Assessment of Cross-Reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP-10 at the T-Cell Epitope Level[▽]

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Cross-reactivity between *Mycobacterium kansasii* ESAT-6 and CFP-10 homologues and their *M. bovis* counterparts can confound the interpretation of immunodiagnostic tests for tuberculosis. *M. kansasii* is a nontuberculous mycobacterial species cultured from skin test-positive cattle in Great Britain. Using peptides derived from *M. bovis* and *M. kansasii* ESAT-6 and CFP-10 regions that differ between these species, we investigated the species specificity and cross-reactivity at the level of individual bovine T-cell epitopes. Our results demonstrated that all peptides tested are fully cross-reactive, with the exception of one ESAT-6-derived peptide that harbored an *M. bovis*-specific epitope(s) when it was recognized in the context of bovine leukocyte antigen (BoLA)-DQ but that was cross-reactive with its *M. kansasii* homologues when it was restricted by BoLA-DR. This observation further highlights that prediction of species specificity by comparing sequence identity/homology alone is not sufficient and that individuals with diverse major histocompatibility complex constellations need to be tested to characterize the cross-reactivity or species specificity of peptide-based reagents.

The incidence of bovine tuberculosis (BTB) in Great Britain has been increasing steadily over the last two decades (10). At present the BTB control program involves regular skin testing with the single intradermal comparative tuberculin test, followed by the compulsory slaughter of cattle with a positive result (19). Tuberculin specificity can also be affected by infection of cattle by nontuberculous mycobacterial (NTM) species, as can be seen by the frequent background responses to avian tuberculin that is being used to control for such environmental sensitization. Encouragingly, when antigens like ESAT-6 and CFP-10 are used in the BOVIGAM gamma interferon (IFN-γ) test, they have also been shown to enhance the specificity of the tuberculin-based test per se in this respect (7, 8, 20, 21, 26, 31). However, some NTM species, like *Mycobacterium kansasii*, *M. gordonae*, *M. marinum*, *M. szulgai*, *M. flavescens*, and *M. gastri*, contain genes for ESAT-6 and CFP-10 homologues (4, 20). Cattle infected with such ESAT-6- or CFP-10-expressing NTM species could give rise to false-positive responses to these antigens. This has been demonstrated in a recent study by Waters et al. (32), who demonstrated potent cross-reactive IFN-γ responses with the *M. bovis* ESAT-6 and CFP-10 protein species in cattle experimentally infected with *M. kansasii*. Genes for *M. kansasii* homologues have also been described for other *M. tuberculosis* antigens, such as MPB83, TB10.4, and TB10.3 (24). *M. kansasii* can cause disease in cattle presenting with lesions in the pulmonary tract and associated lymph nodes (18). This organism can also cause pulmonary disease in immunocompetent humans and disseminated disease almost ex-

clusively in immunocompromised individuals (3, 12). In vitro responses to *M. tuberculosis* ESAT-6 and CFP-10 in humans infected with *M. kansasii* have been reported, again suggesting cross-reactivity between the *M. tuberculosis* and *M. kansasii* protein homologues. Interestingly, *M. kansasii* is highly heterogenic, with five subtypes/genotypes being described (2). Subtype I has been found exclusively in humans; in contrast, subtype II can be isolated from both human and environmental samples, whereas subtypes III to V are rarely found in humans but were present in environmental samples (2, 4). The amino acid sequences of the ESAT-6 and CFP-10 homologues of these *M. kansasii* genotypes have been defined and were found to be highly homologous with those of *M. tuberculosis* (6). Armed with this sequence information, we decided to investigate the potential species specificity and cross-reactivity between the *M. bovis* and *M. kansasii* ESAT-6 and CFP-10 homologues at the molecular level, i.e., at the level of the individual peptide epitopes recognized by CD4+ T cells isolated from cattle infected with *M. bovis*. We also investigated with this synthetic peptide-based approach whether *M. bovis*-specific reagents could be defined.

MATERIALS AND METHODS

Antigens and peptides. Bovine tuberculin (purified protein derivative [PPD] B) and avian tuberculin (PPD A) were supplied by the Tuberculin Production Unit at the Veterinary Laboratories Agency (VLA), Weybridge, Surrey, United Kingdom, and were used in vitro in IFN-γ enzyme-linked immunospot (ELISPOT) assays or enzyme immunoassays (EIAs) at 10 μg/ml. Staphylococcal enterotoxin B was used as a positive control at 1 μg/ml. Synthetic peptides derived from the sequences of *M. bovis* and *M. kansasii* ESAT-6 and CFP-10 (see Table 1 for the sequences) were synthesized by the facility of the University of Leiden (Leiden, The Netherlands) by solid-phase synthesis and purified, and their quality was assessed as described previously (24). The peptides were used at 25 μg/ml in the IFN-γ ELISPOT assay. Recombinant *M. tuberculosis* ESAT-6 and CFP-10 were kindly provided by M. Singh (Gesellschaft für Biotechnolo-

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TABLE 1. Peptides used in this study

Protein and peptide	Peptide sequence[a]	Species (Mka genotype)[b]
ESAT-6		
1	EAYQGVQQKWDATATE	M. bovis
1A	--------------Q-	M. kansasii (I-V)
2	KWDATATELNNALQNL	M. bovis
2A	------Q---------	M. kansasii (I-IV)
2B	------Q---S-----	M. kansasii (V)
3	LNNALQNLARTISEAG	M. bovis
3A	--------S-------	M. kansasii (I-IV)
3B	--S-----S-------	M. kansasii (V)
CFP-10		
4	QEAGNFERISGDLKTQ	M. bovis
4A	-----------M---	M. kansasii (IV)
5	IDQVESTAGSLQGQWR	M. bovis
5A	--------A---A---	M. kansasii (I-V)
6	GSLQGQWRGAAGTAAQAA	M. bovis
6A	A---A------------	M. kansasii (I-V)
6B	A---A------A------	M. kansasii (IV)
7	AGTAAQAAVVRFQEAANK	M. bovis
7A	--A--------------	M. kansasii (IV)
8	VVRFQEAANKQKQELDEI	M. bovis
8A	-----------A--E--	M. kansasii (I-V)
9	QKQELDEISTNIRQAGVQYS	M. bovis
9A	--A----E-----------	M. kansasii (I-V)
10	NIRQAGVQYSRADEEQQQ	M. bovis
10A	----------K------	M. kansasii (I-V)

[a] -, amino acid residue in M. kansasii-derived peptide identical to the residue in the M. bovis sequence. Amino acids are represented by the one-letter code.
[b] M. kansasii genotype according to Arend et al. (4).

gische Forschung, Braunschweig, Germany) and were used at 5 μg/ml in in vitro assays (IFN-γ EIA and ELISPOT assay).

Cattle used in this study. Heparinized blood samples were obtained from adult tuberculin skin test-positive cattle (n = 15) from herds with ongoing BTB cases. Seven animals were selected due to the presence of strong IFN-γ responses after stimulation with recombinant ESAT-6 and/or CFP-10; IFN-γ was evaluated with the BOVIGAM EIA kit (see below). These cows were of five different breeds (Holstein-Friesian, Charolais, Devon, British Friesian, and Aberdeen Angus) and were obtained from five herds from distinct geographical locations within Great Britain. The disease status of the animals was confirmed postmortem by the presence of lesions in lymph nodes and/or lungs typical of BTB and by the culture of M. bovis from tissues. The animals were held at VLA under the provision of a Home Office project license granted under the Animals (Scientific Procedures) Act, 1986. This license and the experiments listed in it were approved by the local ethical review committee.

Whole-blood IFN-γ assay (BOVIGAM assay) (33). To select animals for further study, blood samples were collected from M. bovis-infected cattle and were placed into heparinized Vacutainer tubes (Becton Dickinson, Oxford, United Kingdom). Briefly, whole-blood cultures were performed in 96-well tissue culture plates (flat bottom) in the presence of tuberculin and recombinant ESAT-6 and CFP-10. After 24 h of culture at 37°C, the plasma supernatants (100 μl/well) were harvested and stored at −20°C. The BOVIGAM IFN-γ ELISA was performed according to the supplier's instructions (Prionics, Schlieren, Switzerland). Animals showing recombinant ESAT-6- or CFP-10-specific changes in the values of the optical density at 450 nm of >0.5 were selected for this study.

Ex vivo IFN-γ ELISPOT assay (28). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Histopaque-1077 (Sigma) gradient centrifugation and were immediately cultured in RPMI 1640 tissue culture medium (Life Technologies, Paisley, Scotland, United Kingdom) supplemented with 10% fetal calf serum (Sigma Aldrich, Poole, United Kingdom), nonessential amino acids (Sigma Aldrich), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. The ex vivo (direct) ELISPOT assay results were enumerated as described earlier (28). Briefly, ELISPOT assay plates (Immunobilon-P polyvinylidene difluoride membranes; Millipore, Molsheim, France) were coated overnight at 4°C with the bovine IFN-γ-specific monoclonal antibody 5D10 (BioSource, Wheatley, United King-

dom). Unbound antibody was removed by washing, and the wells were blocked with 10% fetal calf serum in RPMI 1640 medium. PBMCs suspended in tissue culture medium were then added (2×10^5 PBMCs/well) and cultured in the presence of PPD B, recombinant proteins, or synthetic peptides for 24 h at 37°C in a humidified 5% CO_2 incubator. Spots were developed with rabbit serum specific for IFN-γ prepared at VLA, followed by incubation with an alkaline phosphatase-conjugated monoclonal antibody specific for rabbit immunoglobulin G (Sigma Aldrich). The spot-forming cells (SFCs) were visualized with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium substrate (Sigma Aldrich). The SFCs in the wells with medium only were subtracted from the SFCs in wells with antigen or peptides (ΔSFCs/well). ΔSFCs/well of >10 were considered positive. For blockage of the bovine leukocyte antigen (BoLA) presentation of peptides, monoclonal antibodies specific for BoLA-DR and BoLA-DQ were purchased from VRMD, Pullman, WA. Each antibody was preincubated with PBMCs in the ELISPOT assay plate at 5 μg/ml for 1 h at 37°C, after which the relevant peptide was added to the wells and the plates were incubated as described above for the standard IFN-γ ELISPOT assays (13).

Hain genotype system. Forty-six frozen nontuberculous isolates from the Weybridge TB Section culture collection were used. Once they were thawed, 300 μl of each isolate was sown onto one Lowenstein-Jensen–pyruvate medium slope and one Lowenstein-Jensen–glycerol medium slope, and the slopes were incubated at 37°C. Once the isolate had grown sufficiently on the medium, 1 μl was harvested into 100 μl of high-pressure liquid chromatography-grade water and heat killed at 80°C for half an hour. The resulting heat-killed isolate was then used to perform PCR analysis. The Hain genotype kit (Hain Lifescience, Nehren, Germany) (22) was used to test the samples, according to the supplier's instructions. A positive result was obtained when the staining reaction of the kit produced readable bands on the test strip, and the results were interpreted by using the supplier's template.

RESULTS AND DISCUSSION

NTM in cattle in Great Britain. Only a small percentage of mycobacterial isolates obtained from the tissues of cattle slaughtered under the test and slaughter control policy in Great Britain are NTM (ca. 0.8% of total isolates from 2004 to 2006). The majority of these NTM isolates belong to the M. avium/M. intracellulare (MAI) complex (for example, 54% of the NTM isolates in Great Britain from 2004 to 2006). The remaining NTM isolates remained unclassified or comprised a number of different mycobacterial species. Recently, the species of 46 non-MAI NTM isolates (5 from cows with visible lesions typical of BTB and 41 from cattle presenting without visible lesions postmortem) were determined by using the Hain genotype system (22). The results indicated that 22/46 of the non-MAI NTM isolates tested were M. kansasii, including isolates from two of five of the lesioned animals. Genotyping revealed that all M. kansasii isolates found in these cattle were subtype I or II, which could be isolated from humans in a study in Holland (4). The other species found were M. celatum (5/46); M. nonchromogenicum (5/46); M. gordonae (5/46); M. szulgai (4/46); M. fortuitum (2/46); and M. scrofulaceum, M. intermedium, and M. shimodei (1/46 each). These results do not constitute the findings of a representative study of NTM isolates in Great Britain. They do demonstrate, however, that M. kansasii can be isolated from cattle tissues. As stated above, apart from M. kansasii, M. gordonae and M. szulgai were also isolated, and these species also contain genes for ESAT-6 homologues (4) and most likely also genes for CFP-10 homologues. Therefore, these three NTM species can potentially express these cross-reactive antigens. M. kansasii isolates have also been identified in an analysis of NTM cattle isolates from Northern Ireland, although the dominant NTM species were part of the MAI complex (46%) and the M. terrae complex (39%), with 8% of the isolates being M. kansasii (20).

A

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            75                    53                              60
1  M T E Q Q W N F A G I E A A A S A I Q G N V T S I H S L L D E G K Q S L T K L A A A W G G S G S E A Y Q G V Q
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

            66          63                  50
56 Q K W D A T A T E L N N A L Q N L A R T I S E A G Q A M A S T E G N V T G M F A   M. bovis
56 . . . . . . . . . Q . . . . . . S . . . . . . . . . . . . . . . . . . . . . . .   MKA-1
56 . . . . . . . . . Q . . . . . . S . . . . . . . . . . . . . . . . . . . . . . .   MKA-II
56 . . . . . . . . . Q . . . . . . S . . . . . . . . . . . . . . . . . . . . . . .   MKA-III
56 . . . . . . . . . Q . . . . . . S . . . . . . . . . . . . . . . . . . . . . . .   MKA-IV
56 . . . . . . . . . Q . . . S . . S . . . . . . . . . . . . . . . . . . . . . . .   MKA-V
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B

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            100
1  M A E M K T D A A T L A Q E A G N F E R I S G D L K T Q I D Q V E S T A G S L Q G Q W R G A A G T A A Q A A V
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . . . . .
1  . . . . . . . . . . . . . . . . . . . M . . . . . . . . . . . . . . . . A . . . A . . . . . . A . . . . .
1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . A . . . . . . . . . . . .

            70                      83
56 V R F Q E A A N K Q K Q E L D E I S T N I R Q A G V Q Y S R A D E E Q Q Q A L S S Q M G F   M. bovis
56 . . . . . . . . . . . A . . E . . . . . . . . . . . . K . . . . . . . . . . . . . . . . .   MKA-1
56 . . . . . . . . . . . A . . E . . . . . . . . . . . . K . . . . . . . . . . . . . . . . .   MKA-II
56 . . . . . . . . . . . A . . E . . . . . . . . . . . . K . . . . . . . . . . . . . . . . .   MKA-III
56 . . . . . . . . . . . A . . E . . . . . . . . . . . . K . . . . . . . . . . . . . . . . .   MKA-IV
56 . . . . . . . . . . . A . . E . . . . . . . . . . . . K . . . . . . . . . . . . . . . . .   MKA-V
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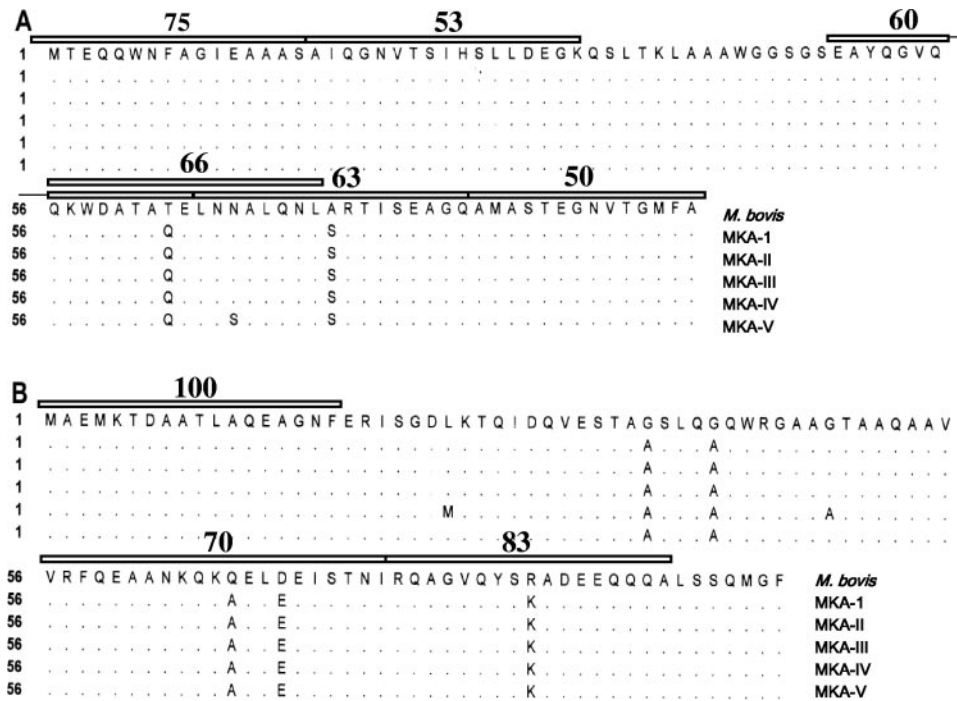
FIG. 1. Alignment of the amino acid sequences and positions of dominant T-cell epitopes of ESAT-6 (A) and CFP-10 (B) of *M. bovis*/*M. tuberculosis* and *M. kansasii* genotypes I to V (MKA-1 to MKA-V). Amino acids are represented by the one-letter code. Promiscuous recognition of *M. bovis*-derived ESAT-6 and CFP-10 peptides (defined as giving rise to T-cell responses in ≥50 of the cattle tested that recognized *M. bovis* ESAT-6 or CFP-10) is represented by open bars above the sequences (27, 31). The proportions of animals (responder frequencies) that recognized these peptides are shown above the respective bars (27, 31). (Adapted from reference 4 with permission of the publisher.)

These data highlighted the potential need for assessment of the cross-reactivities of the *M. kansasii* and *M. bovis* homologues of ESAT-6 and CFP-10, although as referred to above, the actual percentage of NTM species in general and *M. kansasii* in particular compared to that of *M. bovis* among isolates from cattle is low. However, an additional observation is that *M. kansasii* can also be isolated from humans without disease: one-third of isolates have been reported to represent colonization rather than infection (1), although it is not known if this could lead to T-cell responses. Colonization in the absence of disease also occurs in cattle (20) and could therefore comprise a possible source of false-positive responses to tuberculin and antigens like ESAT-6 and CFP-10, with resulting greater potential financial and epidemiological impacts beyond those from the relatively few clinical cases. Due to the potential geographical tropism of *M. kansasii* infections (2), such "false-positive" responders are unlikely to be equally distributed throughout Great Britain and could therefore be a problem of particular herds. Indeed, herds with consistent and protracted cases of animals that are skin test reactors, that do not have lesions typical of BTB, and from whose tissues *M. bovis* cannot be cultured have been observed. The reasons for these so-called nonspecific skin test reactions are not known, and there is yet no evidence that they are due to *M. kansasii* infection. Nevertheless, it would be desirable to be able to develop immunodiagnostic reagents that can distinguish between *M. bovis*- and *M. kansasii*-infected cattle.

Comparison of sequence homologies between *M. bovis*/*M. tuberculosis* and *M. kansasii* CFP-10 and ESAT-6 homologues in relation to the positions of dominant promiscuous bovine T-cell epitopes. The sequences of the *M. kansasii* homologue genes of ESAT-6 and CFP-10 of all five *M. kansasii* genotypes and the deduced amino acid sequences have been reported previously (4). The *M. bovis* and *M. tuberculosis* CFP-10 and ESAT-6 molecules are identical (9, 14) and are henceforth referred to in this paper as *M. bovis* homologues. Comparison of the *M. kansasii* homologues with the respective *M. bovis* sequences demonstrated a high degree of identity (>95%) at the amino acid level (4). For example, the ESAT-6 homologues differ only at two positions (*M. kansasii* subtypes I to IV) or three positions (*M. kansasii* subtype V) (Fig. 1), whereas the CFP-10 homologues differed between the *M. bovis* protein and the different *M. kansasii* subtypes by five to seven residues (Fig. 1). As previous studies have shown, ESAT-6- and CFP-10-derived epitopes are recognized promiscuously by class II-restricted T cells from tuberculous cattle (27), and it is therefore possible to assess the potential impact of the high degree of sequence identity between the *M. kansasii* and *M. bovis* homologues in relation to these immunodominant epitopes. The epitopes within the sequences of CFP-10 and ESAT-6 that were recognized by CD4$^+$ T cells from >50% of the cattle tested are shown in Fig. 1 (27, 29, 31; H. M. Vordermeier et al., unpublished data). The amino acid sequences of the majority of these epitopic regions are fully identical between the protein homologues (i.e., the N-terminal epitopes from both ESAT-6

SFC/well


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1.  EAYQGVQQKWDATATE
1A. -------------Q-

2.  KWDATATELNNALQNL
2A. ------Q---------
2B. ------Q---S-----

3.  LNNALQNLARTISEAG
3A. --------S-------
3B. -S------S-------
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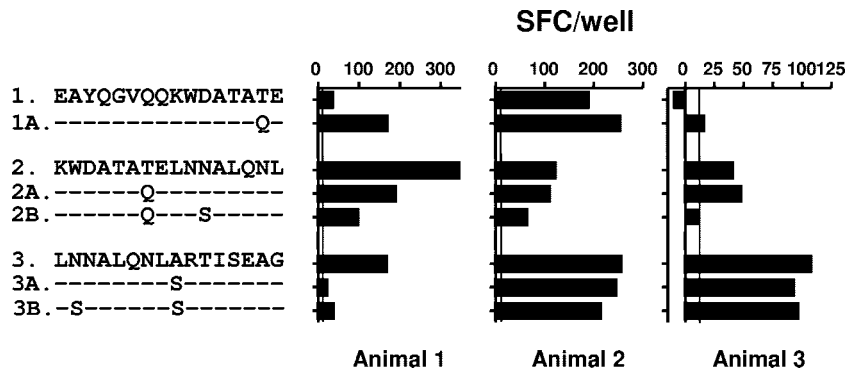
Animal 1   Animal 2   Animal 3

FIG. 2. Recognition of *M. bovis* and *M. kansasii* ESAT-6-derived peptides by T cells from cattle with BTB. PBMCs from naturally infected cattle were stimulated with synthetic peptides (see Table 1 for sequence details). The readout system indicates the IFN-γ production measured by the ex vivo ELISPOT assay. The results are expressed as the mean SFCs/well, and the standard errors of the means for all values are <20% of the means. Horizontal lines indicate the cutoff for positivity (10 ΔSFCs/well).

and CFP-10). Full cross-recognition of these sequences after *M. bovis* or *M. kansasii* infection is therefore almost inevitable. In contrast, other dominant epitopes differed at between one and two amino acid residues between the *M. bovis*- and *M. kansasii*-derived proteins (e.g., within ESAT-6 peptides 49 to 64) (Fig. 1), and these differences could be sufficient to impart species specificity to such peptides. The unambiguous prediction of T-cell cross-reactivity is difficult even in situations with high degrees of sequence identity between epitope regions. For example, while we could demonstrate that a high degree of sequence identity between sequence regions from unrelated mycobacterial antigens (>50% in the 16- to 20-mer regions) indicated cross-reactivity at the epitope level in cattle, other peptides that displayed similar degrees of sequence identity were not cross-reactive (17). Conversely, the molecular basis of T-cell cross-reactivity between two unrelated mycobacterial proteins can depend on the sequence identity of 1 residue of 8 to 10 residues spanning the epitope (16). Moreover, changing one single residue within a 20-mer amino acid peptide containing a T-cell epitope can result in the peptide derivative not being recognized by T cells specific for the wild-type peptide (30), e.g., by abrogating major histocompatibility complex

(MHC) binding. To highlight this point, we performed single-alanine mutation analysis of ESAT-6 peptides 49 to 64. While single substitutions of most residues with alanine did not affect or only marginally affected T-cell recognition (data not shown), replacement of T^{63} with alanine resulted in a complete loss of T-cell responses (data not shown). Interestingly, T^{63} in the *M. bovis* sequence has been replaced by a glutamine residue in the *M. kansasii* homologues (4). Therefore, despite the high degrees of sequence homology between the *M. bovis* and *M. kansasii* homologues of CFP-10 and ESAT-6, it seemed of interest to investigate the potential species specificity and cross-reactivity at the level of individual peptides recognized by CD4^{+} T cells isolated from cattle infected with *M. bovis* and to determine whether a synthetic peptide-based approach could define "*M. bovis*-specific" peptides useful for the differentiation between *M. kansasii* and *M. bovis* infections.

Recognition of *M. bovis*- and *M. kansasii*-derived ESAT-6 and CFP-10 peptides by T cells from cattle with BTB. Synthetic peptides representing the regions of *M. kansasii* ESAT-6 and CFP-10 with sequence differences compared to the sequences of their *M. bovis* counterparts were prepared (five and eight peptides, respectively; Table 1). The recognition of these pep-

SFC/well


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4.  QEAGNFERISGDLKTQ
4A. ------------M---

5.  IDQVESTAGSLQGQWR
5A. --------A---A---

6.  GSLQGQWRGAAGTAAQAA
6A. A---A-------------
6B. A---A------A------

7.  AGTAAQAAVVRFQEAANK
7A. --A---------------

8.  VVRFQEAANKQKQELDEI
8A. ------------A--E--

9.  QKQELDEISTNIRQAGVQYS
9A. --A---E-------------

10. NIRQAGVQYSRADEEQQQ
10A.----------K-------
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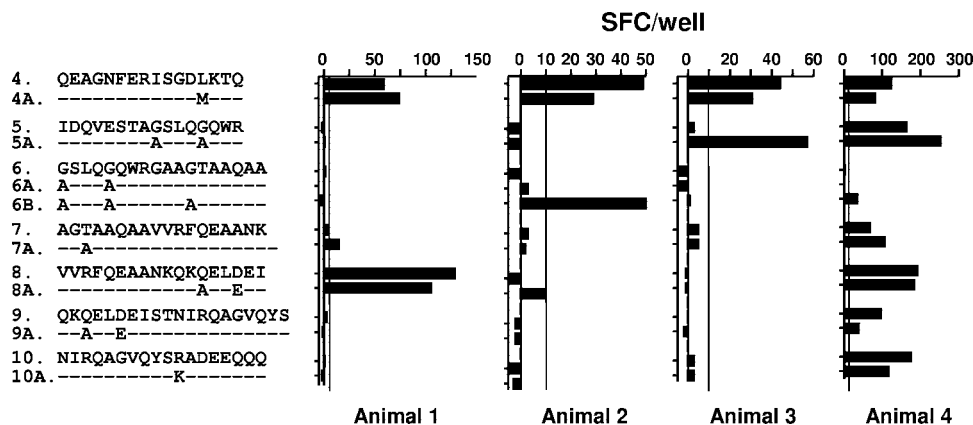Animal 1   Animal 2   Animal 3   Animal 4

FIG. 3. Recognition of *M. bovis* and *M. kansasii* CFP-10-derived peptides by T cells from cattle with BTB. PBMCs from naturally infected cattle were stimulated with synthetic peptides (see Table 1 for sequence details). The readout system indicates the IFN-γ production measured by the ex vivo ELISPOT assay. The results are expressed as the mean SFCs/well, and the standard errors of the means for all values are <20% of the means. Horizontal lines indicate the cutoff for positivity (10 ΔSFCs/well).
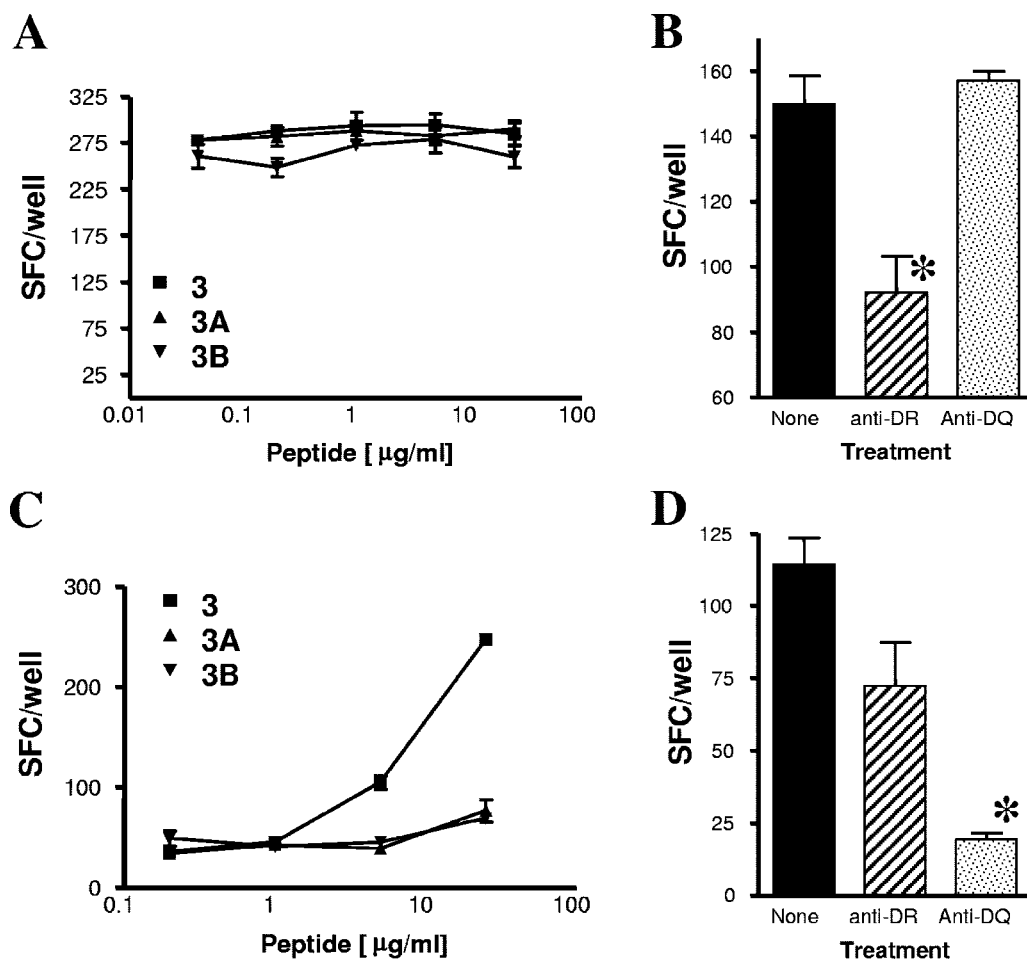
FIG. 4. BoLA class II restriction of the recognition of *M. bovis* ESAT-6 peptide 3. Cross-reactive recognition of peptides 3, 3A, and 3B in animal 2 (A) was BoLA-DR restricted (B). *M. bovis*-specific recognition of peptide 3 in animal 1 (C) was BoLA-DQ restricted (D). Anti-BoLA-DR or anti-BoLA-DQ monoclonal antibody was added to peptide 3-stimulated PBMC cultures (B and D). The results are expressed as the mean numbers of IFN-γ-producing SFCs/well ± standard errors of the means. *, $P < 0.05$ compared to the results for the other groups (Student's *t* test).

tides was compared to the recognition of the *M. bovis*-derived peptides (Table 1): PBMCs from tuberculous cattle known to recognize either CFP-10 or ESAT-6 (or both) were prepared and stimulated with these peptides to determine the IFN-γ responses by ELISPOT assay. The results of these experiments are summarized in Fig. 2 (ESAT-6) and Fig. 3 (CFP-10). With the exception of *M. bovis* CFP-10-derived peptide 6, all 10 *M. bovis*-derived peptides were recognized by at least one cow, thereby demonstrating the presence of epitopes (Fig. 2 and 3). The results for ESAT-6-derived peptides are shown in Fig. 2. Peptides 1 and 1A and peptides 2, 2A, and 2B, encompassing two of the three sequence regions of ESAT-6 with differences between *M. bovis* and *M. kansasii*, were recognized by at least one animal tested, thereby confirming immunological cross-reactivity at the epitope level. The most interesting results were obtained with peptides 3, 3A, and 3B (representing sequences derived from *M. bovis*, *M. kansasii* subtypes I to IV, and *M. kansasii* subtype V, respectively) (Fig. 2). In animal 3 only *M. bovis*-derived peptide 3 was recognized, as shown in Fig. 2 and 4C, thus suggesting species-specific responses to this peptide. In contrast, however, the majority of cattle tested (three of five

cattle tested; see the results for animals 2 and 3 in Fig. 2 and Fig. 4A) recognized peptides 3, 3A, and 3B equally well; i.e., they displayed fully cross-reactive responses.

The *M. kansasii* CFP-10-derived peptides (peptides 4A to 10A) were all recognized by at least one tuberculous cow tested, thus demonstrating full cross-reactivity between *M. bovis* and *M. kansasii* CFP-10 peptide homologues (Fig. 3). Interestingly, in animal 2 *M. kansasii*-derived peptide 6B (representing subtype IV) was recognized, whereas *M. bovis*-derived peptide 6 was not (Fig. 3). A similar observation was made in respect to the recognition of peptides 5 and 5A by T cells from animal 3; peptide 5A was strongly recognized, while the corresponding *M. bovis* peptide was not (although both peptide species were recognized in animal 4) (Fig. 3).

It has been shown that promiscuously recognized peptides can harbor different sets of epitopes that can be recognized in the context of different MHC class II alleles (e.g., for peptides from a mycobacterial antigen) (30), thus accounting for their promiscuous recognition across a range of different MHC class II alleles. We therefore hypothesized that the "*M. bovis* specificity" of ESAT-6 peptide 3 in some animals, in contrast to the

cross-reactivity with peptides 3A and 3B in others, may be due to the recognition of distinct epitopes within peptide 3 that are recognized in the context of different BoLA class II types or alleles. Thus, we next defined the restriction elements used in ESAT-6 peptide 3 recognition by blocking the IFN-γ responses with monoclonal antibodies specific for BoLA-DR and BoLA-DQ. Two animals were selected: animal 2, which recognized peptides 3, 3A, and 3B in a cross-reactive manner (Fig. 4A), and animal 1, which recognized only peptide 3 (Fig. 4C). Interestingly, recognition of peptide 3 was BoLA-DR restricted in the animal that recognized both the *M. bovis*- and the *M. kansasii*-derived peptides (Fig. 4B), whereas the "species-specific" recognition of peptide 3 in animal 1 was restricted by BoLA-DQ (Fig. 4D). Similar results were obtained with a second similar pair of cattle tested (data not shown). The BoLA complex consists of one DR gene pair and up to two DQ gene pairs per haplotype and is highly polymorphic (11, 15). We had previously shown that identical peptides from mycobacterial antigens can be recognized in cattle in the context of both BoLA-DR and BoLA-DQ molecules (13), as had been reported previously, for example, for the antigen part of a vaccine against foot-and-mouth disease virus (25). Our results therefore confirm and extend these earlier observations. In addition, our data on cross-reactivity when peptide 3 was recognized in the context of BoLA-DR or when "*M. bovis* specificity" was recognized in the context of BoLA-DQ suggest that this is because the 20-mer peptide harbors distinct epitope regions that bind differentially to BoLA-DR or BoLA-DQ. Interestingly, peptides that are derived from mycobacterial antigens such as CFP-10 and that are recognized by human CD4$^+$ cells in the context of HLA-DR and HLA-DQ have also been described (5, 23).

In summary, this study has extended studies by Waters et al. (32) by assessing the T-cell cross-reactivity between the *M. bovis*/*M. tuberculosis* and the *M. kansasii* homologues of ESAT-6 and CFP-10 at the peptide/epitope level by testing synthetic peptides derived from both mycobacterial species. Generally, we found, with the possible exception of ESAT-6 peptide 6, complete cross-reactivity between the homologue peptides in infected cattle. It is therefore unlikely that one can design peptide-based reagents that would allow the immunological differentiation of cattle infected with *M. bovis* or *M. kansasii* even if further antigens like MPB83 (34) and TB10.4 were to be considered. Furthermore, we could show that identical peptides can be recognized in the context of BoLA-DR and BoLA-DQ and that the restriction element used determined whether such peptides are "*M. bovis* specific" or cross-reactive. This observation introduces a further complication in attempts to predict species specificity by comparing sequence identity/homology alone and highlights that empirical experimental studies with individuals that express diverse BoLA class II haplotypes are required to assess T-cell cross-reactivity.

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