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Citation for published version:
Graham, SP, Pelle, R, Yamage, M, Mwangi, DM, Honda, Y, Mwakubambanya, RS, de Villiers, EP, Abuya, E, Awino, E, Gachanja, J, Mbwika, F, Muthiani, AM, Muriuki, C, Nyanjui, JK, Onono, FO, Osaso, J, Riitho, V, Saya, RM, Ellis, SA, McKeever, DJ, MacHugh, ND, Gilbert, SC, Audonnet, JC, Morrison, WI, van der Bruggen, P & Taracha, EL 2008, 'Characterization of the fine specificity of bovine CD8 T-cell responses to defined antigens from the protozoan parasite Theileria parva' Infection and Immunity, vol. 76, no. 2, pp. 685-694. DOI: 10.1128/IAI.01244-07

Digital Object Identifier (DOI):
10.1128/IAI.01244-07

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

OPEN ACCESS

Characterization of the Fine Specificity of Bovine CD8 T-Cell Responses to Defined Antigens from the Protozoan Parasite *Theileria parva*[▽]

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Immunity against the bovine intracellular protozoan parasite *Theileria parva* has been shown to be mediated by CD8 T cells. Six antigens targeted by CD8 T cells from *T. parva*-immune cattle of different major histocompatibility complex (MHC) genotypes have been identified, raising the prospect of developing a subunit vaccine. To facilitate further dissection of the specificity of protective CD8 T-cell responses and to assist in the assessment of responses to vaccination, we set out to identify the epitopes recognized in these *T. parva* antigens and their MHC restriction elements. Nine epitopes in six *T. parva* antigens, together with their respective MHC restriction elements, were successfully identified. Five of the cytotoxic-T-lymphocyte epitopes were found to be restricted by products of previously described alleles, and four were restricted by four novel restriction elements. Analyses of CD8 T-cell responses to five of the epitopes in groups of cattle carrying the defined restriction elements and immunized with live parasites demonstrated that, with one exception, the epitopes were consistently recognized by animals of the respective genotypes. The analysis of responses was extended to animals immunized with multiple antigens delivered in separate vaccine constructs. Specific CD8 T-cell responses were detected in 19 of 24 immunized cattle. All responder cattle mounted responses specific for antigens for which they carried an identified restriction element. By contrast, only 8 of 19 responder cattle displayed a response to antigens for which they did not carry an identified restriction element. These data demonstrate that the identified antigens are inherently dominant in animals with the corresponding MHC genotypes.

Major histocompatibility complex (MHC) class I-restricted CD8 T-cell responses have been shown to play an important role in the immune response against a number of protozoan infections, including *Plasmodium* spp. (3, 22) and *Theileria* spp. (14) infections. *Theileria parva* causes an often fatal lymphoproliferative disease of cattle known as East Coast fever (16). Like *Plasmodium* spp., *T. parva* initially infects and replicates in nucleated cells, after which it invades erythrocytes. However, the ability of *T. parva* to transform host lymphocytes and undergo rapid multiplication in the transformed cells, as well as the limited replicative capacity of the intraerythrocytic piroplasm stage, represent fundamental differences from the corresponding plasmodial stages. Cattle that recover from an initial exposure to *T. parva*, either spontaneously or as a result of chemotherapy, are solidly immune to homologous and, in certain instances, heterologous challenge (17). The long duration of immunity to East Coast fever also contrasts with immunity to malaria, which develops only after several years of exposure and wanes in the absence of exposure to parasite challenge (9). The ability to culture the schizont stage of the parasite in continuously growing lymphoblastoid cell lines allows detailed in vitro analyses of T-cell responses to the whole parasite; this system has been used extensively to

monitor responses in animals undergoing immunization and challenge and to examine the specificity of the responses (6, 7, 8, 10, 11, 15, 19, 20).

Evidence that immunity to *T. parva* is mediated by MHC class I-restricted CD8 T cells specific for the intralymphocytic schizont stage of the parasite is based on observations on the kinetics of CD8 T-cell responses (15) and the demonstration that immunity can be transferred adoptively with CD8 T-cell-enriched populations from immune cattle (13). Parasite-specific CD8 T-cell responses induced by immunization with a single parasite strain show differential degrees of recognition of heterologous parasite strains, and a close correlation between the specificity of CD8 T-cell responses and the cross-immunity profiles of distinct parasite strains has been observed previously (8, 19). Analyses of CD8 T-cell responses of cattle of defined MHC genotypes have suggested that strain specificity is due, in part, to the focusing of the responses of individual animals on a limited number of immunodominant peptides and MHC determinants (8, 19).

Recently, six *T. parva* antigens recognized by CD8 T cells have been identified using CD8 T-cell lines from immune cattle to screen a parasite cDNA library and a set of cDNAs selected on the basis of bioinformatic analyses of the parasite genome (10, 11). All but one of the CD8 T-cell lines used for screening identified a single antigen, and lines from cattle of different MHC genotypes tended to identify different antigens. Additional analyses of a number of these target antigens have demonstrated that they are polymorphic (R. Pellé, unpublished data). The identification of schizont antigens provides an opportunity not only to explore vaccination, but also to dissect further the specificity of CD8 T-cell responses in order to understand better the basis of the strain specificity of immunity to *T. parva*. Here, we report the identification of antigenic epitopes in the six CD8 T-cell target antigens and their MHC restriction specificities. By examining responses of cattle to immunization with live parasites or a cocktail of the CD8 T-cell target antigens, we also provide evidence that these antigens are highly dominant in animals with the corresponding MHC genotypes.

MATERIALS AND METHODS

Cattle and cell lines. All animal experimentation procedures were reviewed and approved by the International Livestock Research Institute (ILRI) Institutional Animal Care and Use Committee or the United Kingdom Home Office. Boran (*Bos indicus*), Jersey (*Bos taurus*), Holstein-Friesian (*Bos taurus*), and crossbred cattle with no prior exposure to *T. parva* were recruited for the study. Cattle of defined MHC genotypes were included. MHC class I types were determined using a combination of serological typing with alloantisera (21) and/or monoclonal antibodies (5) and allele-specific PCR (4). Most of the animals expressing one of three fully defined class I haplotypes (*B. taurus* A10 and A18 and *B. indicus* A10-KN104) were the progeny of bulls known to carry these haplotypes, and this group included several animals produced by father-daughter mating that were homozygous for the MHC type. For experiments involving the immunization of cattle with *T. parva* antigens, the expression of specific class I alleles was also confirmed functionally by testing the ability of cells from individual typed animals to present peptide to CD8 T-cell lines of the appropriate MHC restriction specificity. Lymphoblasts obtained by the stimulation of peripheral blood mononuclear cells with concanavalin A (Sigma, Poole, United Kingdom) (6) were pulsed with titration preparations of antigenic peptides for 1 h at 37°C and then fixed in 0.1% glutaraldehyde as described previously (12). Cells (2.5×10^4/well) were cocultured with antigen-specific CD8 T-cell lines, and recognition was assessed by a gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay (10).

Cattle were immunized against the *T. parva* Muguga stock by the infection-and-treatment method and then challenged after 3 months with the same parasite stock described previously (19). *T. parva*-specific polyclonal CD8 T-cell lines and clones were established from the peripheral blood of immunized animals by repeated restimulation with irradiated autologous infected lymphoblasts as previously described (6). After three rounds of in vitro restimulation, CD8 T cells were purified by magnetic cell sorting with a MACS system according to the instructions of the manufacturer (Miltenyi Biotec, Gergisch Gladbach, Germany). CD8 T cells were sorted indirectly using a monoclonal antibody specific for bovine CD8 (IL-A105; ILRI, Nairobi, Kenya), followed by incubation with goat anti-mouse immunoglobulin G microbeads (Miltenyi Biotec). CD8 T cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (tested for bovine viral diarrhea virus and *Mycoplasma* spp.; Perbio Science UK, Ltd., Cramlington, United Kingdom), 10 U of recombinant human interleukin-2 (Sigma)/ml, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, 50 μg of gentamicin/ml, 5×10^{-5} M 2-mercaptoethanol, and 2 mM L-glutamine and were stimulated every 14 days with irradiated autologous *T. parva*-infected lymphoblasts at a responder-to-stimulator ratio between 2:1 and 10:1 (6). The specificities of the CD8 T-cell lines were assessed using a chromium-51 release assay (6). CD8 T-cell lines were harvested 6 to 8 days poststimulation and cocultured for 4 h with chromium-51 (Amersham)-labeled *T. parva*-infected lymphoblasts, peptide-pulsed skin fibroblasts, or transfected COS-7 cells (10). The degree of lysis of target cells was calculated as follows: % lysis = 100 × (test release − spontaneous release)/(maximum release − spontaneous release).

Alternatively, CD8 T-cell specificity was assessed by measuring the release of IFN-γ by an ELISPOT assay (10) or, in limited instances, by a bioassay (1). CD8 T cells were harvested 7 to 14 days poststimulation and resuspended at 2 × 10^5/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5 U of recombinant human interleukin-2 (Sigma)/ml and were cocultured with autologous immortalized skin fibroblasts or COS-7 cells pulsed with synthetic peptides or transfected with plasmids expressing parasite antigens. Experiments directed at epitope mapping and the identification of MHC class I restriction elements utilized CD8 T cells from eight cattle, including the cell lines and the clone used previously to identify the six *T. parva* antigens (Table 1; 10). The generation of the clonal CD8 T-cell line from animal D409 was described previously (8).

T. parva antigens. Six *T. parva* antigens, Tp1, Tp2, Tp4, Tp5, Tp7, and Tp8, previously identified to be the targets of CD8 T-cell responses of *T. parva*-immune cattle, were used in this study (Table 1) (10). cDNA corresponding to the full-length open reading frame (ORF) for Tp2 was cloned into the eukaryotic expression plasmid vector pTargeT (Promega, Madison, WI), while the full-length ORFs for Tp1, Tp4, and Tp5 were cloned into the eukaryotic expression plasmid vector pcDNA3 (Invitrogen, Paisley, United Kingdom) (10). Partial cDNAs encoding amino acids 1 to 291 of the 721 amino acid residues of Tp7 and all but the first 22 amino acid residues of Tp8 were also cloned into pcDNA3.

Cloning and functional screening of BoLA class I cDNA. mRNA was purified from bovine peripheral blood mononuclear cells by using the mRNA DIRECT kit (Dynal Biotech, Oslo, Norway). Reverse transcriptase PCR was conducted using degenerate primers designed with sequences from the partially conserved regions of published bovine leukocyte antigen (BoLA) class I cDNA sequences (5′-ATG[A/G]GGGCCGCGA[A/G]CCC[T/A]-3′ and 5′-TCA[A/C][A/G]CTTTA GGAAC[C/T][G/A]TG[A/C]G-3′). PCR products were cloned into pTargeT (Promega), and orientation was confirmed by PCR with a T7 forward primer and a pan-BoLA class I gene reverse primer (5′-CCAGGTATCTGCGGAGCC-3′). Plasmid DNA from all positive clones was purified with a QIAprep Spin miniprep kit (QIAGEN, Crawley, United Kingdom), and aliquots of 50 ng/well were used to transfect 2×10^4 COS-7 cells/well in 96-well flat-bottom plates. Twenty-four hours posttransfection, COS-7 cells were detached and the surface expression of BoLA class I was assessed by staining with a pan-BoLA class I monoclonal antibody (IL-A88; ILRI, Nairobi, Kenya) (5) and analysis by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA). COS-7 cells were cotransfected with positive cDNA clones (100 ng/well) and the appropriate CD8 T-cell target antigen cDNA (100 ng/well), and the recognition of transfectants by CD8 T-cell lines was assessed by an IFN-γ ELISPOT assay as described previously (10). For cytotoxicity assays, COS-7 cells transfected with BoLA and CD8 T-cell target antigen cDNAs and *T. parva*-infected cells were labeled with chromium-51 (Amersham Biosciences Europe GmbH, Freiburg, Germany) and lysis by parasite-specific T-cell lines was assessed by measuring isotope release after incubation for 4 h at 37°C (6).

Mapping and identification of antigenic peptides. Exonuclease III digestion was used to generate truncated cDNA clones to assist in mapping the epitope-encoding region of the Tp1 gene. Recombinant Tp1 plasmid DNA was digested with ApaI to generate exonuclease III-resistant protruding 3′ termini. The lin-

TABLE 1. Summary of CD8 T-cell target *T. parva* antigens and cattle from which antigen-specific CD8 T-cell lines were generated

Antigen	GenBank accession no. of corresponding gene sequence	No. of amino acids[a]	Functional annotation[b]	Antigen-specific CD8 T-cell donor[c]	MHC class I serotype of donor animal[d]
Tp1	XP_762973	543	Hypothetical protein	BV115	A10/A18
Tp2	XP_765583	174	Hypothetical protein	BW002	−/−
				BW013	A10/−
				BW014	A10/−
				D409[e]	A7/A10-KN104
Tp4	XP_763228	579	ε-TCP1	BX063	A10-KN104[f]
Tp5	XP_765334	155	eIF-1A	BV050	−/−
Tp7	XP_764810	721	Hsp90	BW012	−/−
Tp8	XP_764709	440	Cysteine proteinase	BX063	A10-KN104[f]

[a] The size of the full-length protein predicted by each gene sequence is given.
[b] Abbreviations: ε-TCP1, eta subunit of T complex protein 1; eIF-1A, translation elongation initiation factor 1A; Hsp90, heat shock protein 90.
[c] Parasite-specific polyclonal CD8 T-cell lines were generated from cattle following infection-and-treatment immunization and challenge.
[d] MHC class I type determined on the basis of parentage and the reactivity of cells with monoclonal antibodies. Minus signs denote unknown haplotypes.
[e] A parasite-specific CD8 T-cell clone was isolated from this animal (8).
[f] Homozygous MHC serotype.

earized plasmid DNA was purified by phenol-chloroform extraction and digested with XhoI, which cut at a unique vector cloning site to generate exonuclease III-sensitive recessed 3′ termini. The linearized DNA was purified as described above, and 100-ng aliquots were digested with 1 U of exonuclease III (New England BioLabs, Beverly, MA) in 20-μl reaction mixtures at 37°C for various times (from 0 to 30 min). Reactions were stopped by 10 min of incubation at 75°C. DNA was ethanol precipitated, washed with 70% ethanol, air dried, and redissolved in 20 μl of 1× Mung Bean nuclease reaction buffer (New England BioLabs). Five units of Mung Bean nuclease was added to each reaction mixture, and the mixtures were incubated at 30°C for 1 h to remove single-stranded DNA extensions and create blunt ends that could be ligated. The DNA was purified, precipitated, washed, and dissolved in 10 μl of sterile distilled water. For re-ligation, 5 μl of DNA was mixed with 1 μl of 10× T4 DNA ligase buffer, 3 μl of water, and 1 μl of T4 DNA ligase (New England BioLabs) and the mixture was incubated at 16°C overnight. Competent *Escherichia coli* DH5α cells were then transformed with 2 μl of the ligation mixture, plated onto agar plates containing ampicillin, and grown overnight. Plasmid DNA from single bacterial colonies was purified by using the Wizard Plus SV miniprep DNA purification kit (Promega), and inserts were excised by digestion with BamHI and NotI. Plasmid clones with cDNA inserts of different sizes were selected for IFN-γ ELISPOT screening. Oligonucleotide primers were designed to allow the amplification of three overlapping fragments of a 387-bp region of Tp1 cDNA, six overlapping fragments of Tp4 cDNA, four overlapping fragments of Tp7 cDNA, and six overlapping fragments of Tp8 cDNA, which were cloned into pTargeT (Promega) (see Fig. 1). The recognition of the truncated subclones by CD8 T-cell lines was then analyzed by an IFN-γ ELISPOT assay. Synthetic overlapping peptides spanning the entire lengths of Tp2 (12-mers offset by two residues) and Tp5 (11-mers offset by two residues) and a defined epitope-containing region of Tp1 (12-mers offset by two residues) were obtained from Mimotopes (Clayton, Australia), while peptides spanning epitope-containing regions of Tp4, Tp7, and Tp8 (15-mers offset by four residues) were obtained from Pepscan Systems B.V. (Lelystad, The Netherlands). Peptides were dissolved in a solution of 50% (vol/vol) DNA synthesis-grade acetonitrile-water (Applied Biosystems, Warrington, United Kingdom), aliquoted, and stored at −20°C.

Autologous immortalized skin fibroblasts (10) were cocultured with parasite-specific CD8 T cells in the presence of peptides at a concentration of 1 μg/ml, and recognition was assessed by an IFN-γ ELISPOT assay. Based on the sequences of positive overlapping peptides, individual 8-, 9-, 10-, and 11-mer peptides were synthesized, and titration preparations of peptides were tested using an IFN-γ ELISPOT assay. To test for cytotoxic activity, immortalized skin fibroblasts were pulsed overnight with peptides at a concentration of 1 μg/ml and labeled with chromium-51 (Amersham) and the degree of lysis by CD8 T-cell lines was measured (6).

Assessment of the specificity of CD8 T-cell responses following heterologous primer-booster immunization of cattle with recombinant plasmid DNA and viruses. Recombinant pSG2 DNA vaccine plasmids, canary pox (CP) virus, and the modified vaccinia virus Ankara strain (MVA) expressing Tp1, Tp2, Tp4, Tp5, and Tp8 ORFs were used in a heterologous primer-booster regimen to immunize

calves that had been identified as expressing a BoLA class I allele capable of presenting one or more of the defined antigens to CD8 T cells (10). The recombinant DNA, CP virus, and MVA constructs expressing the five single antigens were prepared separately and inoculated at different sites on each animal. Cattle were primed with 0.5 mg of each recombinant DNA plasmid by intradermal injection or with 10^8 PFU of each recombinant CP virus by subcutaneous injection. After 4 weeks, cattle were inoculated subcutaneously with 5×10^8 PFU of each MVA recombinant. Control calves received sham inoculations with phosphate-buffered saline. Three weeks after the MVA booster, cattle were challenged with a lethal dose of *T. parva* sporozoites (1:20 dilution of *T. parva* Muguga stock sporozoite stabilate no. 4133; ILRI, Nairobi, Kenya). Purified CD8 T cells (2.5×10^5/well) and monocytes (2.5×10^4/well) were cocultured with pools of overlapping synthetic peptides (at a 1-μg/ml final concentration) covering the entire antigens (Tp2 12-mers offset by two residues [Mimotopes], Tp5 11-mers offset by two residues [Mimotopes], and Tp1, Tp4, and Tp8 16-mers offset by four residues [Pepscan Systems]), and responses were measured by an IFN-γ ELISPOT assay (10). Responses were classified as antigen specific when the number of spot-forming cells (SFC) in wells containing peptide was three times greater than that obtained with T cells in medium alone. For all responses, the number of SFC among peptide-stimulated cells was >2 standard deviations (95% confidence interval) above the mean number of SFC among unstimulated cells.

Statistical analysis. An analysis of variance for the evaluation of fixed effects on different traits was performed by using SAS Release 8.2 (SAS Institute Inc., Cary, NC).

Nucleotide sequence accession numbers. The nucleotide sequences of the BoLA class I cDNAs for T2c, T5, and T7 have been deposited in GenBank under accession numbers EU189194, EU189195, and EU189196, respectively.

RESULTS

Identification of epitopes on *T. parva* antigens recognized by CD8 T cells. A two-step approach to the identification of the CD8 T-cell epitopes on *T. parva* antigens was adopted, utilizing CD8 T-cell lines from *T. parva*-immune animals in an IFN-γ ELISPOT assay. The first step employed a molecular approach to map the epitope-containing regions, and the second step involved the screening of overlapping synthetic peptides for fine mapping and definition of the antigenic peptides. Due to their small size, Tp2 and Tp5 were omitted from the first step and peptides spanning the entire proteins were utilized.

Screening for CD8 T-cell recognition of autologous immortalized skin fibroblasts transfected with cDNA clones encoding segments of antigens Tp1, Tp4, Tp7, and Tp8 identified regions

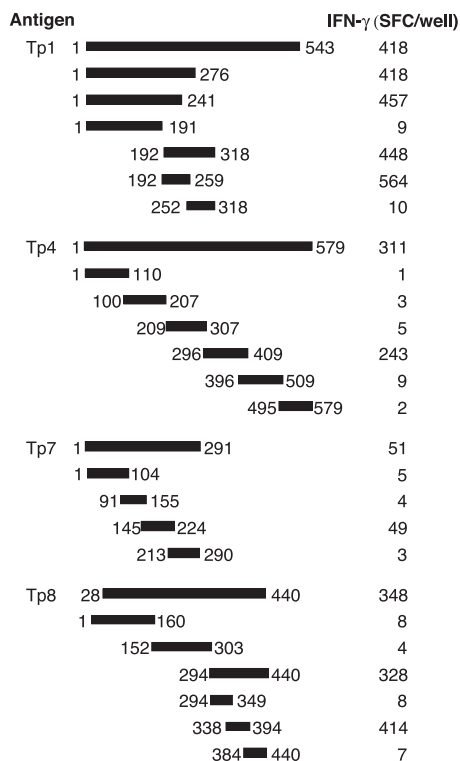| Antigen		IFN-γ (SFC/well)
Tp1	1 — 543	418
	1 — 276	418
	1 — 241	457
	1 — 191	9
	192 — 318	448
	192 — 259	564
	252 — 318	10
Tp4	1 — 579	311
	1 — 110	1
	100 — 207	3
	209 — 307	5
	296 — 409	243
	396 — 509	9
	495 — 579	2
Tp7	1 — 291	51
	1 — 104	5
	91 — 155	4
	145 — 224	49
	213 — 290	3
Tp8	28 — 440	348
	1 — 160	8
	152 — 303	4
	294 — 440	328
	294 — 349	8
	338 — 394	414
	384 — 440	7

FIG. 1. Mapping of CD8 T-cell epitopes on defined *T. parva* antigens. The recognition of autologous immortalized skin fibroblasts transiently transfected with full-length, partial-length, exonuclease III-digested, or cloned fragments of cDNA encoding Tp1, Tp4, Tp7, and Tp8 by antigen-specific CD8 T-cell lines (those from animals BV115 [Tp1], BX063 [Tp4 and Tp8], and BW012 [Tp7]) was assessed by an IFN-γ ELISPOT assay. The results of one of two independent experiments are shown. The data are the mean numbers of $SFC/10^4$ CD8 T cells for triplicate cultures. The amino acids included in the antigen peptides are indicated.

encoding antigenic peptides (Fig. 1). For one of the antigens, Tp1, a set of four truncated cDNA clones that had been digested to various lengths by exonuclease III and Mung Bean nuclease was used. Screening of these clones with a Tp1-specific CD8 T-cell line showed that the antigenic peptide(s) lay between amino acid residues 192 and 241. The presence of an epitope(s) in this region was confirmed by screening a further three overlapping cDNA subclones spanning the corresponding part of the gene. Sets of sequential overlapping cDNA clones were used to locate the epitope-containing regions in a further three antigens, Tp4, Tp7, and Tp8. In the case of Tp7, these clones corresponded to the first 291 amino acids of the protein (full length, 721 amino acids), as the original cDNA clone identified by the screening of a parasite cDNA library with CD8 T cells encodes only this part of the protein (10). The screening of sets of six, four, and three overlapping fragments of Tp4, Tp7, and Tp8 cDNAs, respectively, with the respective specific CD8 T-cell lines established the epitope-containing regions as amino acids 296 to 409 of Tp4 ($Tp4_{296-409}$), $Tp7_{145-224}$, and $Tp8_{294-440}$. The screening of a further three short overlapping cDNA clones corresponding to residues 294 to 440 of Tp8 further resolved the epitope-containing region to $Tp8_{338-394}$.

Libraries of overlapping synthetic peptides covering the de-fined epitope-containing regions of Tp1, Tp4, Tp7, and Tp8, along with the entire lengths of Tp2 and Tp5, were screened to determine the sites of the epitopes. Three polyclonal CD8 T-cell lines and one cloned antigen-specific CD8 T-cell line, derived from different cattle, were used to screen the Tp2 antigen, whereas a single polyclonal T-cell line was used for each of the other antigens. CD8 T-cell reactivity against two overlapping peptides of Tp1, Tp4, Tp5, and Tp7 and against three overlapping Tp8 peptides was observed (data not shown). The four CD8 T-cell lines used to screen Tp2 peptides identified four distinct epitope-containing sites (data not shown).

The minimal-length antigenic peptide that gave maximal CD8 T-cell reactivity was determined for each antigenic site by screening titration preparations of shorter peptides spanning the epitope-containing regions with an IFN-γ ELISPOT assay (Fig. 2). This screening resulted in the identification of the minimal-length antigenic peptides as the 11-mer $Tp1_{214-224}$ (VGYPKVKEEML; $P < 0.01$) and the 9-mers $Tp4_{328-336}$ (TGASIQTTL; $P < 0.01$), $Tp5_{87-95}$ (SKADVIAKY; $P < 0.01$), $Tp7_{206-214}$ (EFISFPISL; $P < 0.01$), and $Tp8_{379-387}$ (CGAELNHFL; $P < 0.01$) (Fig. 2). The Tp2-specific CD8 T cells from animals BW002, BW013, D409, and BW014 identified two 11-mers, $Tp2_{27-37}$ (SHEELKKLGML) and $Tp2_{49-59}$ (KSSHGMGKVGK), and two 9-mers, $Tp2_{96-104}$ (FAQSLVCVL) and $Tp2_{98-106}$ (QSLVCVLMK), respectively ($P < 0.01$) (Fig. 2).

The ability of the nine identified minimal-length antigenic peptides to elicit cytotoxic activity in specific CD8 T-cell lines was examined using autologous immortalized skin fibroblasts pulsed with peptide, and the resultant cytotoxicity was compared with that obtained using parasitized cells as targets (Fig. 3). In all cases, the degree of lysis of peptide-pulsed cells was greater than (for T-cell lines from animals BV115 [$Tp1_{214-224}$], BW014 [$Tp2_{98-106}$], BV050 [$Tp5_{87-95}$], and BX063 [$Tp8_{379-387}$]) or comparable to (for T-cell lines from animals BW002 [$Tp2_{27-37}$], BW013 [$Tp2_{49-59}$], D409 [$Tp2_{96-104}$], BX063 [$Tp4_{328-336}$], and BW012 [$Tp7_{206-214}$]) that of infected cells, further indicating that these peptide sequences are likely to represent the natural epitopes ($P < 0.01$).

Identification of BoLA restriction of CD8 T-cell epitopes on *T. parva* antigens. Only a few of the MHC class I genes expressed by the animals used in these experiments had been identified at the outset of the studies (10). The *B. taurus* haplotype carrying the serologically defined class I specificity A18 was known to express a single class I gene, N*01301, and this gene had been used in the antigen-screening protocol that resulted in the identification of Tp1. A second haplotype, found in *B. indicus* cattle, carries the A10 and KN104 serological specificities, which are encoded by the N*00101 and N*00301 class I genes, respectively; these genes were also used in the antigen-screening protocol that identified the Tp4 and Tp8 antigens. Subsequent experiments involving cotransfection with the class I and antigen genes demonstrated that both Tp4 and Tp8 are presented by the N*00101 gene product. The recognition of the epitopes identified in Tp1, Tp4, and Tp8 in the context of these class I molecules was confirmed by testing COS-7 cells transfected with the individual class I genes and CD8 target antigen cDNAs in an IFN-γ ELISPOT assay (Fig. 4).

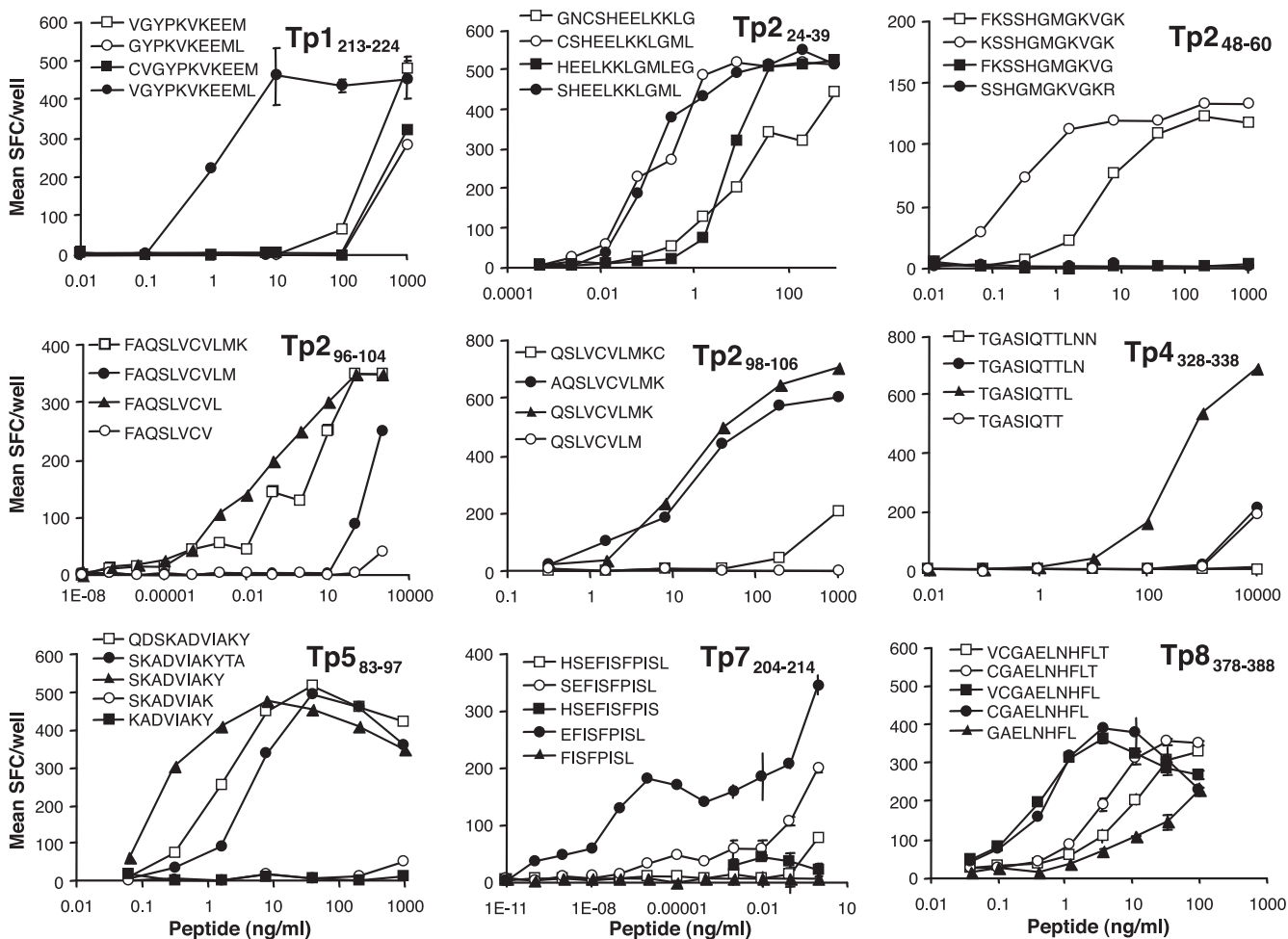The MHC restriction specificities of antigen recognition by

FIG. 2. Identification of CD8 T-cell epitopes on defined *T. parva* antigens. Titration preparations of overlapping synthetic peptides were used to pulse immortalized skin fibroblasts, and the recognition by antigen-specific CD8 T cells (those from animals BV115 [Tp1$_{213-224}$], BW002 [Tp2$_{24-39}$], BW013 [Tp2$_{48-60}$], D409 [Tp2$_{96-106}$], BW014 [Tp2$_{97-107}$], BX063 [Tp4$_{328-338}$ and Tp8$_{378-388}$], BV050 [Tp5$_{83-97}$], and BW012 [Tp7$_{204-214}$]) was assessed using an IFN-γ ELISPOT assay. Minimal-length antigenic peptides were identified as the shortest peptides that induced CD8 T-cell reactivity at the lowest concentration. The results of one of two independent experiments are shown. The data are the mean numbers of SFC/10^4 CD8 T cells ± standard errors of the means for triplicate cultures.

the remaining T-cell lines were determined by isolating a set of cloned full-length class I MHC heavy-chain cDNAs from each of the donor animals, cotransfecting COS-7 cells with these cDNAs and the respective CD8 target antigen cDNAs, and screening for recognition by antigen-specific CD8 T-cell lines. The nucleotide sequences of class I cDNAs giving positive responses were then determined.

Using this approach, we identified class I cDNAs capable of presenting the antigens for all of the remaining T-cell lines. As an example, the screening of cDNA clones from animals BW013 and BW014, which recognized the Tp2 antigen, detected 2 of 17 and 1 of 9 class I cDNA clones, respectively, that were effective at presenting Tp2 to the antigen-specific CD8 T-cell lines from these animals. All three positive cDNAs were shown to correspond to the class I allele N*01201 (data not shown), which is expressed by *B. taurus* animals carrying the A10 class I serological specificity; the latter specificity is encoded by a second gene of this haplotype (N*00102), which differs in sequence from the gene encoding A10 (N*00101) in

B. indicus. The four epitopes in Tp2, which had been identified using T-cell lines from four different cattle, were found to be restricted by three different class I alleles. Tp2$_{49-59}$ and Tp2$_{98-106}$, which were identified using T-cell lines from *B. taurus* A10$^+$ animals, were confirmed to be restricted by N*01201; Tp2$_{27-37}$ and Tp2$_{96-104}$ were restricted by two novel alleles, one designated N*04001 and the other temporarily designated BoLA-T2c, derived from animals BW002 and BX017, respectively (Fig. 4). The epitopes identified in Tp5 and Tp7, Tp5$_{87-95}$ and Tp7$_{206-214}$, were found to be restricted by two novel alleles, temporarily designated BoLA-T5 and BoLA-T7, respectively, derived from animals BV050 and BW012 (Fig. 4). Cytotoxicity assays were also performed with cells cotransfected with the identified class I cDNAs and the corresponding CD8 T-cell target antigen cDNAs, and in all cases the results confirmed those obtained in the IFN-γ ELISPOT assay (data not shown).

To determine whether the epitopes identified in the course of these studies were consistently recognized by CD8 T cells from animals carrying the respective MHC class I restriction
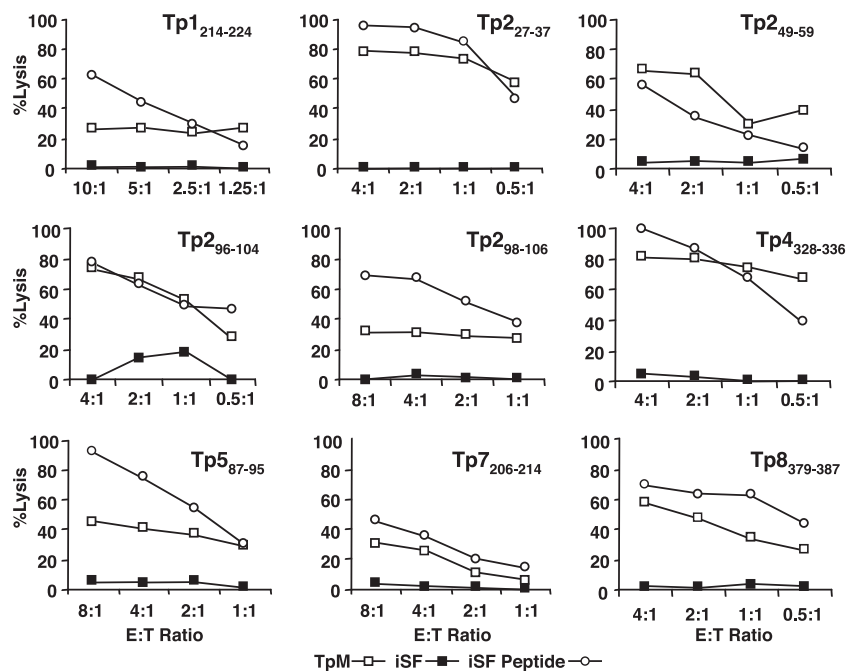
FIG. 3. CD8 cytotoxic-T-lymphocyte activity against epitope-pulsed immortalized skin fibroblasts and *T. parva*-infected cells. The lysis of *T. parva*-infected cells and peptide-pulsed and unpulsed immortalized skin fibroblasts by antigen-specific cytotoxic T lymphocytes (those from animals BV115 [Tp1$_{214-224}$], BW002 [Tp2$_{27-37}$], BW013 [Tp2$_{49-59}$], D409 [Tp2$_{96-104}$], BW014 [Tp2$_{98-106}$], BX063 [Tp4$_{328-336}$ and Tp8$_{379-387}$], BV050 [Tp5$_{87-95}$], and BW012 [Tp7$_{206-214}$]) was evaluated using a ^{51}Cr release assay. The results of one of two independent experiments are shown. The data are the means ± standard errors of the means for triplicate cultures. E:T ratio, effector-to-target cell ratio; TpM, *T. parva*-infected cells; iSF, unpulsed immortalized skin fibroblasts; iSF peptide, peptide-pulsed immortalized skin fibroblasts.

elements, analyses of responses were extended to additional *T. parva*-immune animals expressing three of the class I alleles, namely, N*00101, N*01301, and N*01201, associated with the serological specificities A10-KN104 (*B. indicus*), A18 (*B. taurus*), and A10 (*B.taurus*), respectively. In each instance, these groups included two or three animals known, on the basis of typing and breeding records, to be homozygous for the MHC type. The results are summarized in Table 2. All five A18$^+$ animals examined were found to respond to Tp1$_{214-224}$. Similarly, all five *B. taurus* A10$^+$ animals gave significant responses to both of the epitopes identified in the Tp2 antigen. Of seven *B. indicus* A10-KN104$^+$ cattle examined, all gave significant responses to the Tp8$_{379-387}$ epitope, but responses to Tp4$_{328-336}$ were detected in only four animals, three of which were homozygous for this MHC haplotype. Thus, only one of the four animals heterozygous for the MHC type recognized Tp4$_{328-336}$.

Responses induced by immunization of cattle with a mixture of *T. parva* antigens expressed individually in vaccine constructs. Access to animals subjected to primer-booster immunization with five of the *T. parva* antigens by using separate vaccine constructs for each antigen (10) provided an opportunity to ascertain whether such immunization results in responses to all of the antigens or only those corresponding to the MHC alleles expressed by the animals. Four groups of six cattle, each expressing one of four selected class I alleles (N*01301, N*01201, N*00101, and T5), were used in these studies. Within each group, three of the animals were immunized with plasmid DNA preparations and three were immunized with recombinant CP viruses expressing five of the *T.*

parva antigens (Tp1, Tp2, Tp4, Tp5, and Tp8). After 4 weeks, all animals received a booster dose of recombinant MVA expressing the same antigens and were then challenged with *T. parva* sporozoites after a further 3 weeks. Specific CD8 T-cell responses to the five antigens were examined every 7 days by using pools of overlapping peptides in an IFN-γ ELISPOT assay (Table 3). The responses to Tp2 were generally poor, with only four animals, including two of the six animals carrying the identified restriction element (N*1201), giving significant responses. The majority of the animals carrying the identified class I restriction elements for the remaining four antigens generated significant responses to the respective antigens. Thus, all six T5$^+$ animals responded to Tp5, all N*01301$^+$ animals responded to Tp1, and five of six N*00101$^+$ animals responded to both Tp4 and Tp8. By contrast, many of the animals failed to give significant responses to the antigens for which they did not carry an identified class I restriction element. Thus, none of the remaining 18 animals responded to Tp5, and only one and two N*00101-negative animals responded to Tp4 and Tp8, respectively. A larger number (6 of 18) of the N*01301-negative animals responded to Tp1. Overall, in 11 of the 19 animals that responded to one or more antigens, the significant response was focused entirely on the antigen or antigens for which the animal carried the identified class I restriction element. Moreover, the magnitudes of responses to antigens for which the animals carried identified class I restriction elements were in most instances greater than those of responses to other antigens. To illustrate this finding, the CD8 T-cell responses of the six BoLA allele N*00101$^+$ animals are depicted in Fig. 5. After priming and booster
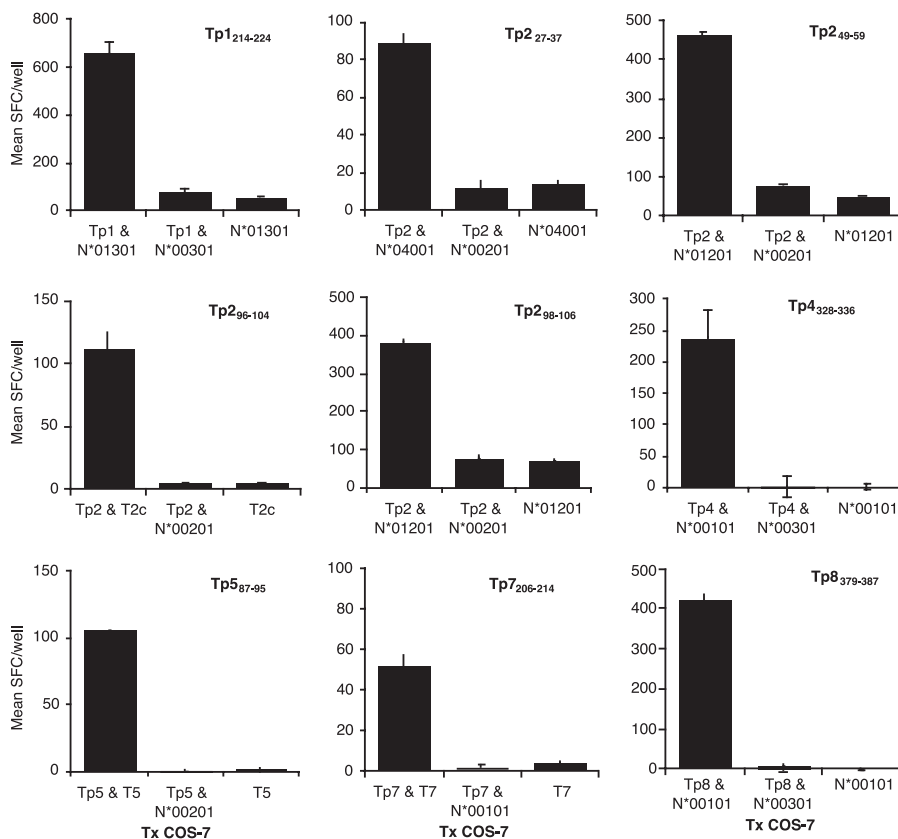
FIG. 4. BoLA restriction of CD8 T-cell epitopes from defined *T. parva* antigens. The recognition of COS-7 cells transiently cotransfected with CD8 T-cell target antigen cDNA and test BoLA cDNA or with CD8 T-cell target antigen cDNA and irrelevant BoLA cDNA or transfected with test BoLA cDNA alone by antigen-specific cytotoxic T lymphocytes (those from animals BV115 [$Tp1_{214-224}$], BW002 [$Tp2_{27-37}$], BW013 [$Tp2_{49-59}$], D409 [$Tp2_{96-104}$], BW014 [$Tp2_{98-106}$], BX063 [$Tp4_{328-336}$ and $Tp8_{379-387}$], BV050 [$Tp5_{87-95}$], and BW012 [$Tp7_{206-214}$]) was assessed using an IFN-γ ELISPOT assay. The results of one of three independent experiments are shown. The data are the mean numbers of SFC/10^4 CD8 T cells ± standard errors of the means for triplicate cultures. Tx COS-7, COS-7 cell transfection.

immunizations, responses were dominated by Tp8, with significant responses to Tp4 also being observed in five of six animals, although with one exception (that in animal BX225), these responses were of lesser magnitude than the Tp8-specific responses. Three animals mounted a response to a third antigen, Tp1, and this Tp1 response was most significant in animal BX225. Following challenge infection, only one animal, BX219, showed a clear recall of the responses, although the peak was below that observed after the MVA booster. The magnitudes of the responses of the other animals were more variable than those of the BoLA allele N*00101$^+$ animals, but the evidence of boosting of responses in the N*00101-negative animals by challenge was clearer, and in most instances, the hierarchy of dominance was maintained after challenge (data not shown). This strong association between the expression of the defined class I restriction element and the antigen specificity of the CD8 T-cell response indicates that the respective antigens are inherently dominant antigens in animals with the corresponding MHC genotypes.

DISCUSSION

Despite the vast pool of potentially antigenic peptides, data from previous studies of cattle immunized by infection and

treatment suggested that the CD8 T-cell response is restricted to a limited number of immunodominant antigens (8, 19). Thus, evidence of a hierarchy in dominance in MHC restriction of the response was obtained; responses were often restricted in their parasite strain specificity and, in animals of different MHC genotypes, frequently exhibited different patterns of parasite strain specificity, indicating that they were directed against different antigens. Based on these findings, it was hypothesized that immunodominance was a key factor in determining the parasite strain specificity of immunity. The results of antigen identification studies, which used polyclonal CD8 T-cell lines from *T. parva*-immune animals of different MHC genotypes to screen a schizont cDNA library and a set of parasite genes selected by bioinformatic analyses, provided further support for this idea. With one exception, T cells from each animal identified only a single antigen, and T-cell lines from animals of different MHC genotypes tended to identify different antigens (10).

The present study set out to identify the epitopes recognized in the *T. parva* antigens and to determine their MHC restriction specificities, in order to facilitate further dissection of the specificity of CD8 T-cell responses in immune cattle and to allow the development of assays and tools, such as class I tetramers, for monitoring the responses. Nine epitopes in six *T.*

TABLE 2. Recognition of defined *T. parva* CD8 T-cell epitopes by cattle with defined BoLA types immunized by infection and treatment

Animal	MHC class I serotype[b]	MHC class I restricting allele[c]	Response to epitope[d]:				
			Tp1$_{214-224}$	Tp2$_{49-59}$	Tp2$_{98-106}$	Tp4$_{328-336}$	Tp8$_{379-387}$
BV115	A10/A18	N*01301	+				
D232	A18/−	N*01301	+				
633	A18/A14	N*01301	+				
468[a]	A18	N*01301	+				
641[a]	A18	N*01301	+				
BV115	A10/A18	N*01201		+	+		
BW013	A10/−	N*01201		+	+		
BW014	A10/−	N*01201		+	+		
592[a]	A10	N*01201		+	+		
1011[a]	A10	N*01201		+	+		
BX063[a]	A10-KN104	N*00101				+	+
BX064[a]	A10-KN104	N*00101				+	+
BX065[a]	A10-KN104	N*00101				+	+
BZ136	A10-KN104/−	N*00101					+
BZ143	A10-KN104/−	N*00101					+
BZ144	A10-KN104/−	N*00101				+	+
BZ145	A10-KN104/−	N*00101					+

[a] Homozygous for MHC serotype.
[b] A minus sign indicates that the MHC serotype is unknown.
[c] The restricting alleles N*01301, N*01201, and N*00101 are associated with the serotypes *B. taurus* A18, *B. taurus* A10, and *B. indicus* A10-KN104, respectively.
[d] CD8 T-cell responses to defined antigenic peptides were assessed by IFN-γ release and/or cytotoxicity, and plus signs indicate significant responses.

TABLE 3. Antigen specificity of CD8 T-cell IFN-γ responses following immunization with defined *T. parva* antigens

MHC class I allele (associated serotype)	Immunization[a]	Animal	Specificity of response to[b]:				
			Tp1	Tp2	Tp4	Tp5	Tp8
N*01301 (A18)	CP virus-MVA	BZ027	+				
	CP virus-MVA	BZ039	+				
	CP virus-MVA	BZ018	+				
	DNA-MVA	BZ023	+				
	DNA-MVA	BY100	+				
	DNA-MVA	BZ040	+				
N*00101 (A10-KN104)	CP virus-MVA	BX223			+		+
	CP virus-MVA	BX216	+		+		+
	CP virus-MVA	BX215			+		+
	DNA-MVA	BX219	+		+		+
	DNA-MVA	BX220		+			
	DNA-MVA	BX225	+		+		+
T5 (undefined)	CP virus-MVA	BZ016		+		+	
	CP virus-MVA	BZ017	+			+	
	CP virus-MVA	BZ022				+	
	DNA-MVA	BZ019				+	
	DNA-MVA	BZ025	+			+	
	DNA-MVA	BZ013				+	
N*01201 (A10)	CP virus-MVA	BY142					
	CP virus-MVA	BY206	+	+	+		+
	CP virus-MVA	BZ026					
	DNA-MVA	BY001		+			+
	DNA-MVA	BY138					
	DNA-MVA	BZ020					

[a] Cattle were immunized with antigens Tp1, Tp2, Tp4, Tp5, and Tp8 by using heterologous primer-booster regimes. Cattle were primed with either recombinant CP virus or DNA vectors, and all received a booster immunization with recombinant MVA and then were challenged with a lethal dose of sporozoites.
[b] The detection of a significant antigen-specific CD8 T-cell IFN-γ response, as defined by the criteria described in Materials and Methods, is indicated by a plus sign.

parva antigens, together with their respective MHC restriction elements, were successfully identified. This report provides the first description of CD8 T-cell epitopes and corresponding MHC class I restriction elements in cattle. The epitopes, as defined by the minimal peptide length that induced maximal T-cell reactivity, were either 9-mers or 11-mers. In all but one case, CD8 T cells derived from eight different animals recognized only one of the six antigens. Single epitopes were identified in five of the six antigens; the epitopes in two of these antigens (Tp4 and Tp8) were restricted by the same class I allele, while those in the other three antigens (Tp1, Tp5, and Tp7) were restricted by different class I alleles. The Tp2 antigen was found to contain four epitopes, two of which were restricted by the same MHC class I allele, while the other two epitopes were restricted by different alleles.

The detection of responses to only one of the six antigens, in many cases focused on a single epitope, supports previous evidence that there is a dominance hierarchy in the antigens recognized in CD8 T-cell responses to *T. parva*. Further studies are needed to determine what proportion of the response in individual immune animals is directed at these antigens in order to provide quantitative data on the extent to which these antigens dominate the response. Immunodominance in outbred populations is a well-described phenomenon for CD8 T-cell responses to viruses and cancer, in which the pool of potential antigenic peptides is relatively small in comparison to those in response to complex intracellular bacterial and protozoan pathogens. The screening of *Plasmodium falciparum* and *Mycobacterium tuberculosis* for antigens and epitopes by using CD8 T cells from immunized or exposed humans has shown the recognition of a relatively large number of antigens, rather than a narrow focus on a few immunodominant proteins and epitopes (2, 18). The results of this and earlier studies (8, 19) suggest that immunodominance may play a much greater role in the bovine CD8 T-cell response to *T. parva* than in
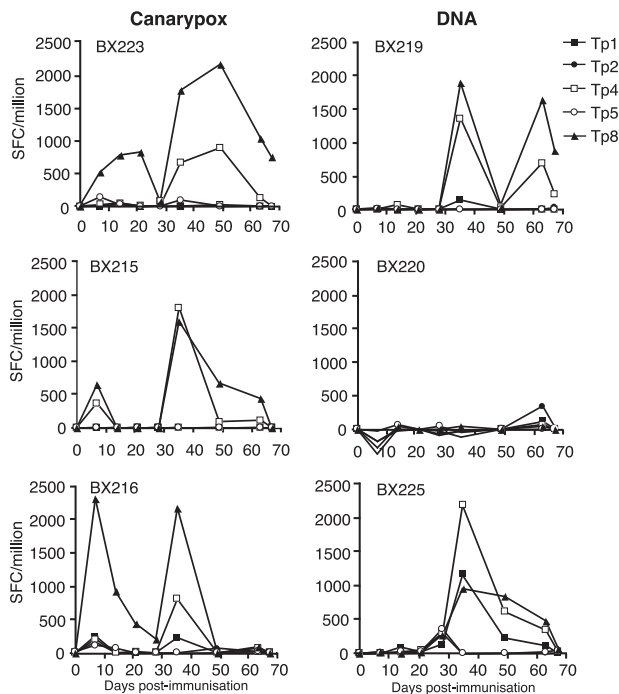
FIG. 5. Antigen specificity of CD8 T cells from BoLA allele N*00101+ cattle vaccinated with defined antigens by using heterologous primer-booster regimens. Three cattle were primed with recombinant CP viruses, and three were primed with plasmid DNA preparations expressing five of the *T. parva* antigens (Tp1, Tp2, Tp4, Tp5, and Tp8). All cattle were immunized with recombinant vaccinia viruses (MVA) expressing the same antigens after 4 weeks and then challenged with *T. parva* sporozoites after a further 3 weeks. IFN-γ responses of purified CD8 T cells to pools of overlapping peptides were measured longitudinally using an ELISPOT assay. Responses presented are the medium corrected numbers of SFC/10^6 CD8 T cells.

the human CD8 T-cell response to pathogens of similar complexity.

The present study provided evidence that *T. parva*-immune cattle carrying defined MHC class I alleles consistently respond to the epitopes corresponding to the respective MHC backgrounds. Animals homozygous or heterozygous for *B. taurus* A18 or A10 and *B. indicus* A10-KN104, which were shown to recognize five epitopes in the Tp1, Tp2, Tp4, and Tp8 antigens, were available for these studies. Responses to the single epitope defined in Tp1 and the two epitopes in Tp2 were consistently detected in all of the *B. taurus* A18 and A10 animals, respectively. Responses to the epitope in Tp8 were also observed in all seven *B. indicus* A10-KN104 animals, but only four of these animals responded to the epitope in Tp4. These findings indicate that the Tp1, Tp2, and Tp8 epitopes are dominant in animals with these MHC genotypes. The variable responses to Tp4$_{328-336}$ suggest that this is a less-dominant epitope; the failure to detect responses in three animals that were heterozygous for the class I restriction element may have been due to the presence of competing dominant epitopes presented via the other MHC haplotype expressed by these animals.

Despite the finding that animals of selected MHC genotypes immunized with live parasites had significant CD8 T-cell responses to only one or, in the case of BoLA allele N*00101+

animals, to one or two of the six antigens, most of the antigens are likely to contain peptides capable of being presented by cells of the different genotypes. Studies of murine and human systems have provided evidence that the relative dominance of particular epitopes can be influenced by a number of factors, including the abundance of processed peptide, the binding affinity of the peptides for the MHC, and the T-cell receptor repertoire (23). The presentation of the different epitopes by the same antigen-presenting cell, i.e., by dedicated antigen-presenting cells following cross-priming or by the parasitized cells themselves, may favor the preferential generation of responses to the most dominant antigens by allowing competition for epitope recognition by T cells. We therefore examined whether or not the dominance of responses to the preferred antigens was retained in animals immunized with five of the antigens incorporated individually into different vaccine constructs. Responses against pools of synthetic peptides spanning entire antigens were measured in order to assess the breadth of the responses. Nineteen of 24 vaccinated animals mounted a CD8 T-cell response, and in all instances, the dominant response was directed against the antigen(s) that was predicted based on the expression of the relevant MHC restriction element. For 11 of the animals, the responses were directed solely against these antigens. Although the assay employed did not allow the target epitopes to be identified, the recognition of the defined epitopes by cattle of the respective MHC genotypes was confirmed for a small number of animals (S. P. Graham, unpublished data). The absence of significant responses to many of the antigens in most animals may reflect a lack of potency of the vaccination protocols rather than an inability of the animals to respond to the antigens. Nevertheless, the findings clearly demonstrate that the preferred antigens are inherently dominant in animals with the respective MHC genotypes. They also suggest that the preferential induction of responses to particular antigens is not due solely to competition for presentation within antigen-presenting cells but rather may reflect the presence within the T-cell repertoire of T cells with high-avidity receptors for the respective epitopes.

The results of these studies are of particular practical significance because of the well-documented antigenic variability among strains of *T. parva*, which results in incomplete cross-protection against different strains (17). Additional preliminary studies of the antigens described herein have confirmed that the defined epitope regions vary in sequence among parasite strains and are differentially recognized by CD8 T cells (R. Pellé and S. P. Graham, unpublished data). Hence, a major challenge in the development of a vaccine based on the induction of CD8 T-cell responses will be to generate responses that are effective against all parasite strains. Further studies are required to provide a more comprehensive picture of the antigen recognition capacity of animals of different MHC genotypes to ascertain whether combinations of antigens that potentially provide broad protection can be identified and used to induce protective CD8 T-cell responses.

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Editor: J. F. Urban, Jr.