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Mapping of Murine Th1 Helper T-Cell Epitopes of Mycolyl Transferases Ag85A, Ag85B, and Ag85C from *Mycobacterium tuberculosis*

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BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were infected intravenously with *Mycobacterium tuberculosis* H37Rv or vaccinated intramuscularly with plasmid DNA encoding each of the three mycolyl transferases Ag85A, Ag85B, and Ag85C from *M. tuberculosis*. Th1-type spleen cell cytokine secretion of interleukin-2 (IL-2) and gamma interferon (IFN-γ) was analyzed in response to purified Ag85 components and synthetic overlapping peptides covering the three mature sequences. Tuberculosis-infected C57BL/6 mice reacted strongly to some peptides from Ag85A and Ag85B but not from Ag85C, whereas tuberculosis-infected BALB/c mice reacted only to peptides from Ag85A. In contrast, spleen cells from both mouse strains produced elevated levels of IL-2 and IFN-γ following vaccination with Ag85A, Ag85B, and Ag85C DNA in response to peptides of the three Ag85 proteins, and the epitope repertoire was broader than in infected mice. Despite pronounced sequence homology, a number of immunodominant regions contained component specific epitopes. Thus, BALB/c mice vaccinated with all three Ag85 genes reacted against the same amino acid region, 101 to 120, that was also immunodominant for Ag85A in *M. bovis* BCG-vaccinated and tuberculosis-infected *H-2*^{d} haplotype mice, but responses were completely component specific. In C57BL/6 mice, a cross-reactive T-cell response was detected against two carboxy-terminal peptides spanning amino acids 241 to 260 and 261 to 280 of Ag85A and Ag85B. These regions were not recognized at all in C57BL/6 mice vaccinated with Ag85C DNA. Our results underline the need for comparative analysis of all three Ag85 components in future vaccination studies.

Secreted and surface-exposed cell wall proteins are major antigens recognized by the protective immune response against tuberculosis, and immunization with whole culture filtrate, a rich source of these extracellular proteins, can protect mice and guinea pigs to some extent against subsequent challenge with the tubercle bacillus *Mycobacterium tuberculosis* (1, 31). A major fraction of the secreted proteins in *M. tuberculosis* culture filtrate is formed by the Ag85 complex, a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C) (42), all of which possess a mycolyl transferase enzyme activity required for the biogenesis of cord factor (trehalose-dimycolate) (3) and are encoded by three paralogous genes located in distinct regions of the bacterial genome (6). These genes show no resemblance in their 5′ upstream region, and they are probably regulated independently at the transcriptional level (6).

Members of the Ag85 family are found in all mycobacteria, and sequence comparison indicates that the Ag85 gene family arose by ancient duplications of an ancestral gene, probably before the emergence of the known mycobacterial species. Genetic disruption of the genes encoding the three Ag85 components of *M. tuberculosis* suggests that Ag85A may be the most essential component for bacterial survival within macrophages (2, 20).

Ag85A induces strong T-cell proliferation and gamma interferon (IFN-γ) production in most healthy individuals infected with *M. tuberculosis* or *Mycobacterium leprae* (23) and in *Mycobacterium bovis* BCG-vaccinated mice (17), and both Ag85A and Ag85B rank among the most promising candidates for future subunit tuberculosis vaccines, as indicated in experimental mouse and guinea pig models. Indeed, vaccination of C57BL/6 mice with naked plasmid DNA encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated immune responses and confer significant protection against aerosol or intravenous challenge with live *M. tuberculosis* H37Rv (18, 21, 37). Also, immunization with purified Ag85B protein in adjuvant has shown some protective efficacy in mice (26) and guinea pigs (16). Recently, it was shown that the protective efficacy of the BCG vaccine can be increased by boosting with purified Ag85A protein (4), by overexpression of the Ag85B component (15), and by priming with DNA encoding Ag85B (12).

So far, murine Th1 T-cell epitopes have been described for Ag85A (8, 19, 36) and for Ag85B (43). In guinea pigs immunized with purified native Ag85A and Ag85B protein in adjuvant (24), a large number of epitopes were defined on both proteins that were all recognized by 20 to 30% of the animals. Concerning T-cell responses in humans, human T-cell epitope mapping of Ag85A has revealed a largely promiscuous recognition pattern, with a majority of healthy *M. tuberculosis*- and *M. leprae*-infected subjects with different HLA haplotypes reacting to a restricted number of peptide regions (23). Similar promiscuous T-cell recognition has also been observed for antigen Ag85B in peripheral blood mononuclear cells or T-cell lines from purified protein derivative-positive donors or BCG-vaccinated subjects (29, 32, 34, 39).

Whereas most immunological studies have focused on Ag85A

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and Ag85B, less is known about the third member of this protein family, Ag85C. We have reported before that the three Ag85 components have different isoelectric points (4.55, 4.35, and 4.15 for Ag85A, Ag85C, and Ag85B, respectively) and that they can be separated by isoelectric focusing (9). Combining this isoelectric focusing separation with Western blot analysis, we demonstrated that Ag85A was the least specific component for serodiagnosis of tuberculosis because low-level immunoglobulin G antibodies were also present in control serum samples from healthy persons (41). In contrast, antibodies against the Ag85B and Ag85C component could only be detected in serum samples from tuberculous and lepromatous leprosy patients (40, 41).

In an enzyme-linked immunosorbent assay (ELISA) with purified Ag85 components from *M. tuberculosis* culture filtrate, serum samples from more tuberculosis patients reacted to Ag85A and Ag85B than to Ag85C (25), but mice vaccinated with plasmid DNA encoding Ag85A, Ag85B, or Ag85C all produced significant levels of Ag85-specific antibodies (38). This suggests that the weak recognition of Ag85C by patients may be caused by lower expression levels rather than by lower immunogenicity of the molecule. The Ag85C molecule was also reported to have a lower secretion index than the Ag85A and Ag85B molecules (42), which are found predominantly in culture filtrate (14), and this might influence the in vivo availability of Ag85C for antigenic processing in the phagosome.

As these three proteins are highly paralogous (about 77% shared amino acids between Ag85A and Ag85B and about 71% shared amino acids with Ag85C), one could expect them to have common immunodominant epitopes. In order to analyze this in further detail, we performed a comparative murine Th1 T-cell epitope in BALB/c and C57BL/6 mice infected with *M. tuberculosis* or vaccinated with plasmid DNA encoding Ag85A, Ag85B, or Ag85C from *M. tuberculosis* with synthetic, overlapping peptides spanning the entire mature sequences of the three proteins.

MATERIALS AND METHODS

Mice. BALB/c (*H-2*^{d}) and C57BL/6 (*H-2*^{b}) mice were maintained by strict brother-sister mating in the animal facilities of the Pasteur Institute of Brussels from breeding pairs obtained from Bantin and Kingman Universal Ltd. (London, United Kingdom).

Infection and vaccination. Mice were inoculated intravenously with 10^6 CFU of luminescent *M. tuberculosis* H37Rv (35), grown as a surface pellicle on synthetic Sauton medium for 14 days, and stored in 20% glycerol in frozen aliquots at −70°C. Spleens were removed 3 months after infection. DNA vaccination was performed by three intramuscular injections of 100 μg of purified plasmid DNA encoding a secreted form of the three Ag85 genes from *M. tuberculosis* in V1J.ns-tPA vector at 3-week intervals, as reported before (18, 27). Spleens were removed 3 weeks after the last DNA immunization.

Antigens. Individual Ag85A, Ag85B, and Ag85C components and antigen 85 complex (a mix of more or less equal parts of Ag85A, Ag85B, and Ag85C) were purified by sequential chromatography on phenyl-Sepharose, DEAE-Sephacel, and Sephadex G75 from 2-week-old *M. bovis* BCG (strain GL2) cultures grown as a surface pellicle on synthetic Sauton medium and checked by isoelectric focusing (7, 9). All antigens were used at a final concentration of 5 μg/ml.

Alignment of amino acid sequences of mature Ag85A, Ag85B, and Ag85C from *M. tuberculosis*. The aligned amino acid sequences of Ag85A (295 amino acids), Ag85B (285 amino acids), and Ag85C (294 amino acids) from *M. tuberculosis* are shown in Table 1. Sequence information was obtained from the SWISS-PROT database: accession number P17944 for Ag85A, P31952 for Ag85B, and P31953 for Ag85C The three amino acids essential for the catalytic mycolyl transferase function are shown in bold. Amino acids 33 and 34 are deleted in Ag85C, and these positions were left open to permit optimal alignment, resulting in a final numbering of 296 residues.

The sequences of Ag85A and Ag85C are 100% identical for *M. tuberculosis* and *M. bovis* BCG, whereas the sequence data of Ag85B for *M. bovis* BCG have to be considered with some caution. Indeed, the sequence reported by Matsuo et al. in 1988 for *M. bovis* Tokyo differs from the Ag85B sequence of *M. tuberculosis* at position 100 (shift from phenylalanine in *M. tuberculosis* to leucine in *M. bovis* BCG Tokyo), positions 163 and 164 (proline and serine in *M. tuberculosis* deleted in BCG Tokyo), and positions 250 and 251 (asparagine and alanine in *M. tuberculosis* versus lysine and proline for BCG Tokyo) (28). However, the Ag85B sequence from the unfinished *M. bovis* genome (Sanger 1765, contig 265) is completely identical to the Ag85B sequence from *M. tuberculosis*, and for positions 250 to 251, the sequence of the Ag85B gene of at least five *M. bovis* BCG strains (including GL2) is also identical to the one from *M. tuberculosis* (V. Rosseels, unpublished data). The complete genome sequence of *M. bovis* BCG 1173P2 (from which the Brussels GL2 strain was derived) has not been published yet.

Peptide synthesis. Peptides spanning the entire mature 295-amino-acid Ag85A sequence of *M. tuberculosis* were synthesized as 20-mers overlapping by 10 residues, with the exception of the 19-mer from 35 to 53, which was resynthesized in this form for economy reasons, and the carboxy-terminal peptide containing amino acids 275 to 295 (19). Peptides spanning the entire 285-amino-acid Ag85B sequence from *M. tuberculosis* were synthesized as 18-mers overlapping by nine residues, with the exception of a 21-mer spanning amino acids 240 to 260 and the carboxy-terminal 15-mer from 271 to 285. Peptides spanning the entire mature 294-amino-acid Ag85C sequence of *M. tuberculosis* were synthesized as 20-mers overlapping by 10 residues with the exception of two 18-mers (amino acids 31 to 50 and 41 to 60; residues 33 and 34 are lacking in Ag85C) and the carboxy-terminal 16-mer from 281 to 296 (Table 1). All peptides were used at a final concentration of 10 μg/ml.

Cytokine production. Spleen cells (4×10^6 white blood cells/ml) from four mice per group were tested individually for cytokine response to native Ag85 complex and its purified components (see Table 3) and as a pool for peptide mapping (see Fig. 1, 2, and 3 and Tables 4 and 5) (19). Supernatants were harvested after 24 h (interleukin-2 [IL-2]) and 72 h (IFN-γ), when peak values of the respective cytokines can be measured. Supernatants from at least three separate wells were pooled and stored frozen at −20°C until assayed. Experiments were performed at least three times, and data from one representative experiment are reported.

IL-2 assay. IL-2 activity was quantified with a bioassay, measuring uptake of tritiated thymidine by the IL-2-dependent CTLL-2 cell line as described before (17). IL-2 levels are expressed as mean counts per minute (cpm) of samples tested in duplicate. Variation between duplicates was <10%. A standard recombinant murine IL-2 preparation of 10,000 arbitrary units per vial was obtained from the National Institute for Biological Standards and Control (London, United Kingdom; code 93/566), and serial twofold dilutions of the standard were run in parallel with the assays. The standard curve corresponding to the IL-2 results of Fig. 3 is given in Table 2. IL-2 responses were considered positive when the stimulation index (cpm for spleen cell cultures stimulated with peptide or antigen divided by cpm for unstimulated spleen cell cultures) was greater than 2.

IFN-γ assay. IFN-γ activity was quantified by sandwich ELISA with coating antibody R4-6A2 and biotinylated detection antibody XMG1.2 (both from Pharmingen). The sensitivity of the ELISA was about 10 pg/ml.

RESULTS

Ag85-specific Th1-type cytokine secretion in mice vaccinated with Ag85 DNA. Spleen cells from BALB/c and C57BL/6 mice vaccinated with Ag85 DNA produced significant levels of IL-2 and IFN-γ in response to native Ag85 and its three purified components (Table 3). Overall, IL-2 levels were more or less comparable for both mouse strains and for all three genes. IFN-γ levels tended to be higher in C57BL/6 than in BALB/c mice. BALB/c and C57BL/6 mice vaccinated with Ag85C DNA reacted only very weakly with purified native Ag85A and Ag85B. In contrast, cross-reactive IFN-γ (and to a lesser extent IL-2) responses were found to the three protein antigens following vaccination with Ag85A or Ag85B DNA.

IFN-γ-inducing T-cell epitopes of Ag85A, Ag85B, and Ag85C in spleen cell cultures from BALB/c and C57BL/6 mice infected with *M. tuberculosis* or vaccinated with DNA encoding

TABLE 2. IL-2 standard curve

IL-2 (A.U./ml)[a]	Mean cpm ± SD
500	208,088 ± 22,218
250	198,411 ± 27,635
125	186,590 ± 22,789
62.5	121,050 ± 15,419
31.25	85,756 ± 18,129
15.6	48,572 ± 7,299
7.8	32,068 ± 1,693
3.9	14,109 ± 2,445
1.9	8,679 ± 1,075
0.98	3,785 ± 603
0.49	1,558 ± 421
0.24	530 ± 71

[a] A.U., arbitrary units.

Ag85 components. As shown in Fig. 1, spleen cells from BALB/c mice infected with tuberculosis 3 months previously (white bars) only produced significant levels of IFN-γ (>500 pg/ml) in response to peptides from Ag85A. Three IFN-γ-inducing peptide regions were identified on Ag85A in these tuberculosis-infected BALB/c mice: amino acids 101 to 120 (previously described as being immunodominant in BCG-vaccinated *H-2*^{d} haplotype mice [19]), amino acids 151 to 170, and amino acids 191 to 210. Spleen cells from tuberculosis-infected C57BL/6 mice (Fig. 2, white bars) reacted strongly (approximately 2,000 pg/ml) to the peptide composed of amino acids 241 to 260 at the carboxy-terminal end of Ag85A and Ag85B and only weakly to peptides from Ag85C. We have previously reported that amino acids 241 to 260 of Ag85A contain a Th1 T-cell epitope in BCG-vaccinated mice with the *H-2*^{b} haplotype as well (19). Similar results were obtained after 1 and 5 months of tuberculosis infection (data not shown). Spleen cells from mice vaccinated with empty vector and from naive mice did not react to the Ag85 peptides (data not shown).

In contrast to tuberculosis-infected animals, robust IFN-γ responses could be detected in spleen cell cultures from BALB/c and C57BL/6 mice vaccinated with DNA encoding either of the three Ag85 components (Fig. 1 and 2, black bars). Experiments were performed at least three times. The same peptide regions were identified in corresponding experiments, although the relative intensity of the immune response to the different peptides varied somewhat from one experiment to another. Confirming previous findings for Ag85A (8, 36), DNA vaccination induced a broader IFN-γ-inducing epitope repertoire in *H-2*^{b} and in *H-2*^{d} haplotype mice than infection with *M. tuberculosis*. It is unlikely (but cannot be excluded) that aerosol infection with *M. tuberculosis* might have generated a broader spleen cell immune response than intravenous infection, but this remains to be examined.

All peptides that induced IFN-γ in tuberculosis-infected mice were also recognized in DNA-vaccinated animals. Thus, DNA-vaccinated BALB/c mice reacted to various extents to the peptides spanning amino acids 100 to 120, amino acids 151 to 170, and 191 to 210. Moreover, DNA-vaccinated BALB/c mice produced high IFN-γ levels (but little IL-2; see Fig. 3) in response to a peptide spanning amino acids 60 to 80, containing predicted L^d and K^d binding motifs on Ag85A/B and Ag85C, respectively. Finally, BALB/c mice vaccinated with

TABLE 1. Amino acid sequences of mature Ag85A, Ag85B, and Ag85C of *M. tuberculosis*[a]

Antigen	Sequence
	1 100
Ag85A	FSRPGLPVEY LQVPSPSMGR DIKVQFQSGG ANSPALYLLD GLRAQDDFSG WDINTPAFEW YDQSGLSVVM PVGGQSSFYS DWYQPAGKA GCQTYKWETF
Ag85B	FSRPGLPVEY LQVPSPSMGR DIKVQFQSGG NNSPAVYLLD GLRAQDDYNG WDINTPAFEW YYQSGLSIVM PVGGQSSFYS DWYSPACGKA GCQTYKWETF
Ag85C	FSRPGLPVEY LQVPSASMGR DIKVQFQGGG PH..AVYLLD GLRAQDDYNG WDINTPAFEE YYQSGLSVIM PVGGQSSFYT DWYQPSQSNG QNYTYKWETF
	101 200
Ag85A	LTSELPGWLQ ANRHVKPTGS AVVGLSMAAS SALTLAIYHP QQFVYAGAMS GLLDPSQAMG PTLIGLAMGD AGGYKASDMW GPKEDPAWQR NDPLLNVGKL
Ag85B	LTSELPQWLS ANRAVKPTGS AAIGLSMAGS SAMILAAYHP QQFIYAGSLS ALLDPSQGMG PSLIGLAMGD AGGYKAADMW GPSSDPAWER NDPTQQIPKL
Ag85C	LTREMPAWLQ ANKGVSPTGN AAVGLSMSGG SALILAAYYP QQFPYAASLS GFLNPSEGWW PTLIGLAMND SGGYNANSMW GPSSDPAWKR NDPMVQIPRL
	201 296
Ag85A	IANNTRVWVY CGNGKPSDLG GNNLPAKFLE GFVRTSNIKF QDAYNAGGGH NGVFDFPDSG THSWEYWGAQ LNAMKPDLQR AL.GATPNTG PAPQGA
Ag85B	VANNTRLWVY CGNGTPNELG GANIPAEFLE NFVRSSNLKF QDAYNAAGGH NAVFNFPPNG THSWEYWGAQ LNAMKGDLQS SL.GAG~~~~ ~~~~~~
Ag85C	VANNTRIWVY CGNGTPSDLG GDNIPAKFLE GLTLRTNQTF RDTYAADGGR NGVFNFPPNG THSWPYWNEQ LVAMKADIQH VLNGATPPAA PAAPAA

[a] The three amino acids essential for enzymatic mycolyl transferase function are indicated in bold.

TABLE 3. Ag85-specific Th1-type cytokine secretion in BALB/c and C57BL/6 mice vaccinated with DNA encoding the three Ag85 components

Mice	Vaccination	Mean IL-2 (cpm) ± SD[a]			Mean IFN-γ (pg/mL) ± SD[b]		
		Ag85A DNA	Ag85B DNA	Ag85C DNA	Ag85A DNA	Ag85B DNA	Ag85C DNA
BALB/c	Medium	2,595 ± 512	3,752 ± 1,118	2,939 ± 663	<50	<50	<50
	Ag85A[c]	**22,090 ± 9,898**	5,784 ± 1,118	4,005 ± 1,146	**1,092 ± 435**	293 ± 80	142 ± 104
	Ag85B	4,742 ± 1,657	**8,519 ± 2,972**	3,202 ± 328	266 ± 104	**472 ± 233**	142 ± 90
	Ag85C	7,301 ± 2,780	4,306 ± 770	**10,307 ± 1,809**	523 ± 163	482 ± 122	**396 ± 99**
	Ag85	13,237 ± 4,041	7,392 ± 1,329	7,481 ± 1,560	779 ± 78	699 ± 284	378 ± 93
C57BL/6	Medium	1,848 ± 230	2,116 ± 406	1,897 ± 232	<50	<50	<50
	Ag85A	**21,308 ± 1,568**	16,300 ± 4,764	2,788 ± 593	**2,737 ± 226**	1,517 ± 462	<50
	Ag85B	7,714 ± 2,051	**13,319 ± 4,765**	2,571 ± 271	1,018 ± 432	**2,300 ± 711**	287 ± 191
	Ag85C	7,343 ± 3,432	6,072 ± 1,730	**6,202 ± 2373**	1,170 ± 661	612 ± 136	**2,392 ± 713**
	Ag85	16,487 ± 4,687	12,860 ± 4,297	4,252 ± 755	2,300 ± 700	1,926 ± 829	934 ± 390

[a] Mean IL-2 levels measured in 24-h culture supernatant of spleen cells from mice vaccinated with DNA and unstimulated (medium) or restimulated in vitro with Ag85 components. Four mice were tested individually in each group. Data from corresponding DNA and antigen pairs are given in bold.
[b] Mean IFN-γ levels measured in 72-h culture supernatant of spleen cells from mice vaccinated with DNA and unstimulated (medium) or restimulated in vitro with Ag85 components. Four mice were tested individually in each group.
[c] Antigen used for in vitro restimulation (5 μg/ml).

Ag85B and Ag85C produced high levels of IFN-γ in response to a peptide spanning amino acids 260 to 280.

C57BL/6 mice vaccinated with Ag85A reacted most strongly to amino acids 261 to 280 in the carboxy-terminal half of the protein, whereas C57BL/6 mice vaccinated with Ag85C DNA reacted most strongly to peptides in the amino-terminal half, i.e., amino acids 1 to 20, 21 to 40, and 81 to 100. C57BL/6 mice vaccinated with Ag85B DNA reacted strongly to amino acids 82 to 99 in the amino-terminal half and to amino acids 240 to 260 in the carboxy-terminal half. Although results differed slightly from one experiment to another, IFN-γ responses in Ag85A DNA-vaccinated C57BL/6 mice were generally lower to amino acids 240 to 260 than to the more-carboxy-terminal peptide spanning amino acids 261 to 280. The IFN-γ response of C57BL/6 mice vaccinated with Ag85B resembled more the response of tuberculosis-infected mice, with highest titers following stimulation with amino acids 240 to 260. C57BL/6 mice vaccinated with Ag85C did not react to these two carboxy-terminal peptides at all.

IL-2-inducing T-cell epitopes of Ag85A, Ag85B, and Ag85C in spleen cell cultures from BALB/c and C57BL/6 mice infected with *M. tuberculosis* or vaccinated with DNA encoding Ag85 components. IL-2 production was very low in spleen cell cultures from tuberculosis-infected mice in response to these synthetic peptides (data not shown). In contrast, positive IL-2 production (stimulation index, >2; cpm values, generally >10,000) was detected in spleen cell culture supernatants from BALB/c (Fig. 3, white bars) and C57BL/6 (Fig. 3, black bars) mice vaccinated with DNA encoding either of the three Ag85 components and restimulated with the respective peptides. As for IFN-γ production, the peptide spanning amino acids 101 to 120 induced elevated levels of IL-2 in BALB/c mice vaccinated with DNA encoding each of the three genes. Spleen cells from BALB/c mice also strongly recognized amino acids 191 to 210 following vaccination with Ag85A and carboxy-terminal region 260 to 280 following vaccination with Ag85B and Ag85C.

In C57BL/6 mice, strong IL-2 levels were detected in response to the two carboxy-terminal peptides of Ag85A and Ag85B, amino acids 241 to 260 and amino acids 261 to 280, respectively. As for IFN-γ, IL-2 responses were generally higher to amino acids 261 to 280 following vaccination with Ag85A DNA and higher to amino acids 241 to 260 following vaccination with Ag85B DNA. C57BL/6 mice vaccinated with Ag85C DNA did not produce IL-2 in response to these carboxy-terminal peptides, but in contrast, they produced elevated IL-2 levels to the amino-terminal peptide spanning amino acids 21 to 40 of Ag85C. Finally, C57BL/6 mice vaccinated with Ag85B and Ag85C DNAs demonstrated a strong IL-2 response following stimulation with amino acids 81 to 100 and amino acids 141 to 160.

Definition of cross-reactive and component-specific Th1 epitopes on Ag85 complex proteins. Although the three Ag85 components are highly homologous, a number of their amino acids are different, and therefore, recognition of the same peptide region on the three components does not necessarily indicate that immune responses are genuinely cross-reactive. Spleen cells from BALB/c mice vaccinated with each of the three Ag85 genes produced IFN-γ (but no IL-2) in response to amino acids 61 to 80 (Table 4). A second cross-reactive IFN-γ response was detected to amino acids 191 to 210, a peptide region also recognized on Ag85A by tuberculosis-infected BALB/c mice. Interestingly, BALB/c mice vaccinated with Ag85A or Ag85C produced IFN-γ in response to the corresponding peptide from all three proteins, but vaccination with the Ag85B DNA failed to induce an IFN-γ response to this peptide. Elevated IL-2 responses to amino acids 191 to 210 were only found in BALB/c mice immunized with Ag85A (Table 4). Finally, amino acids 101 to 120 induced very strong IFN-γ and IL-2 production in BALB/c mice vaccinated with all three genes, but the epitopes were found to be completely component specific.

In C57BL/6 mice (Table 5), two $CD4^+$ T-cell epitopes present in amino acids 241 to 260 and 261 to 280 induced cross-reactive immune responses for Ag85A and Ag85B, respectively. No cross-reactive immune response to these two peptides could be found in C57BL/6 mice vaccinated with Ag85C DNA.

Predicted and experimentally defined Th1 T-cell epitopes on Ag85A, Ag85B, and Ag85C for *H-2*^{d} and *H-2*^{b} haplotype mice. The predicted murine $CD4^+$ T-cell epitopes according to alpha-helical periodicity, Rothbard and Taylor motifs, and $I\text{-}A^d$ and $I\text{-}E^d$ binding motifs on Ag85A, Ag85B, and Ag85C by the

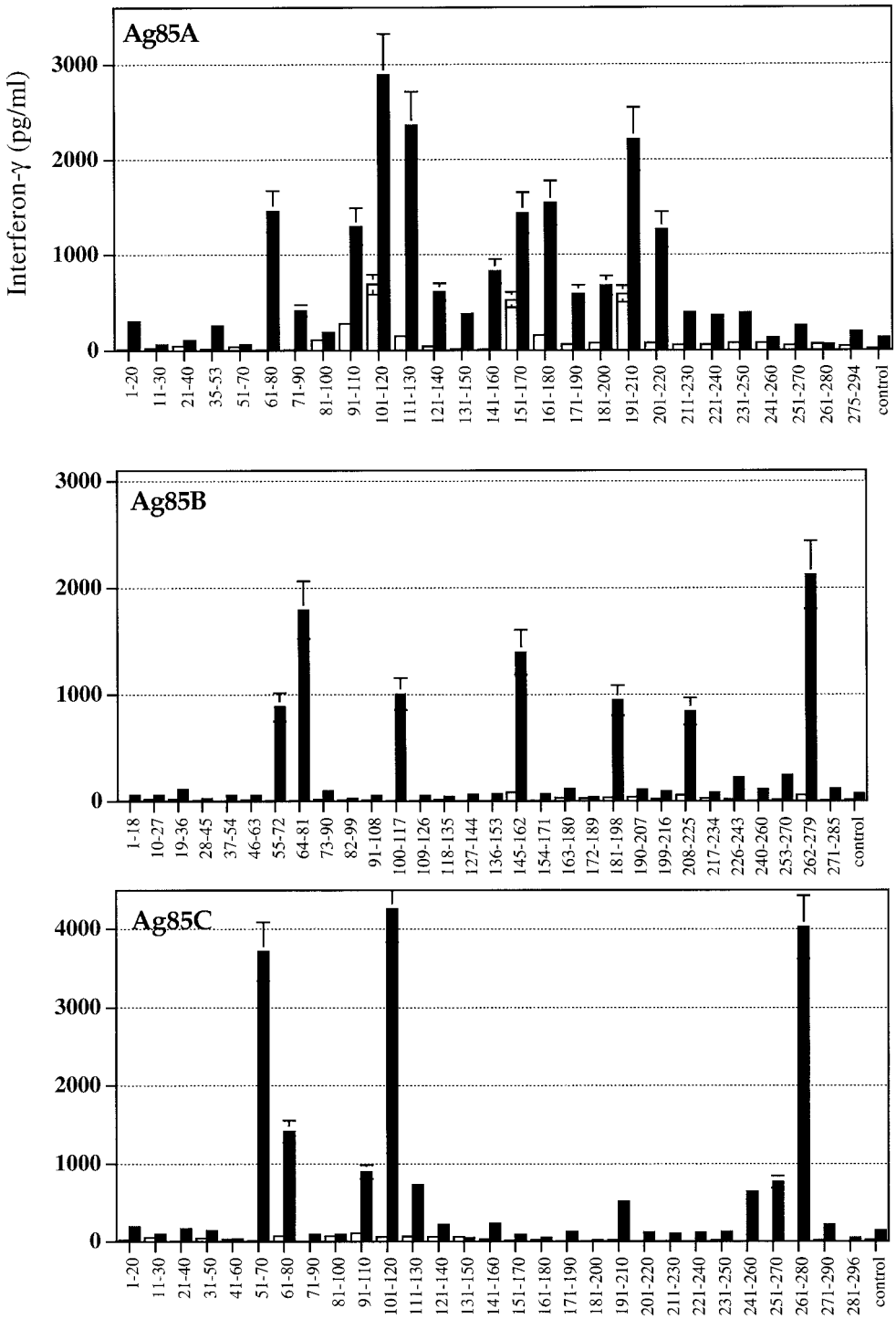

FIG. 1. IFN-γ-inducing epitopes of Ag85A, Ag85B, and Ag85C in tuberculosis-infected and DNA-vaccinated BALB/c mice. Mean IFN-γ levels detected in 72-h culture supernatant of spleen cells from BALB/c mice infected intravenously with *M. tuberculosis* 3 months previously (white bars) or vaccinated with Ag85A, Ag85B, or Ag85C DNA (black bars) and stimulated in vitro with synthetic overlapping peptides covering the mature sequences of Ag85A, Ag85B, and Ag85C from *M. tuberculosis*. Spleen cells from four mice were pooled in each group, and supernatants from at least three separate wells were pooled for assay.

Tsites program (11) are given in Fig. 4A, 4B, and 4C. Experimentally defined Th1 epitopes (inducing both IL-2 and IFN-γ) in BALB/c mice are shown in red, those defined in C57BL/6 mice are shown in green, those overlapping in both mouse strains are shown in blue. Although most of the experimentally defined epitopes were also predicted by Tsites, the predictive value of the program was imperfect, as many more epitopes were predicted than identified and at least two epi-

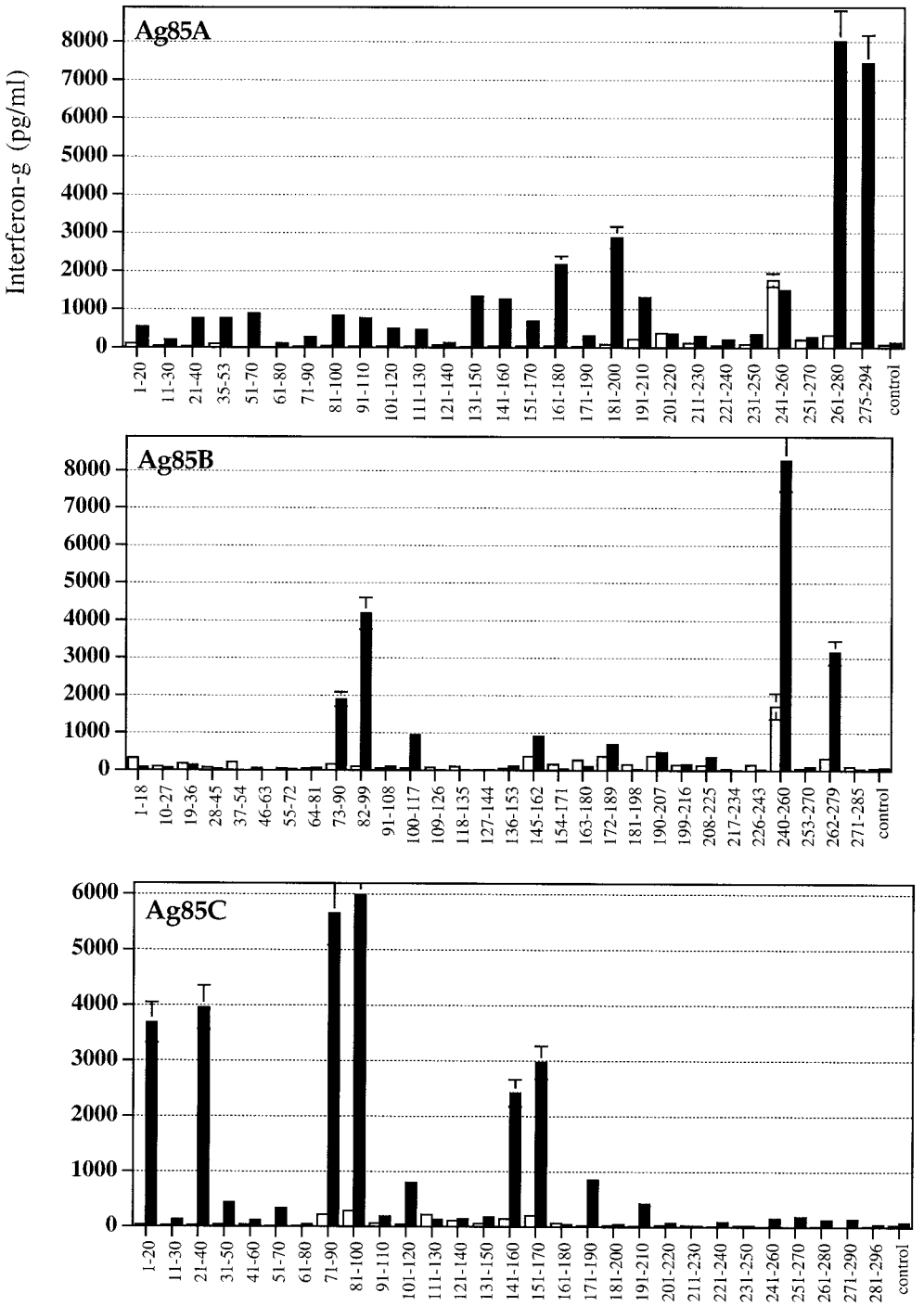

FIG. 2. IFN-γ-inducing epitopes of Ag85A, Ag85B, and Ag85C in tuberculosis-infected and DNA-vaccinated C57BL/6 mice. Mean IFN-γ levels detected in 72-h culture supernatant of spleen cells from C57BL/6 mice infected intravenously with *M. tuberculosis* 3 months previously (white bars) or vaccinated with Ag85A, Ag85B, or Ag85C DNA (black bars) and stimulated in vitro with synthetic overlapping peptides covering the mature sequences of Ag85A, Ag85B, and Ag85C from *M. tuberculosis*. Spleen cells from four mice were pooled in each group, and supernatants from at least three separate wells were pooled for assay.

topes were defined (on the Ag85C molecule) that were not predicted at all.

DISCUSSION

Members of the mycolyl transferase family of *M. tuberculosis*, also called the Ag85 complex, rank among the most promising tuberculosis vaccine candidates (22). As the three Ag85 components (Ag85A, Ag85B, and Ag85C) are highly homologous, one could expect them to share most of their immunodominant epitopes. In order to analyze this in detail, we vaccinated mice of two commonly used haplotypes, *H-2*^{d} and *H-2*^{b}, with plasmid DNA encoding each of the three Ag85 compo-

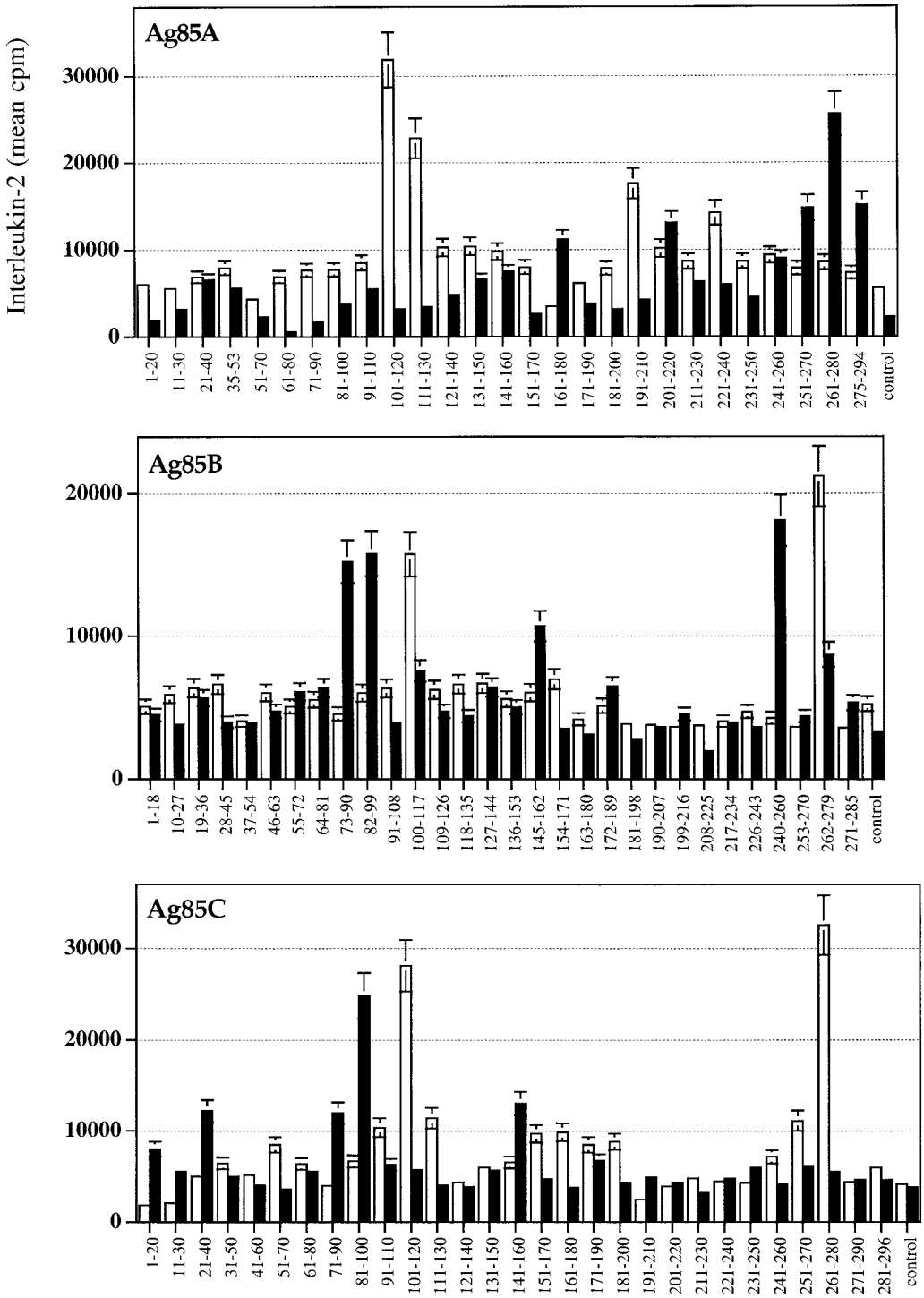

FIG. 3. IL-2-inducing epitopes of Ag85A, Ag85B, and Ag85C in DNA-vaccinated BALB/c and C57BL/6 mice. Mean IL-2 levels detected in 24-h culture supernatant of spleen cells from BALB/c (white bars) and C57BL/6 (black bars) mice vaccinated three times with DNA and stimulated in vitro with synthetic overlapping peptides covering the mature sequence of Ag85A, Ag85B, and Ag85C 3 weeks after the third DNA injection. Spleen cells from four mice were pooled in each group, and supernatants from at least three separate wells were pooled for assay.

nents from *M. tuberculosis*. We have previously shown that vaccination with DNA encoding Ag85A can be used for the mapping of $CD4^+$ and $CD8^+$ T-cell epitopes and that this type of vaccination is more potent than vaccination with BCG or infection with tuberculosis because it induces a stronger response and a broader epitope repertoire (8, 36). DNA vaccination enabled us to define IL-2- and IFN-γ-inducing epitopes on Ag85A, Ag85B, and Ag85C in both mouse strains. In a previous preliminary study, we were unable to detect Th1-type immune responses following Ag85C DNA vaccination (27).

TABLE 4. Cross-reactivity of three immunodominant Th1 T-cell epitopes of Ag85A, Ag85B, and Ag85C in DNA-vaccinated BALB/c mice[a]

Peptide	Mean IL-2 (cpm) ± SD			Mean IFN-γ (pg/ml)		
	Ag85A DNA	Ag85B DNA	Ag85C DNA	Ag85A DNA	Ag85B DNA	Ag85C DNA
None (medium control)	5,422 ± 229	3,868 ± 560	4,699 ± 118	62	75	50
Ag85A 61–80	**7,319 ± 95**	5,955 ± 21	6,069 ± 686	**590**	1,863	311
Ag85B 64–81	7,645 ± 605	**5,359 ± 479**	5,364 ± 689	674	**1,670**	584
Ag85C 61–80	5,442 ± 65	4,352 ± 261	**5,591 ± 176**	713	767	**720**
Ag85A 101–120	**24,988 ± 927**	4,174 ± 201	8,182 ± 13	**2,077**	88	235
Ag85B 100–117	5,126 ± 506	**10,428 ± 1,475**	4,636 ± 422	834	**1,058**	524
Ag85C 101–120	7,432 ± 821	5,024 ± 412	**23,463 ± 843**	357	106	**3,822**
Ag85A 191–210	**21,012 ± 765**	5,789 ± 553	7,884 ± 949	**976**	143	556
Ag85B 190–207	8,637 ± 675	**5,648 ± 299**	5,596 ± 605	1,003	**42**	129
Ag85C 191–210	9,266 ± 650	4,716 ± 557	**6,227 ± 234**	853	55	**517**

[a] IL-2 and IFN-γ levels in 24-h and 72-h spleen cell culture supernatants from BALB/c mice vaccinated with DNA encoding Ag85A, Ag85B, or Ag85C and restimulated in vitro with medium or synthetic peptides covering overlapping regions of Ag85A, Ag85B, or Ag85C. Results are presented as mean values for pooled cells from four mice per group. Data from corresponding DNA and peptide pairs are indicated in bold.

However, as we did not use specific Ag85C peptides at that time but only Ag85 complex purified from culture filtrate (which is known to contain little Ag85C protein unless spiked) (14), these data are not really in contradiction with our present findings.

BALB/c mice vaccinated with each of the three plasmids reacted against a peptide spanning amino acids 100 to 120, previously reported as being immunodominant for Ag85A in DNA- and BCG-vaccinated and in tuberculosis-infected *H-2*^{d} haplotype mice (8, 19). Detailed analysis showed that the Th1 T helper response to this peptide region is specific for each Ag85 component. It is probable that the shift from histidine (amino acid 114) in Ag85A to alanine in Ag85B and glycine in Ag85C is responsible for this specificity. Tsites predictions were partially concordant with the experimental findings. Indeed, Tsites predicted an I-E^d binding site on Ag85A in this region and an I-A^d binding site on Ag85B, but no motif was predicted for Ag85C. The fact that *M. tuberculosis*-infected BALB/c mice did not react to the putative I-A^d binding motif on the Ag85B molecule (nor to any Ag85B peptide for that matter) confirms previous speculations that BALB/c mice may have a relative lack of I-A^d and would, at least in tuberculosis infection, preferentially use their I-E^d heterodimer for antigen presentation (30). Interestingly, amino acids 100 to 120 of Ag85B are also recognized in a promiscuous manner by T cells from a majority of purified protein derivative-positive human volunteers (29, 39).

The region spanning amino acids 61 to 80 induced weak IL-2 but strong IFN-γ responses in BALB/c mice vaccinated with all three genes. Amino acids 71 to 80 of Ag85A encode an L^d-restricted epitope that can be recognized by CD8$^+$ cytotoxic T lymphocytes in Ag85A DNA (8) and Ag85B DNA-vaccinated but not in Ag85C DNA-vaccinated BALB/c mice (data not shown), which is surprising because the sequences of the three genes are identical between positions 70 and 79. As the 61 to 80 region also contains a predicted K^d epitope spanning amino acids 61 to 69 (8), with a higher half-life dissociation score for Ag85C (2,880) than for Ag85A (600), it is possible that the IFN-γ response to amino acids 61 to 80 of Ag85C is produced by K^d-restricted cytotoxic T lymphocytes, but this remains to be examined.

The peptide spanning amino acids 241 to 260 in the carboxy-terminal half of Ag85A and Ag85B was recognized by C57BL/6 mice infected with tuberculosis or vaccinated with DNA encoding Ag85A and Ag85B but not with DNA encoding Ag85C. This region was already identified on the Ag85A molecule in BCG- and Ag85A DNA-vaccinated mice (19, 36). Also, Yanagisawa et al., who performed a helper T-cell epitope mapping of Ag85B (called MPT59 in that study) on lymph node cells from C57BL/6 mice immunized with heat-killed *M. tuberculosis*, demonstrated that a peptide spanning amino acids 240 to 254 was immunodominant, contained a motif specific for I-A^b, and could trigger $v_\beta11^+$ CD4$^+$ T cells (43).

The two differences between Ag85A and Ag85B at positions 249 (arginine to glycine) and 251 (glycine to alanine) apparently do not affect the binding to the I-A^b molecule, as also suggested by the fact that both molecules present a predicted amphipathic stretch in this region. On the other hand, the four differences between the Ag85A/B and Ag85C molecules in positions 243 (aspartic acid to threonine), 245 (asparagine to alanine), 247 (glycine to aspartic acid), and 250 (histidine to arginine) apparently induce a dramatic change in the structure, leading to loss of the amphipathic character and immunogenicity. For the *H-2*^{b} peptide spanning amino acids 261 to 280 (particularly immunogenic in C57BL/6 mice vaccinated with Ag85A DNA), the Ag85A and Ag85B molecules are identical in their first 15 residues, whereas in the Ag85C molecule, three shifts, from glutamic acid to proline in position 265, from

FIG. 4. Predicted and experimentally defined Th1 T-cell epitopes of Ag85A (A), Ag85B (B), and Ag85C (C) for *H-2*^{d} and *H-2*^{b} haplotypes. The predicted T-cell epitopes are indicated according to alpha-helical periodicity (A), Rothbard and Taylor motifs (R), I-A^d (D), and I-E^d (d) binding motifs. Experimentally defined peptide regions are in red for *H-2*^{d} and in green for *H-2*^{b} haplotype mice and in blue when recognized by both haplotypes. The three amino acids essential for catalytic function are underlined.

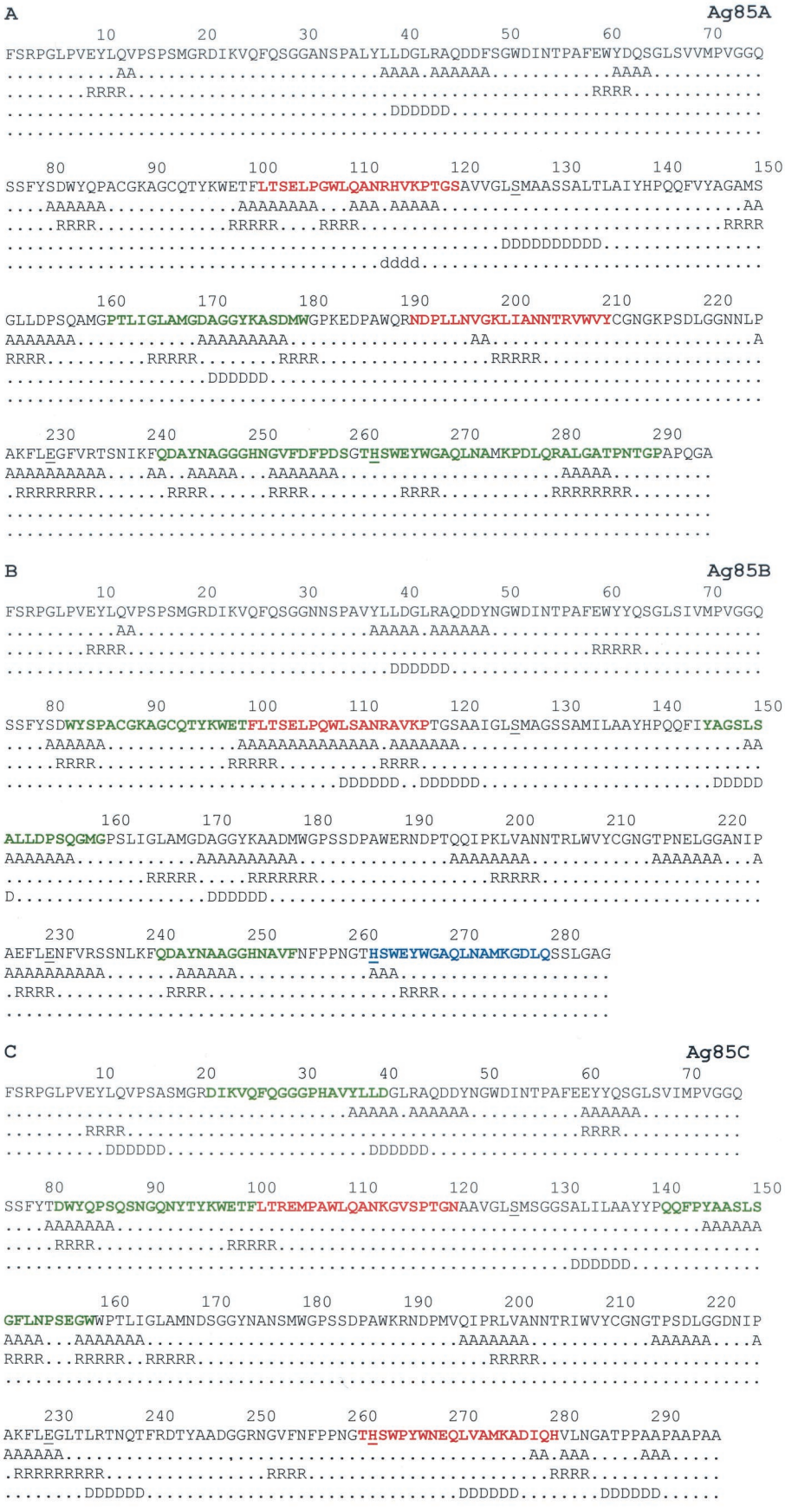
A
Ag85A
B
Ag85B
C
Ag85C

TABLE 5. Cross-reactivity of two immunodominant Th1 T-cell epitopes of Ag85A, Ag85B, and Ag85C in DNA-vaccinated C57BL/6 mice[a]

Peptide	Mean IL-2 (cpm) ± SD			Mean IFN-γ (pg/ml)		
	Ag85A DNA	Ag85B DNA	Ag85C DNA	Ag85A DNA	Ag85B DNA	Ag85C DNA
None (medium control)	3,255 ± 126	3,462 ± 635	4,068 ± 427	124	38	37
Ag85A 241–260	**8,632 ± 526**	4,988 ± 895	4,947 ± 175	**1,457**	12,004	52
Ag85B 240–260	10,430 ± 119	**15,412 ± 1,234**	5,582 ± 76	1,230	**18,069**	19
Ag85C 241–260	3,482 ± 709	3,355 ± 832	**6,276 ± 275**	145	89	**95**
Ag85A 261–280	**13,534 ± 1,146**	6,194 ± 481	6,451 ± 192	**7,448**	1,964	365
Ag85B 262–279	15,128 ± 1,393	**11,716 ± 508**	5,796 ± 790	6,208	**2,414**	128
Ag85C 261–280	5,672 ± 188	4,523 ± 241	**5,175 ± 143**	60	<10	**58**

[a] IL-2 and IFN-γ levels in 24-h and 72-h spleen cell culture supernatants from C57BL/6 mice vaccinated with DNA encoding Ag85A, Ag85B, or Ag85C and restimulated in *in vitro* with medium or synthetic peptides covering overlapping regions of Ag85A, Ag85B, or Ag85C. Results are presented as mean values for pooled cells from four mice per group. Data from corresponding DNA and peptide pairs are indicated in bold.

glycine to asparagine in position 268, and from asparagine to valine in position 272, have apparently destroyed the I-A^b epitope. This loss was also predicted by the disappearance of the amphipathicity and Rothbard motifs. Interestingly, the shift from aspargine to valine at position 272 resulted in the appearance of a predicted I-A^d binding motif on Ag85C, which was indeed confirmed by the strong recognition of the peptide spanning 261 to 280 in BALB/c mice vaccinated with Ag85C DNA. Ag85B DNA-vaccinated BALB/c mice also reacted to this region.

A number of regions on the Ag85A and Ag85B molecules have been predicted by the EpiMer program to contain human T-cell epitopes (24): amino acids 101 to 105, 121 to 134, 148 to 171, 198 to 207, and 270 to 280 for Ag85A and amino acids 65 to 70, 101 to 105, 128 to 133, 146 to 154, and 270 to 280 for Ag85B. With the exception of amino acids 121 to 134 of Ag85A, all these predicted epitopes were present in the peptides identified in our study with DNA-vaccinated BALB/c and/or C57BL/6 mice. For C57BL/6 mice, this is not completely unexpected, as the I-A^b peptide-binding repertoire is known to overlap significantly with that of several common HLA-DR molecules (33).

The Ag85C molecule has not been mapped for human T-cell epitopes so far. Lim et al. reported positive but weak T-cell responses to purified native Ag85C protein, lower in magnitude than to Ag85A and Ag85B, in peripheral blood mononuclear cell cultures from five purified protein derivative-positive subjects (25). Preliminary results on a small number of purified protein derivative-positive volunteers in our lab confirm that T-cell responses to this component are indeed low but that some T-cell epitopes can be identified on the amino-terminal part of the molecule with the Ag85C-overlapping peptides (T. Pierre-Louis and K. Huygen, unpublished data).

In conclusion, DNA vaccination offers a powerful and easy tool for the definition of Th1 T-cell epitopes of mycobacterial antigens. The epitope repertoire was wider than in *M. tuberculosis*-infected mice, particularly for the Ag85C component, for which no epitopes could be defined in tuberculosis-infected mice. This confirms previous findings from our and several other groups that DNA vaccination can elicit immune responses against both dominant and subdominant epitopes (5, 8, 13). In *H-2*^{d} haplotype mice, this broadening was caused to some extent by the generation of Ag85-specific IFN-γ-producing $CD8^+$ T-cell responses that were absent or below the detection level in BCG-vaccinated or tuberculosis-infected mice (8).

In *H-2*^{b} haplotype mice, we have no indications for DNA-induced Ag85A- or Ag85B-specific $CD8^+$ T-cell responses (10), and broadening of the repertoire is the result not so much of the appearance of new epitopes but of the induction of stronger responses to initially subdominant epitopes. Antigenic processing and presentation in DNA-vaccinated mice may be different from that in tuberculosis-infected mice, in the sense that only complete and correctly folded Ag85 proteins would be available during live infection, whereas truncated or differently folded molecules could be generated in the DNA-transfected cells. It is also possible that cross-priming phenomena in DNA vaccination could generate a broader epitope repertoire. The relevance of these T-cell responses against subdominant epitopes is not exactly clear for the moment, but it is tempting to speculate that they could be used to overcome problems in low- or nonresponder individuals.

In conclusion, despite pronounced sequence homology among the three Ag85 members, component-specific immunodominant T-cell epitopes were identified in two mouse strains. It is clear that further comparative studies of the immune response to the three Ag85 components are warranted.

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