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Mapping of TH1 Helper T-Cell Epitopes on Major Secreted Mycobacterial Antigen 85A in Mice Infected with Live *Mycobacterium bovis* BCG

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TH1 cytokine secretion was examined in response to synthetic peptides of the 85A component of the major secreted, fibronectin-binding antigen 85 complex from *Mycobacterium tuberculosis* in seven different mouse strains infected with live *M. bovis* BCG. Twenty-eight overlapping 20-mer peptides covering the complete mature 295-amino-acid (AA) protein were synthesized. Significant interleukin-2 (IL-2) and gamma interferon (IFN-γ) secretion could be measured following in vitro stimulation of spleen cells with these peptides. *H-2*^{d} haplotype mice reacted preferentially against the amino-terminal half of the protein, i.e., against peptide 5 (AA 41 to 60) and especially against peptide 11 (AA 101 to 120), which contained an I-E^d binding motif. *H-2*^{b} haplotype mice, on the other hand, reacted against peptides from both amino- and carboxy-terminal halves of the protein, peptide 25 (AA 241 to 260) being the most potent stimulator of IL-2 and IFN-γ production. (BALB/c × C57BL/6)F$_1$ animals with the *H-2*$^{d/b}$ haplotype weakly recognized peptides specific for both parental lines. Finally, CBA/J (*H-2*^{k}) and major histocompatibility complex class II mutant B6.C.bm12 mice, carrying a mutant I-Aβ^{bm12} allele on an *H-2*^{b} background, reacted only very weakly to the 85A peptides. Reactive T cells isolated from lungs of BCG-infected *H-2*^{b} haplotype mice recognized the same epitopes as spleen cells, especially peptide 25. These data confirm previous findings regarding the powerful IL-2 and IFN-γ-inducing properties of antigen 85 during infection with live *M. bovis* BCG.

Protective immunity against mycobacterial infections is mediated by interactions between specifically sensitized T cells and activated macrophage-effector cells harboring the intracellular bacteria (28). These populations interact by means of a complex network of lymphokines and monokines which is still not completely understood. It has, however, become clear that T cells belonging to the TH1 T-helper-cell subset and secreting the lymphokines interleukin-2 (IL-2), gamma interferon (IFN-γ), and lymphotoxin, rather than IL-4- and IL-5-producing T helper cells of the TH2 subset, are associated with the development of acquired resistance to *Mycobacterium leprae* and *M. tuberculosis* (13, 15, 32).

The specific antigens eliciting these protective TH1 responses are not known, but living organisms have generally been reported to be more effective in the generation of specific acquired resistance than killed mycobacterial preparations (4). It has therefore been argued that secreted antigens present in large amounts in mycobacterial culture filtrates (CF) and only produced by actively metabolizing bacteria could be essential for the induction of protective immunity (2, 6, 27).

One of the major secreted antigens from *M. tuberculosis-M. bovis* BCG CF is a 30- to 32-kDa protein, also called antigen 85 (Ag85) in the Closs reference system for BCG antigens, identified by crossed immunoelectrophoresis (5, 31). Ag85 is actually a protein family with three members, 85A, 85B, and 85C, encoded by three distinct but related genes (7). The three corresponding genes from *M. leprae* have also been cloned and demonstrate about 90% homology with the genes in *M. bovis* BCG and *M. tuberculosis* (which are virtually identical) (9). Ag85 is a widely cross-reactive protein family found in all mycobacterial species, with little known about its function except that it has at least two fibronectin-binding sites and is not a stress protein (1, 29). Recently, a major secreted 67-kDa protein has been identified in another actinomycete, i.e., *Corynebacterium glutamicum*, which shows resemblance to Ag85 in its NH_2 half (18).

We have previously reported that purified Ag85 from *M. bovis* BCG is a potent in vitro IL-2 and IFN-γ inducer in spleen cell cultures from mice infected with live BCG (15). Ag85 is also a strong T-cell antigen in peripheral blood mononuclear cell cultures from healthy purified protein derivative-positive human volunteers and from tuberculosis patients with minimal lung lesions (17). Furthermore, T cells from lepromin-positive healthy subjects and from leprosy patients also react against Ag85 from BCG, and in the latter group a marked parallelism of responsiveness towards whole *M. leprae* and to purified Ag85 from BCG can be observed throughout the clinical spectrum of the disease (19). T cells reactive against Ag85 are generated very early during *M. leprae* infection even before actual development of a positive skin test (lepromin) reaction to *M. leprae* (20).

These results taken together indicate that Ag85 could indeed be a protective antigen in mycobacterial infection. In this paper, we report on the mapping of TH1 cell epitopes (measuring IL-2 and IFN-γ secretion) of the Ag85A component in seven different mouse strains infected with live BCG, using 28 overlapping 20-mer synthetic peptides covering the entire mature 295-amino-acid (AA) 85A protein.

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MATERIALS AND METHODS

Mice. BALB/c (*H-2*^{d}), DBA/2 (*H-2*^{d}), C57BL/6 (B6) (*H-2*^{b}), major histocompatibility complex (MHC)-congenic BALB.B10 (*H-2*^{b}), (BALB/c × C57BL/6)F_1 (*H-2*$^{d/b}$), CBA/J (*H-2*^{k}), and MHC class II mutant B6.C.bm12 (*H-2*^{bm12}) mice were bred in the animal facilities of the Instituut Pasteur van Brabant. Two-month-old female mice (±20 g) were used in all experiments.

Infection. In the initial experiments, mice were inoculated intravenously in a lateral tail vein with 0.5 mg (±4 × 10^6 CFU) of freshly prepared *M. bovis* BCG GL2, grown as a surface pellicle on synthetic Sauton medium for 14 days. Spleens were removed aseptically 3 weeks after infection. At least three animals were pooled in each experimental group. In the experiment defining cross-reactive epitopes, C57BL/6 mice were inoculated intravenously with 10^5 CFU of live *M. kansasii*, *M. fortuitum*, or *M. scrofulaceum*, and spleens (at least three mice per group) were taken after 4 weeks of infection.

Antigens. Ag85 was purified from *M. bovis* BCG CF, as reported previously by sequential hydrophobic chromatography on phenyl-Sepharose, ion exchange on DEAE-Sephacel, and molecular sieving on Sephadex G75 (8). Purified Ag85A and Ag85B were subsequently obtained by isoelectric focusing of the Ag85 complex in a Rotofor Preparative Isoelectric Focusing Cell (Bio-Rad), using a pH 4 to 6 ampholyte gradient.

Peptide synthesis. All peptides were synthesized on TentaGel S-RAM resin (Rapp Polymere, Tübingen, Germany), which is furnished with a linker designed to generate carboxy-terminal amides upon cleavage. Fmoc-a-amino group and *t*-butyl-based side chain protection were used (12). The guanidino group of arginine was protected with the 2,2,5,7,8-pentamethylchroman-6-sulfonyl moiety, trityl protection was used for both histidine and cysteine, and *t*-butyloxycarbonyl protection was used for tryptophan. Most amino acids were coupled as *O*-pentafluorophenyl esters. Peptides were synthesized by using continuous-flow procedures on a Millipore model 9050 PepSynthesizer (Millipore Corp., Bedford, Mass.). Following synthesis, peptides were cleaved from the resin, and side chain protection was removed with 90% trifluoroacetic acid in the presence of the appropriate scavengers. Peptides were precipitated with *t*-butyl-methyl ether and extracted. They were further purified by reversed-phase high-pressure liquid chromatography and subsequently dissolved in RPMI 1640 medium. All peptides were synthesized as 20-mer molecules, overlapping by 10 AA with the neighboring peptides, except for the carboxy-terminal peptide 28, which was a 22-mer peptide covering positions 274 to 295. A schematic representation of the synthetic peptides with the T-cell epitopes predicted according to the TSites program (11) is shown in Fig. 1 (SWISS-PROT accession number P17944).

Spleen cell cytokine production. Three weeks after infection, mice were killed by cervical dislocation and spleens were removed aseptically. Cells were isolated by using a loosely fitting Dounce homogenizer, washed, adjusted to a concentration of 4 × 10^6 cells per ml, and grown in flat-bottomed microwell plates (Nunc) in RPMI 1640 medium supplemented with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), glutamine, 5 × 10^{-5} M 2-mercaptoethanol, antibiotics, and 10% heat-inactivated fetal calf serum. The purified Ag85 complex, its components Ag85A and Ag85B, and the synthetic 85A peptides were added in a volume of 20 to 180 μl of cell suspension. Native Ag85, Ag85A, and Ag85B were used at final concentrations of 5 μg/ml each; peptides were used at 10 μg/ml each. Cells were incubated at 37°C in a humidified CO_2 incubator, and supernatants were harvested aseptically after 24 (IL-2) and 72 (IFN-γ) h. Supernatants from three separate wells were pooled and stored frozen at −20°C until assay. Experiments were repeated at least three times, and the results of one representative experiment are shown.

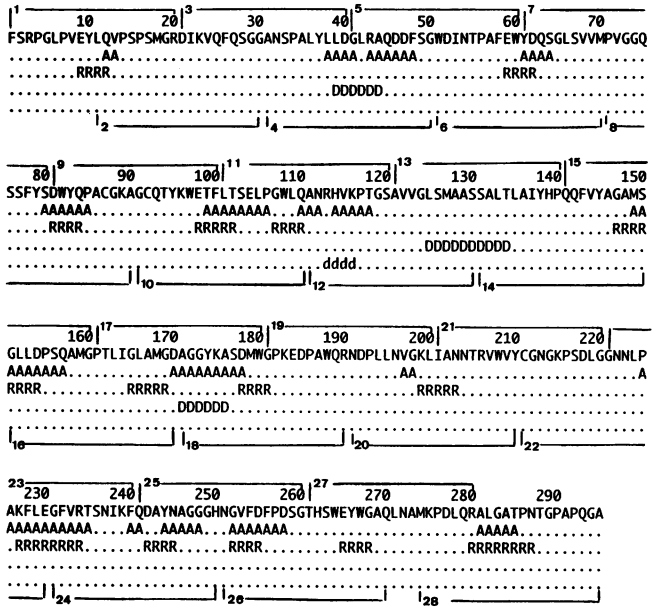

FIG. 1. Schematic representation of the 28 overlapping synthetic peptides from Ag85A with predicted T-cell epitopes according to alpha-helical periodicity and amphipathicity (A), Rothbard and Taylor motifs (R), and I-A^d (D) and I-E^d (d) binding motifs by TSites (11).

Cytokine secretion in the lung. Three weeks after BCG infection, mice were killed by cervical dislocation and lungs were removed aseptically and homogenized in a loosely fitting Dounce homogenizer. Cell suspension was passed over a nylon wool column, and nonadherent cells enriched in T lymphocytes were washed, adjusted to 4 × 10^6 cells per ml, and used for cytokine production in the same conditions as described above for the spleen.

IL-2 assay. IL-2 activity was measured as described before (4). Briefly, 100 μl of culture supernatant was added to 100 μl of IL-2-dependent CTLL-2 cells (1.5 × 10^5/ml) in flat-bottomed microwell plates, and the mixture was incubated for 48 h. Tritiated thymidine was added during the last 17 h of cultures. Cells were harvested in a Titertek Cell Harvester (Skatron), and radioactivity recovered on the filter mats was directly counted in an automated Betaplate (Pharmacia) Scintillation Counter. Results are expressed as mean counts per minute of duplicate cultures. Genuine IL-2 (but not IL-4) was measured in these supernatants since treatment with the anti-IL-2 monoclonal antibody (MAb) S4B6 (rat immunoglobulin G2a) completely abrogated the growth activity. All supernatants were tested in parallel in one assay. Mean counts per minute obtained on serial twofold dilutions of a reference IL-2 preparation (500 IU/ml; Janssen Biochimica) performed in quadruplicate in the same assay, are shown in Table 1.

IFN assay. Antiviral activity was measured on serial twofold dilutions of culture supernatant, using a cytopathic effect reduction assay of vesicular stomatitis virus on mouse L929 fibroblastoid cells, as described before (16). IFN titer is determined as the reciprocal of the last dilution conferring 50% protection against cytopathic effect. Titers are expressed

TABLE 1. IL-2 standard curve

IL-2 (IU/ml)	Mean cpm ± SD[a]
25	122,356 ± 2,614
12.5	106,188 ± 7,104
6.25	81,975 ± 4,870
3.12	50,770 ± 4,190
1.56	25,338 ± 2,894
0.78	11,318 ± 2,237
0.39	4,988 ± 873
0.19	1,752 ± 101
0.10	605 ± 123
0.05	235 ± 46
0.02	122 ± 39

[a] Calculated from serial twofold dilutions of a standard IL-2 preparation, performed in quadruplicate.

as mean $\log_2$ international units of duplicate assays (Gg02-901-533; National Institutes of Health, Bethesda, Md.). Error does not exceed 0.25 $\log_2$ unit in these assays. One $\log_2$ unit equals 6 IU/ml or 2,050 pg/ml compared with the InterTest-γ mouse enzyme-linked immunosorbent assay (Genzyme code 1557-00). The detection limit in the assay was >680 pg/ml. The antiviral activity measured was genuine IFN-γ because it could be completely neutralized by the anti-murine IFN-γ MAb F3 (kindly given to us by H. Heremans, KUL Leuven).

Dot blot assay. B-cell epitopes were examined by using a multidot immunobinding assay, as described before (30). Briefly, 2 μg of each peptide was blotted onto 0.2-μm nitrocellulose membranes (Bio-Rad) with a 96-well Bio-Dot apparatus (Bio-Rad), and membranes were incubated overnight with a 1/10 dilution of one of ten MAbs (17/4, 32/4, HYT27, XD10-3, IIG8-4, VID1-14, VIIIH2-6, VIIH11-4, XF11-7, and 233.27 [10]) or a polyclonal rabbit antibody (8) directed against Ag85. Membranes were washed and incubated for 2 h with alkaline phosphatase-conjugated rabbit anti-mouse (1:1,000) or goat anti-rabbit (1:750) immunoglobulin G antibodies (Dakopatts, Copenhagen, Denmark). After the mixture was washed, nitroblue tetrazolium salt–5-bromo-4-chloro-3-indolyl phosphate substrate was added, and the reaction was stopped after 15 min with distilled water.

RESULTS

Cytokine production in response to Ag85 complex and its purified components 85A and 85B in spleen cell cultures from seven different mouse strains infected with *M. bovis* BCG. Seven different mouse strains were infected with live BCG, and spleen cell cytokine secretion in response to purified Ag85 was analyzed 3 weeks later. Confirming previous results (15, 16), pronounced differences were observed in the amount of cytokines produced (Table 2). *H-2* haplotype profoundly affected the magnitude of the response, $H\text{-}2^b$ haplotype (B6 and BALB.B10 mice) being associated with high and $H\text{-}2^d$ haplotype (BALB/c and DBA/2 mice) being associated with low response. (BALB/c × C57BL/6)F_1 ($H\text{-}2^{d/b}$) animals were found to be low responders. These five mouse strains reacted to both the 85A and the 85B components of the Ag85 complex. CBA ($H\text{-}2^k$) and B6.C.bm12 ($H\text{-}2^{bm12}$) class II mutant mice were found to be high reactors to the total Ag85 complex, but these strains reacted very strongly to the 85B component and only very weakly to 85A. Down-regulation of $L3T4^+$ T cells, using MAb GK1.5, completely abrogated IL-2 and IFN-γ production in response to Ag85 (16). Participation of IL-2-dependent $CD8^+$ cells in the production of IFN-γ cannot be completely excluded, although we have never been able to affect its production in spleen cells from BCG-infected mice by treatment with anti-*Lyt-2* antibodies (data not shown).

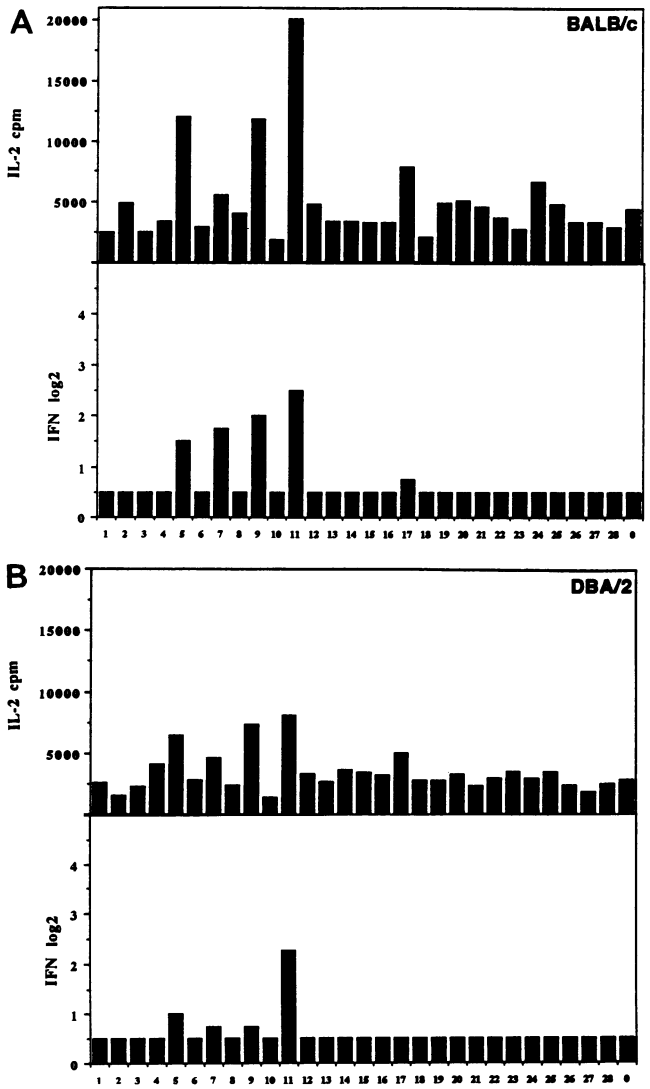

FIG. 2. IL-2 and IFN-γ activities as measured in 24- and 72-h spleen cell culture supernatants, respectively, from BCG-infected BALB/c (A) or DBA/2 (B) mice stimulated with one of the 28 synthetic Ag85A peptides (10 μg/ml) or in unstimulated cultures (0).

Cytokine secretion in response to synthetic overlapping peptides from Ag85A in BALB/c and DBA/2 mice. In line with the response to whole Ag85A, overall T-cell reactivity (especially IFN-γ production) against 85A synthetic peptides was low in the two $H\text{-}2^d$ haplotype mice BALB/c and DBA/2. As shown in Fig. 2, $H\text{-}2^d$ haplotype mice reacted preferentially to some peptides in the amino-terminal half of Ag85A. IL-2 and IFN-γ production was detectable after stimulation with peptides 4 and 5, 7, 9, 11, and 17. The highest reactivity, of a magnitude comparable to reactivity against the natural 85A component, was found in response to peptide 11 (AA 101 to 120). According to the TSites program (11), peptide 11 contains the only $I\text{-}E^d$ binding motif (AA 113 to 116) on Ag85A. We therefore examined the effect of anti-$I\text{-}E^d$ antibodies (MAb 14.4.4 [15]) on IL-2 and IFN-γ production by BCG-sensitized BALB/c spleen cells stimulated with Ag85 or p11.

TABLE 2. Cytokine secretion in response to purified Ag85 and its 85A and 85B components[a]

Mice	IL-2 (cpm)			IFN-γ (log_2)		
	Ag85	85A	85B	Ag85	85A	85B
BALB/c	9,955	9,989	12,258	3.75	2.25	3.25
DBA/2	17,596	7,024	12,079	1.5	1.5	1.75
C57BL/6	30,150	22,402	29,884	5.75	4.5	5.5
BALB.B10	32,356	35,336	23,150	6.25	5.25	5.5
$(C \times B)F_1$[b]	14,899	14,126	7,871	4.25	2.75	4.5
CBA/J	24,658	282	22,303	5.5	<1	5.5
B6.C.bm12	29,632	443	32,860	5.75	<1	6.25

[a] IL-2 and IFN-γ activities as measured in 24- and 72-h culture supernatants, respectively, of spleen cell cultures from mice infected with live BCG 3 weeks earlier and stimulated with Ag85 complex or its two purified components, 85A and 85B (5 μg/ml). Data represent mean values of triplicate assays (standard deviations are 10 to 20% of the mean).

[b] $(C \times B)F_1$, $(BALB/c \times C57BL/6)F_1$.

About 90% of the IL-2 production induced with Ag85 or peptide 11 could be blocked by anti-I-E^d antibodies. IFN-γ production, on the other hand, was reduced to about 25% after stimulation with Ag85, but peptide 11-induced IFN-γ production was not affected by anti-I-E^d antibodies.

Cytokine secretion in response to synthetic overlapping peptides from Ag85A in C57BL/6 and MHC-congenic BALB.B10 mice. Overall reactivity against the synthetic peptides was higher in *H-2*^{b} than in *H-2*^{d} haplotype mice (Fig. 3A and B). Furthermore, these mice reacted to completely different epitopes on the 85A molecule, located in both the amino- and the carboxy-terminal regions of the protein. Substantial IL-2 and IFN-γ production could be observed after stimulation with peptides 6 and 7, 16, 24 and 25, and 27 and 28. The highest reactivity was found after stimulation with peptide 25 (AA 241 to 260). In one experiment, a weak reactivity was also found to peptides 19 and 20. Spleen cells from uninfected B6 mice and from B6 mice immunized with heat-killed BCG did not react to any of these peptides.

Epitope recognition by purified T cells from lungs of BCG-infected BALB.B10 mice. Figure 3C shows the IL-2 and IFN-γ production by nylon-wool-enriched T cells isolated from lungs of BCG-infected BALB.B10 mice 3 weeks after infection. T cells from the lung reacted against the same peptides as T cells from the spleen. In particular, peptide 27 and especially peptide 25 were found to be potent IL-2 and IFN-γ inducers.

Cytokine secretion in response to Ag85A synthetic peptides in (BALB/c × C57BL/6)F_1 mice. Global reactivity in F_1 animals was of low *H-2*^{d} haplotype magnitude (Fig. 4). Both peptides defined in BALB/c parental mice (p9 and p11) and peptides defined in C57BL/6 parental mice (p6, p16, p25, and p27) were weakly recognized. Furthermore, low amounts of IFN-γ were produced in response to peptide 20.

Cytokine secretion in response to Ag85A synthetic peptides in CBA and B6.C.bm12 mice. CBA and B6.Cbm12 strain mice were found to be completely unreactive to the 85A peptides (Fig. 5). In one experiment, peptide 1 (AA 1 to 20) and peptide 18 (AA 171 to 190) induced some IL-2 activity in CBA/J spleen cell supernatants. No IFN-γ activity could be detected. Since CBA and bm12 mutant mice do react to the 85B component from Ag85 complex (Table 2), this finding indicates that 85B-specific T-cell epitopes which do not cross-react with 85A must exist on the former component. Sequence comparison of p25 from 85A and 85B from *M. tuberculosis* showed two significant AA differences at positions 245 (asparagine versus lysine) and 246 (alanine versus proline) that could lead to

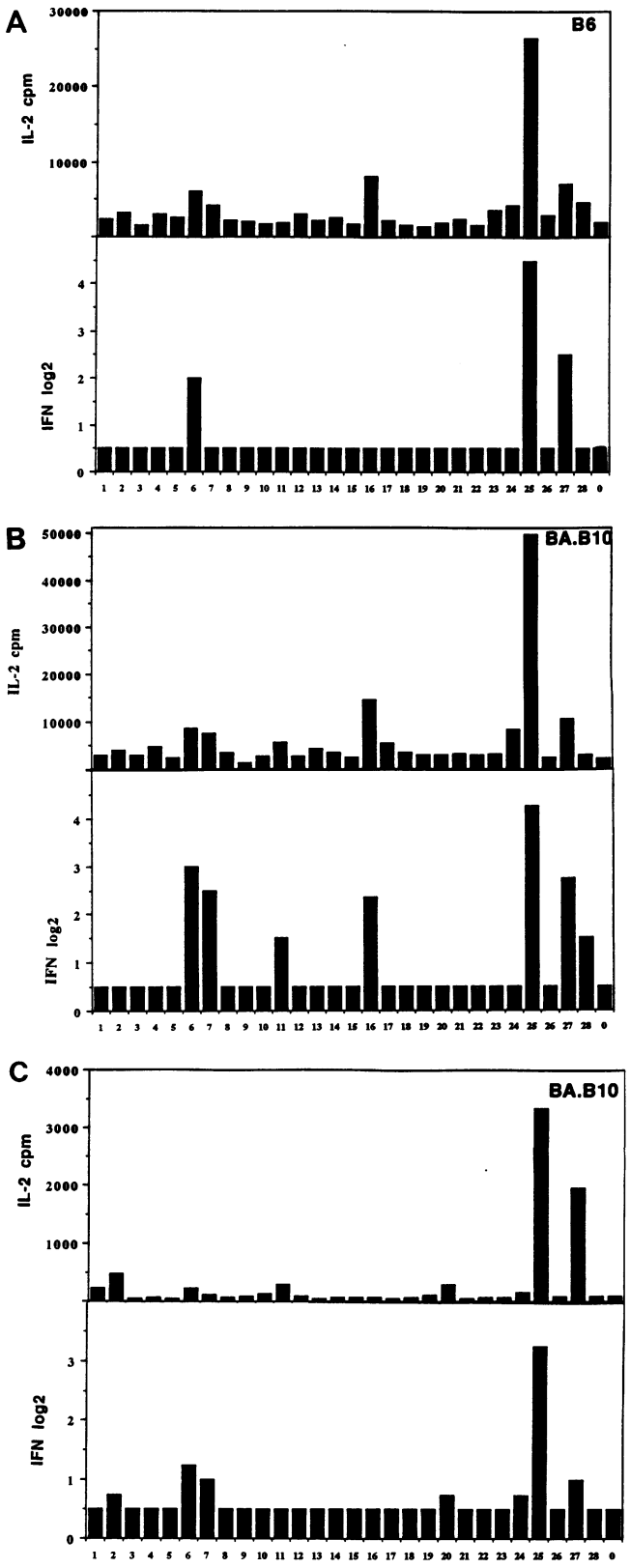

FIG. 3. IL-2 and IFN-γ activities in spleen cell culture supernatants from BCG-infected C57BL/6 (A) or MHC-congenic BALB.B10 (B) mice. (C) IL-2 and IFN-γ activities in culture supernatant from purified lung T cells from BCG-infected BALB.B10 mice.

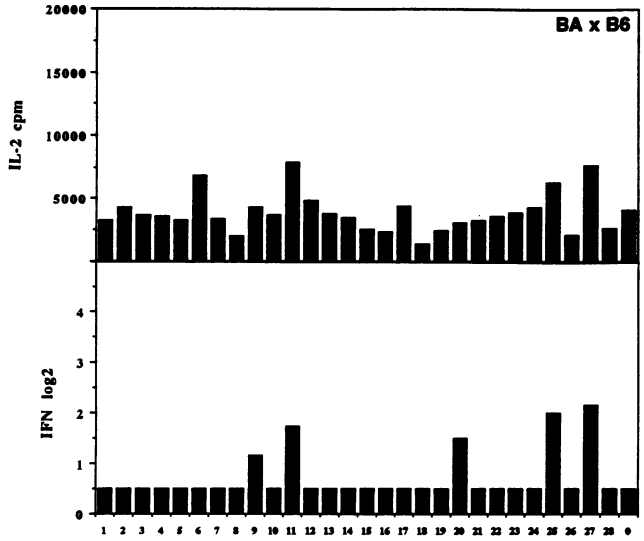

FIG. 4. IL-2 and IFN-γ activities in spleen cell culture supernatants from BCG-infected (BALB/c × C57BL/6)F_1 mice.

profound differences in MHC class II binding. The AA sequence of the *M. tuberculosis* 85C component between positions 241 and 260 was even more different from the 85A product. Interestingly, the corresponding sequence of 85B from *M. kansasii* was more similar to 85A from *M. tuberculosis* (Fig. 6).

Peptide 25 is a cross-reactive TH1 epitope present also on *M. kansasii*. As shown in Table 3, spleen cells from B6 mice infected with *M. kansasii* demonstrated significant IL-2 and IFN-γ secretion against the purified Ag85 complex from BCG. Furthermore, the dominant TH1 epitope of the 85A protein from *M. bovis* BCG-infected mice (i.e., peptide 25) turned out to be as effective an IL-2 and IFN-γ inducer in these mice as in BCG-infected animals. On the other hand, no cross-reactivity could be observed in mice infected with *M. scrofulaceum* or *M. fortuitum*.

Definition of B-cell epitopes on Ag85A. We have recently described the generation of 10 MAbs directed against A85 (10). Using dot blot assays with the 28 overlapping peptides, we tried to define their precise B-cell epitopes on Ag85A, but we have been unable to map the majority of the MAbs tested. For only two MAbs could the relevant epitope be defined: MAb 32/4 (and another subclone, 32-15) reacted against peptide 5, and MAb 17/4 reacted against peptide 27 (Fig. 7). A polyclonal rabbit antibody raised against purified Ag85A (8) reacted very strongly with peptide 28 and weakly with peptide 1 (data not shown).

DISCUSSION

Using spleen cells from mice infected intravenously with live *M. bovis* BCG, we have been able to define potent IL-2- and IFN-γ-inducing epitopes on the 85A component of the Ag85 complex from *M. tuberculosis* CF in animals of *H-2*^{d} and *H-2*^{b} haplotypes. These results extend our previous report on spleen cell cytokine production in BCG-infected mice in which we showed that BCG CF and, in particular, its major secreted protein component Ag85 selectively induce a strong TH1 response in C57BL/6 and MHC-congenic BALB.B10 mice, whereas in BALB/c mice this TH1 response is weaker and partly counterbalanced by TH2 cells (15). We have now found

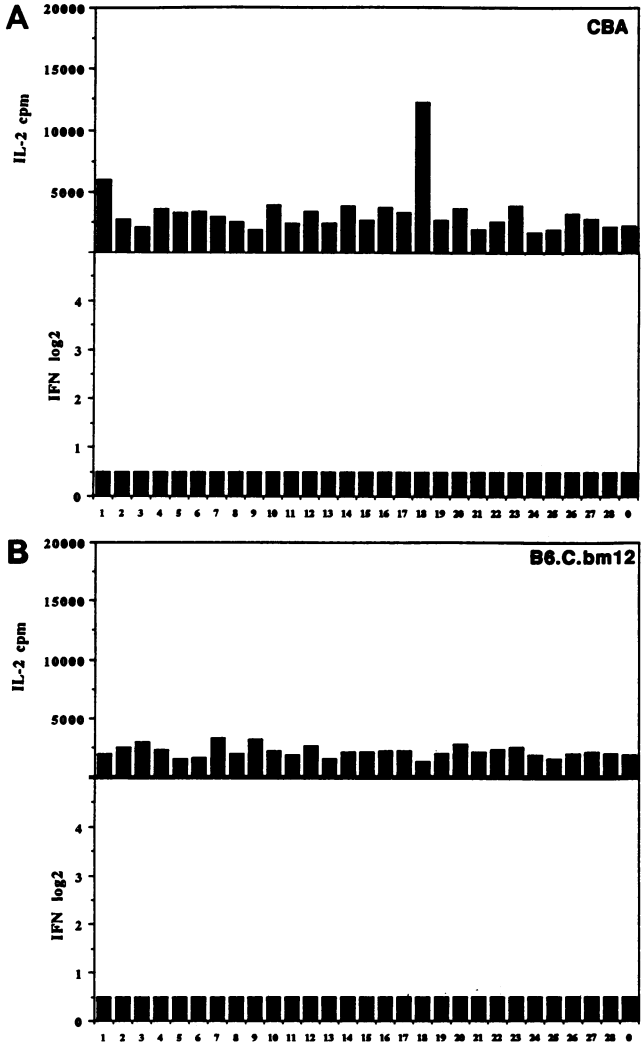

FIG. 5. IL-2 and IFN-γ activities in spleen cell culture supernatants from BCG-infected CBA/J (A) and B6.C.bm12 (B) mice.

that the *H-2*^{d} haplotype is mainly associated with recognition of the amino-terminal part of Ag85A and that peptide 11 (AA 101 to 120) is the most potent inducer of IL-2 and IFN-γ for this haplotype. *H-2*^{b} haplotype mice, on the other hand, react to peptides from both amino- and carboxy-terminal halves of the protein, and here peptide 25 (AA 241 to 260) elicits the strongest cytokine production. Each haplotype has its own specific epitope repertoire and shares none of the stimulatory peptides with the other.

Examination of the 85A sequence for possible T-cell

	241	260
CONSENSUS	Q D A Y N A X G G H N X V F N F P X N G	
85A tub	· · · · · · G · · · · G · · D · · D S ·	
85B tub	· · · · K P A · · · · A · · · · · P · ·	
85C tub	R · T · A · D · · R · G · · · · · P · ·	
85B kan	· · · · · · A · · · · A · · · L D A · ·	

FIG. 6. Sequence comparison of peptide 25 (AA 241 to 260) in Ag85A, -85B, and -85C from *M. tuberculosis* (tub) and Ag85B from *M. kansasii* (kan), according to Content et al. (7).

TABLE 3. Cytokine secretion in C57BL/6 mice infected with other mycobacterial species[a]

Mycobacterial species	Cytokine titer			
	Ag85		Peptide 25 (AA 241–260)	
	IL-2 (cpm)	IFN-γ (log_2)	IL-2 (cpm)	IFN-γ (log_2)
M. kansasii	44,920 ± 4,905	5.87 ± 0.53	27,375 ± 2,914	5.12 ± 0.88
M. scrofulaceum	578 ± 66	1.37 ± 1.23	667 ± 94	<1
M. fortuitum	767 ± 45	<1	744 ± 71	<1

[a] IL-2 and IFN-γ titers in 24- and 72-h spleen cell culture supernatants, respectively, from B6 mice infected 4 weeks previously with 10^5 CFU of the respective mycobacteria and stimulated in vitro with Ag85 (5 μg/ml) or peptide 25 from 85A from *M. tuberculosis* (10 μg/ml). IL-2 titers are means of three assays (mean value in unstimulated cells, ±400 cpm). IFN-γ titers are means of two titrations.

epitopes, using the TSites program (11) combining four predictive algorithms (amphipathicity, Rothbard and Taylor motifs, and I-A^d and I-E^d binding motifs), revealed that all peptides that we found to be stimulatory in cytokine secretion indeed show the predicted sites. On the other hand, two peptides with presumed sites, peptides 13 and 23 (predicted as the strongest T-cell epitope), were not stimulatory at all in our mouse model.

I-A^d binding motifs were found at positions 39 to 44 (present in peptides 4 and 5, together with amphipathic residues), at positions 125 to 134 (peptides 12 and 13), and at positions 171 to 176 (peptides 17 and 18, together with amphipathic and Rothbard motifs). Peptides 4 and 5 and peptide 17 induce cytokine secretion in *H-2*^{d} mice, whereas peptides 12 and 13 do not. Peptide 11 contains the only I-E^d binding motif of Ag85A (AA 113 to 116), and this peptide was found to be the most powerful peptide in BALB/c, DBA/2, and F_1 animals. This peptide also scores very strongly for amphipathicity and has a Rothbard motif at positions 107 to 110. Interestingly, TSites analysis of the Ag85B and Ag85C genes from *M. tuberculosis* indicates that this I-E^d binding region presents instead with an I-A^d binding motif (AA 109 to 114) on Ag85B and with no predicted T site at all on Ag85C. Blocking with anti-I-E^d antibodies showed that peptide 11 is indeed presented to IL-2-producing cells in an I-E context. (As a matter of fact, IL-2 response to whole Ag85 could be blocked, suggesting that *H-2*^{d} mice preferentially use their I-E heterodimer for presentation.) Low proliferation and IFN-γ and IL-2 secretion in BALB/c mice have been observed in response to several antigens in other experimental conditions besides mycobacterial infection, such as infection with *Leishmania major* (22) and contact sensitivity to picryl chloride (3), and there is growing evidence that MHC haplotype is directly regulating this low cytokine response. It has been speculated that low responsiveness might be due to relative lack of I-A^d or a relatively large amount of I-E^d expressed on the antigen-presenting cell (26). Whether or how the preferential I-E usage in response to Ag85 is related to the overall low reactivity of the *H-2*^{d} haplotype remains to be elucidated.

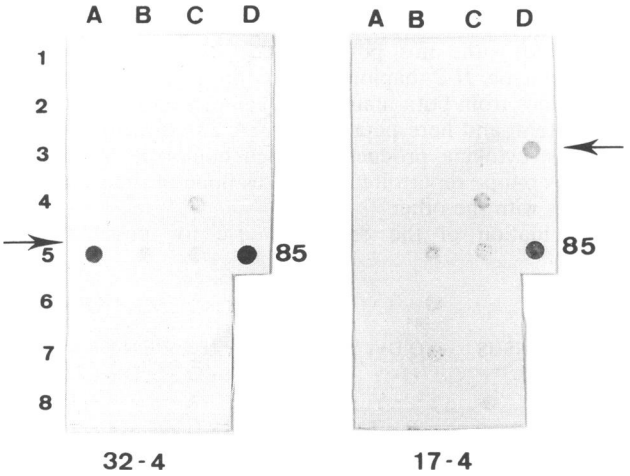

FIG. 7. Dot blot assay against spotted peptides, using MAbs 32/4 and 17/4. Lane A, peptides 1 to 8; lane B, peptides 8 to 16; lane C, peptides 17 to 24; lane D, peptides 25 to 28 and Ag85A (85).

All four peptides recognized by *H-2*^{b} haplotype mice (peptides 6, 16, 25, and 27) contained Rothbard motifs and amphipathic blocks. Some reactivity was also observed in response to the neighboring peptides 7, 24, and 28, indicating that the exact epitopes are located somewhere between AA 60 and 70, 240 and 250, and 274 and 280. *H-2*^{b} haplotype mice do not express the I-E heterodimer at the cell surface because a deletion in the Eα chain makes association with the normal (cytoplasmic) Eβ chain impossible (24). Antigen presentation in this haplotype is therefore exclusively controlled by an I-A heterodimer, and thus peptides 6, 16, 25, and 27 can all be considered to be presented in the context of I-A^b.

Results obtained in MHC class II mutant B6.C.bm12 mice give further insight into how Ag85A epitopes are recognized. This mutant bm12 haplotype is thought to have originated as the result of a gene conversion event in which a stretch of nucleotides from the Aβ^b allele has been replaced by the corresponding nucleotides from the (silent) Eβ^b allele. This has resulted in AA changes in three positions of the Aβ chain: position 67 Ile > Phe, position 70 Arg > Gln, and position 71 Thr > Lys (14, 21). This mutant bm12 sequence is identical to the Aβ^k sequence, and interestingly, CBA/J mice with the *H-2*^{k} haplotype behaved very similar to B6.C.bm12 mutant mice in our analysis, since overall reactivity to 85A or to its peptides was very low in both strains despite their strong recognition of the 85B component. Therefore, high IL-2 and IFN-γ secretion in response to 85A peptides, so characteristic of the *H-2*^{b} haplotype, seems to result from antigen presentation in an exclusive I-A^b context, whereas antigen presentation in an I-E^b (or I-E^k) context apparently leads to suppression of the response to Ag85A, leaving the reactivity to the 85B component intact.

(BALB/c × C57BL/6)F1 mice showed a low reactivity to Ag85 comparable to BALB/c parental values. Peptide reactivity was also low and directed against peptides defined in both parental strains. MHC class II molecules are codominantly expressed in F_1 mice and hence, besides I-E^d expression, the Eβ^b molecule could also associate with the Eα^d molecule, perhaps contributing in this way to the low BALB/c-type responsiveness observed in F_1 animals.

Whereas Ag85A presents with a number of potent TH1 T-helper-cell stimulatory epitopes, only two B-cell epitopes could be defined with MAbs (peptides 5 and 27) and one could be defined with a hyperimmunized rabbit polyclonal antibody (peptide 28). We failed to define the B-cell epitopes recognized by a panel of other MAbs which appear to be directed against tertiary structures on the native antigen rather than against linear structures on denatured protein or peptides. Recently, Ohara et al. reported on the cloning of the 85B antigen from *M. avium* and the mapping of B-cell epitopes on the 85B protein by using deletion constructs (25). Besides the identification of three species-specific B-cell epitopes, they defined three antigenic determinants common to *M. tuberculosis*, *M. kansasii*, and *M. avium*. Interestingly, one of the regions bearing a common determinant was localized between positions 51 and 123 (AA 11 to 83 in the mature protein), and this region contains the peptide 5 sequence (AA 41 to 60) which was recognized by our widely cross-reactive MAb 32/4.

Ag85 complex is a family of related proteins with extensive homologies in various mycobacterial species. Using a panel of MAbs against Ag85 from BCG, we have shown that most B-cell epitopes can be defined as cross-reacting epitopes present on Ag85 homologs from the majority of mycobacterial species (10). Here we report that cross-reactivity also exists at the T-cell level, albeit to a lesser extent, as infection of B6 mice with *M. kansasii* (but not with *M. fortuitum* or *M. scrofulaceum*) resulted in very strong recognition of Ag85 from BCG and its dominant peptide 25.

The potent TH1 stimulatory character of Ag85 was further underscored by our findings on cytokine secretion in the lung. T cells reactive to Ag85A could be isolated from lung tissue of BCG-infected mice, and epitope analysis showed that they were directed against the same peptides as those recognized by spleen cells. In particular, peptide 25 induced significant amounts of IL-2 and IFN-γ following stimulation of $H\text{-}2^b$ haplotype mice. Although the number of CFU of BCG recovered from the lungs is low following intravenous infection (about 10-fold less than the CFU number in the spleen), these data suggest that a considerable proportion of Ag85A-reactive T cells home to the lung tissue during mycobacterial infection. This is reminiscent of the previous findings of Manca et al., who reported on sequestration in the lungs of Ag85A-reactive T cells in patients with tuberculous pleuritis (23).

In conclusion, powerful IL-2- and IFN-γ-inducing epitopes were identified on Ag85A by using spleen and lung cells from mice infected with live but not killed BCG. These observations underline the immunodominant and potentially protective character of Ag85A. Vaccination experiments with these defined peptides (and delivered to the appropriate T-helper subset by the correct antigen-presenting cell) are now needed to further elucidate the role of Ag85A in protective immunity against mycobacteria.

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