TCR diversity of T cells responding to immunodominant epitopes has the potential to influence the strain specificity of the response. Studies of $CD8^+$ T cell responses to immunodominant epitopes in SIV, HIV and HCMV have provided evidence that clonally diverse responses have a greater ability to recognise variant epitopes expressed by heterologous strains and thereby control infection by preventing emergence of 'escape mutants' (Douek et al., 2002; Lopes et al., 2003; Meyer-Olson et al., 2004; Price et al., 2004; Davenport et al., 2007).

Most studies of immunodominant $CD8^+$ T cell responses have focused on viral or bacterial pathogens. Previous work by members of our group has provided evidence that immunodominance is also a feature of $CD8^+$ T cell responses to the bovine protozoan parasite *Theileria parva* (Goddeeris et al., 1990; Taracha et al., 1995b; Morrison 1996). Parasite-specific $CD8^+$ T cells have been shown to be key mediators of immunity to *T. parva* in animals immunised with live parasites (McKeever et al., 1994) and correlation between the strain specificity of protection *in vivo* and $CD8^+$ T cell responses has been demonstrated (Taracha et al., 1995a). We are currently investigating the role of immunodominance in determining parasite-strain specificity of $CD8^+$ T cell responses to *T. parva*, utilising recently identified parasite antigens and epitopes (Graham et al., 2006). As part of these investigations we wish to examine the clonal composition and TCR diversity of the $CD8^+$ T cell response against both the whole pathogen and defined epitopes. The recent publication of sequence data for an extended set of bovine TCRβ cDNAs (Houston, 1997; Houston and Morrison, 1999; Houston et al., 2005) has increased the known Vβ repertoire to 43 genes distributed over 16 subfamilies and made possible the development of appropriate techniques for analysing the TCR repertoire in bovine T cell responses.

In this paper we describe i) the development and validation of Vβ subfamily-specific semi-nested PCR assays for currently identified bovine Vβ subfamilies and ii) the establishment of a CDR3β heteroduplex assay that can be employed to identify clonal expansions within complex bovine T cell populations. By applying these techniques we have been able to provide preliminary evidence that the $CD8^+$ T cell response to *T. parva* shows substantial TCR diversity and is polyclonal but dominated by a limited number of large clonal expansions.

2. Materials and methods

2.1. Animals

Ten Holstein–Friesian cattle were used in this study. Animals were selected on the basis of their class I MHC types, determined by a combination of serological typing with class I-specific monoclonal antibodies (Ellis et al., 2005) and class I allele-specific PCR (Ellis et al., 1998). They included two animals, 592 and 468, defined as homozygous for the A10 and A18 bovine MHCI haplotypes respectively. Blood was collected and peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as described previously (Goddeeris and Morrison, 1988).

2.2. Theileria parva-reactive $CD8^+$ T cell lines

Parasite-reactive $CD8^+$ T cell lines were generated from cattle that had been immunised against *T. parva* by the 'infection and treatment' method (Radley et al., 1975), using the protocol described by Goddeeris and Morrison (1988). In brief, PBMC were stimulated 3 times at weekly intervals by co-culture with gamma-irradiated autologous *T. parva*-infected cells. Prior to the third stimulation cell lines were depleted of $CD4^+$ T cells and γδ T cells by antibody and complement-mediated lysis using monoclonal antibodies IL-A12 (CD4-specific, IgG2a) and CC15 (WC1-specific, IgG2a) and rabbit serum as the source of complement. During the third stimulation the culture medium was supplemented with 100 U/ml recombinant human IL-2 (Chiron Corporation, Emeryville, CA., USA). The resulting cell lines were analysed by flow cytometry to confirm effective depletion of $CD4^+$ and γδ T cells. $CD8^+$ T cell clones were generated from these cell lines by limiting dilution and maintained employing the conditions detailed in Goddeeris and Morrison (1988).

2.3. PCR assays

Total RNA was extracted from PBMC, T cell lines and clones using Tri-reagent (Sigma-Aldrich, Poole, Dorset, UK) and cDNA subsequently synthesised using the Reverse Transcription System (Promega, Madison, WI, USA) with priming by the Oligo $(dT)_{15}$ primer, according to the manufacturers' instructions. Vβ subfamily-specific 5′ primers (Table 1) and Cβ gene-specific 3′ primers (BCext — CTG GAC CTG CTT CCT GTT CAC, BCint — GGTCAGCTC CAC GTG GTC and BCON-4 — CTC TGC TTC CGA GGG TTC) were designed based on sequences available at the commencement of the study. All primers used in this study were manufactured by MWG Biotech (Ebersberg, Germany).

Single-round PCRs were conducted in reactions composed of DNA template, 10 pmol each of the relevant 5′ and 3′ primers, 0.5 units of Biotaq (Bioline, London, UK) and 2 μl SM-