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Influence of Genes from the Major Histocompatibility Complex on the Antibody Repertoire against Culture Filtrate Antigens in Mice Infected with Live *Mycobacterium bovis* BCG

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C57BL/10 and C57BL/6 mice (H-2^b); B10 congenic mice with f, k, p, q, r, and s H-2 haplotypes; B10 mice with recombinant g2, o2, a, h2, h4, i5, and bq1 H-2 haplotypes; and B6 mice with major histocompatibility complex (MHC) mutant bm1 and bm13 (class I) and bm12 (class II) haplotypes were infected intravenously with 4 × 10^6 CFU of live *Mycobacterium bovis* BCG and examined by Western immunoblot analysis for serum antibodies against BCG culture filtrate antigens, following a boost injection with live BCG or with BCG culture filtrate. Parental B10 and B6 mice reacted very intensely with three culture filtrate protein bands with estimated molecular masses of 37, 38, and 40 kDa. Response against the 40-kDa protein was stronger following a boost injection with live BCG than following a boost with culture filtrate. Sera from mice with f, p, i5, bm1, and bm13 haplotypes reacted strongly, with both the 37-38- and 40-kDa antigens, and sera from mice with q and bq1 haplotypes showed a somewhat weaker reaction. Sera from mice with r, s, and bm12 haplotypes reacted against the 37-38-kDa antigen but not against the 40-kDa antigen, and sera from mice with the h2 haplotype reacted only with the 40-kDa antigen but not with the 37-38-kDa antigen. Sera from mice with the k, g2, o2, a, and h4 haplotypes showed, at most, a very weak reaction with the 37-38- and 40-kDa antigens. These results demonstrate that MHC genes profoundly affect the antibody repertoire used against culture filtrate antigens in mice infected with live *M. bovis* BCG. In particular, as shown in mice with the recombinant H-2 haplotype and in class II mutant bm12 mice, the I-A heterodimer controls the recognition of the immunodominant 40-kDa antigen. By using crossed immunoelectrophoresis, this 40-kDa antigen was identified as antigen 88 according to the reference system of Closs et al. for BCG antigens.

Acquired protective immunity against mycobacterial infections is the result of interactions between specifically sensitized T lymphocytes and macrophages harboring the infectious organism (31, 33). Little is known, however, about the precise antigens involved in protective immunity against such pathogens. On the basis of a generally recognized difference in the efficacy of live and dead mycobacterial vaccines to generate protective immunity (11, 34), it has been argued that secreted antigens, present in large amounts in mycobacterial culture filtrates (CFs) (3, 16), rather than structural components may be essential for the induction of protection. In this respect, a number of authors have reported on the potent T-cell-stimulatory properties of whole mycobacterial CF in mice (4), guinea pigs (38), and humans (12, 22) and of a number of purified CF proteins, such as the 30-32-33-kDa fibronectin-binding antigen 85 (24, 27, 28) and the 38-kDa phosphate-binding glycolipoprotein, also called antigen 78 (37, 39).

Following intravenous infection with live *Mycobacterium bovis* BCG, inbred laboratory mice produce substantial amounts of antibodies against these CF proteins (26). We have previously reported that the repertoire of these antibodies is dependent on the mouse genotype, since BALB/c sera react with a wide range of CF antigens but preferentially with the 65-kDa heat shock protein and the 30-32-33-kDa fibronectin-binding protein and C57BL/6 sera demonstrate a more restricted antibody response directed predominantly against three CF antigens with estimated molecular masses of 37, 38, and 40 kDa. Analysis of the antibody repertoire in wild house mice (*Mus musculus domesticus*) has demonstrated a strong individual variation in the recognition pattern of these CF antigens following infection with live BCG (25).

Antibodies are generally thought to be of little importance in protection against mycobacteria and may even enhance bacterial replication (15, 18). Nevertheless, a role of immunoglobulins in opsonization of mycobacteria or of certain isotypes in antibody-dependent cell-mediated cytotoxicity cannot be excluded (32). Moreover, antibody formation against protein antigens is a T-cell-dependent phenomenon, and hence analysis of the antibody repertoire may give more precise information on the type of antigens against which reactive helper T cells are generated in a particular mouse strain. Using BCG-infected B10-congenic mice with different H-2 haplotypes, B10 mice with recombinant H-2 haplotypes, and B6 mice with mutant major histocompatibility complex (MHC) class I and II haplotypes, we analyzed the regulatory control of MHC on antibody formation against BCG CF antigens. We also describe the production of eight new monoclonal antibodies (MAbs) against BCG CF antigens, using spleen cells from BCG-infected mice with the H-2^b haplotype. Using two MAbs directed against the immunodominant 40-kDa antigen, we have identified this protein as antigen 88 according to the reference system of Closs et al. (9) for BCG antigens.

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TABLE 1. H-2 haplotypes of mouse strains used

Mouse strain	H-2 haplotype[a]	Allele at H-2 locus				
		K	A	E	S	D
C57BL/10	b	b	b	NE[b]	b	b
B10.M	f	f	f	NE	f	f
B10.BR	k	k	k	k	k	k
B10.P	p	p	p	p	p	p
B10.Q	q	q	q	NE	q	q
B10.S	s	s	s	NE	s	s
B10.RIII	r	r	r	r	r	r
B10.GD	g2	d	d	NE	b	b
B10.OH	o2	d	d	d	d	k
B10.A	a	k	k	k	d	d
B10.A(2R)	h2	k	k	k	d	b
B10.A(4R)	h4	k	k	NE	b	b
B10.A(5R)	i5	b	b	k	d	d
B10.MBR	bq1	b	k	k	k	q
B6.C-H-2bm1	bm1	bm1	b	NE	b	b
B6.C-H-2bm12	bm12	b	bm12	NE	b	b
B6.C-H-2bm13	bm13	b	b	NE	b	bm13

[a] Nomenclature system of Hansen and Sachs (21).
[b] NE, not expressed.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, C57BL/10, BALB.B10, B10.M, B10.BR, B10.P, B10.Q, B10.RIII, B10.S, B10.GD, B10.OH, B10.A, B10.A(2R), B10.A(4R), B10.A(5R), B10.MBR, B6.C-H-2^{bm1}, B6.C-H-2^{bm12}, and B6.C-H-2^{bm13} mice were bred in the Animal Facilities at the Pasteur Institute of Brabant from couples initially obtained from the Netherlands Cancer Institute. We used 2-month-old male mice for our experiments. A list of the mouse strains described and their respective H-2 haplotypes in the naming system of Hansen and Sachs (20) is shown in Table 1.

BCG infection. Mice were inoculated intravenously with 0.5 mg (ca. 4×10^6 CFU) of freshly prepared *M. bovis* BCG GL2 (Pasteur Institute of Brabant) as described previously (26). At 2 months, they received an intravenous boost injection with 0.5 mg of live BCG or 25 μg of BCG CF. They were sacrificed 4 weeks later. The sera from at least four mice per group were pooled and kept frozen at −20°C until assayed. Although small (quantitative) individual differences could on some occasions be observed in these inbred mice, the overall antibody repertoire was found to be identical for all mice within one strain, enabling the use of pooled sera in this analysis (data not shown).

Antigens. CF antigens were obtained from 14-day-old cultures of *M. bovis* BCG GL2, grown as a surface pellicle culture on synthetic Sauton medium. Proteins were concentrated by precipitation with ammonium sulfate (80% saturation) (26).

MAbs. MAbs directed against various CF antigens were prepared by using H-2^b spleen cells from BCG-infected C57BL/6 or BALB.B10 mice, rechallenged 4 weeks prior to fusion with a second intravenous inoculation of BCG (0.5 mg) or a boost injection with BCG CF (25 μg per mouse). Hybridomas were established by fusion with the Sp2-0 myeloma cell line, as described previously (10). Ten microtiter plates (96 microwells each) were screened by Western immunoblot analysis of pooled supernatants from 12 microwells, followed by individual analysis of the wells from the positive rows. Finally, selected hybridoma cell lines were cloned by limiting dilution.

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as described previously (26). Briefly, CF antigens were separated under reducing conditions on 12.5% polyacrylamide gels and transferred to nitrocellulose sheets. Nitrocellulose strips (containing ca. 30 μg of CF per strip) were incubated with sera (diluted 1:50) or MAbs (diluted 1:10), and reactivity was analyzed by using peroxidase-conjugated rabbit anti-total-mouse immunoglobulin G (IgG) antibody, α-chloronaphthol, and H_2O_2.

CIE. Crossed immunoelectrophoresis (CIE) was performed as described in detail previously (9). Briefly, BCG culture filtrate was applied in circular wells in the lower gel, alone or together with the respective MAbs. Protein was separated by electrophoresis in one dimension. Next, separated proteins were forced to migrate through the intermediate gel, containing rabbit anti-mouse antibodies, and through the upper gel, containing rabbit anti-BCG antibodies. Precipitate lines were stained with Coomassie brilliant blue, and the control CIE pattern was compared with the MAb-influenced pattern to identify the relevant antigen.

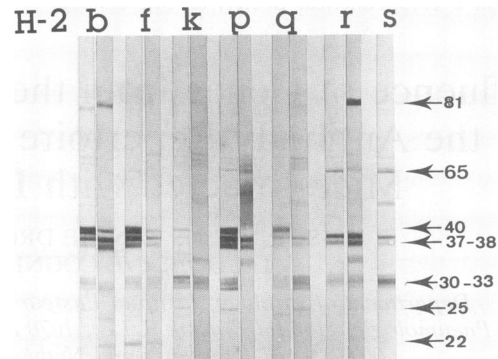

FIG. 1. Western blot analysis of serum antibodies directed against BCG CF antigens in B10 congenic mice with various H-2 haplotypes, infected with BCG and boosted with BCG (left lane for each strain) or CF (right lane for each strain) 2 months later. Lanes: b, C57BL/10; f, B10.M; k, B10.BR; p, B10.P; q, B10.Q; r, B10.RIII; s, B10.S (boost with BCG only).

RESULTS

Western blot analysis of anti-CF antibodies in BCG-infected B10 mice with different H-2 haplotypes. We have previously reported (26) that BCG-infected B10 mice react almost exclusively but very intensely with three CF antigens of 37-38 and 40 kDa after a boost injection with live BCG. Antibodies against the 30-32-33-kDa antigen (antigen 85) are hardly detectable in B10 sera, although this antigen is the major protein component of such CFs (26). We therefore compared antibody reactivity in BCG-infected mice, following a boost injection with live BCG or with purified CF, to determine whether absent reactivity against certain antigens, such as antigen 85, was exclusively controlled by genetic factors or whether it could be modulated by increasing the antigenic dose. Figure 1 shows Western blot patterns of anti-CF antibodies in BCG-infected mice that received a boost injection of live BCG (left lanes) or of total CF protein (right lanes). In most haplotypes, the antibody repertoire was more or less the same after the two types of boost injection, but certain differences could be observed. The 40-kDa protein was always recognized more strongly after boosting with live BCG than after boosting with CF. On the

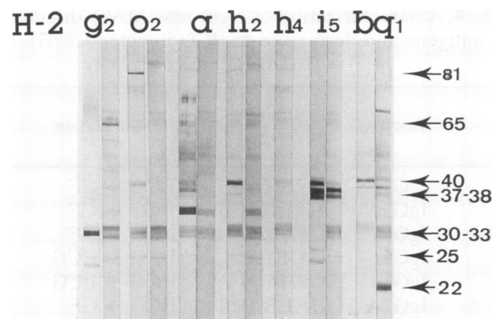

FIG. 2. Western blot analysis of serum antibodies directed against BCG CF antigens in B10 mice with recombinant H-2 haplotypes, infected with BCG and boosted with BCG (left lane for each strain) or CF (right lane for each strain) 2 months later. Lanes: g_2, B10.GD; o_2, B10.OH; a, B10.A; h_2, B10.A(2R); h_4, B10.A(4R) (boost with BCG only); i_5, B10.A(5R); bq_1, B10.MBR.

other hand, antibodies against the 35-kDa protein, characteristic for the response of about 25% of wild house mice (25), were observed only in B10.A (H-2^a) mice after challenge with BCG but also appeared in mice with k, p, and r haplotypes after CF challenge. The H-2 haplotype profoundly influenced the antibody repertoire in these B10 congenic mice. Parental B10 mice and B10.M and B10.P mice reacted strongly (B10.Q mice reacted to a lesser extent) against the 37-38- and 40-kDa proteins after BCG challenge. The 40-kDa antigen was not recognized in B10.P or B10.Q mice after challenge with culture filtrate. B10.S and B10.RIII mice reacted against the 37-38-kDa but not against the 40-kDa protein, and B10.BR mice did not react against either antigen. B10.P, B10.S, and B10.RIII mice also reacted with the 65-kDa heat shock protein (HSP), whereas B10, B10.M, B10.BR, and B10.Q mice were unreactive against this HSP. An 81-kDa antigen was recognized by sera from B10 and B10.RIII mice (especially after a boost with CF), and a 22-kDa antigen was recognized only by B10 and B10.M mice. Finally, a 25-kDa protein and the 30-32-33-kDa protein (antigen 85) were faintly recognized by all sera, after both live BCG and CF boost.

Western blot analysis of anti-CF antibodies in BCG-infected B10 mice with the recombinant MHC haplotype. Analysis of sera from B10 mice with the recombinant MHC haplotype provided further evidence of the remarkable influence of H-2-linked genes on the anti-CF antibody repertoire (Fig. 2). Here again, the most pronounced differences were observed in the recognition of the 37-38- and 40-kDa antigens. B10.A(5R) mice reacted strongly (as did B10.MBR mice, albeit to a lesser extent) against the 37-38- and 40-kDa antigens, and the response against the 40-kDa antigen was strongest after a boost with live BCG. B10.A(2R) mice recognized only the 40-kDa antigen but not the 37-38-kDa antigen. B10.GD, B10.OH, B10.A, and B10.A(4R) mice, finally, showed at most a very weak reaction against the 37-38- and 40-kDa antigens. The 35-kDa antigen was recognized strongly by sera from B10.A mice (challenged with BCG or CF) and by B10.A(2R) mice (challenged with CF only). Antibodies against the 81-kDa antigen were observed in B10.OH mice, those against the 65-kDa HSP were observed in B10.GD mice, and those against the 22-kDa antigen were observed in B10.MBR mice. The 25- and 30-32-33-kDa antigens were again weakly recognized by all sera, after a boost with live BCG or CF. A summary of the reactivities found in Fig. 1 and 2 is given in Table 2.

TABLE 2. Recognition of CF antigens in B10-congenic mice and B10 mice with recombinant H-2 haplotypes

Mouse strain	Recognition of antigen of mol mass (kDa)[a]:					
	22	35	37–38	40	65	81
B10	+	–	+++	+++	–	+
B10.M	+	–	+++	+++	–	–
B10.BR	–	+	–	–	–	–
B10.P	–	+	+++	+++	+	–
B10.Q	–	–	+	+	–	–
B10.S	–	–	+	–	+	–
B10.RIII	–	+	+	–	+	+
B10.GD	–	–	–	–	+	–
B10.OH	–	–	–	–	–	+
B10.A	–	+++	–	–	–	–
B10.A(2R)	–	+	–	+	–	–
B10.A(4R)	–	–	–	–	–	–
B10.A(5R)	–	–	+++	+++	–	–
B10.MBR	+	–	+	+	–	–

[a] Serum reactivity obtained in mice infected with live BCG and boosted 2 months later with live BCG. Abbreviations: +++, strong; +, moderate; –, weak or absent.

Reactivity against the 40-kDa antigen is determined by the MHC class II region. Results obtained in mice with the recombinant H-2 haplotype indicated that the MHC class II region exerted a very powerful control on reactivity against the 40-kDa protein; the I-A^b allele was associated with strong recognition, and the I-A^d and I-A^k alleles resulted in low or absent reactivity (Table 3). Some reactivity against the 40-kDa antigen was also observed in mice carrying D^b or D^q in the presence of I-E^k [B10.A(2R) and B10.MBR] but not in the absence of I-E (B10.GD). Analysis of MHC class I (bml and bm13) and class II (bm12) mutant B6 mice, with mutations in the K, D, or I-A region, respectively, confirmed this hypothesis, since the strong recognition of the 40-kDa protein was almost completely abolished in class II bm12 mutant mice but was strong in class I mutant bml and bm13 mice, similar to the parental B6 phenotype (Fig. 3). The response against the 37-38-kDa antigen was not affected in these mutant mice, whereas bm12 mice were also less reactive against the 81-kDa antigen. Reactivity against the 25- and the 30-32-33-kDa antigens was weak in these three strains.

TABLE 3. Reactivity against the 40-kDa antigen is determined by the MHC class II region

Mouse strain	Reactivity in mice boosted with[a]:		Allele at H-2 locus				
	BCG	CF	K	A	E	S	D
B10	+++	+	b	**b**	NE[b]	b	b
B10.GD	–	–	d	d	NE	b	b
B10.A	–	–	k	k	k	d	d
B10.A(2R)	+	–	k	k	k	d	**b**
B10.A(4R)	–	ND	k	k	NE	b	b
B10.A(5R)	+++	+	b	**b**	k	d	d
B10.MBR	+	+	b	k	k	k	**q**
B6.C-H-2bm12	–	ND	b	bm12	NE	b	b

[a] Serum reactivity obtained in BCG-infected mice, boosted with live BCG or CF. Abbreviations: +++, strong; +, moderate; –, weak or absent; ND, not done.

[b] NE, not expressed.

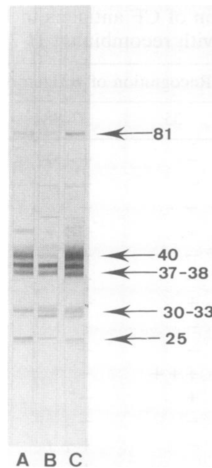

FIG. 3. Western blot analysis of serum antibodies directed against BCG culture filtrate in bm1 (lane A), bm12 (lane B), and bm13 (lane C) MHC mutant B6 mice infected with BCG and boosted with BCG 2 months later.

Identification of culture filtrate antigens with monoclonal antibodies derived from BCG-infected mice. As reported previously (26), MHC-congenic BALB.B10 mice, presenting the H-2^b haplotype on a BALB/c background, reacted as B10 or B6 mice, with strong recognition of both the 37-38- and 40-kDa antigens, whereas BALB/c mice reacted only against the 37-38-kDa protein (Fig. 4, lanes B to D). Furthermore, BALB.B10 mice reacted against the 22- and 81-kDa antigens in the same way as did B6 mice. On the other hand BALB.B10 mice also reacted strongly against the 65-kDa HSP and against the 30-32-33-kDa fibronectin-binding protein, as did BALB/c mice, indicating that the recognition of these two antigens is not controlled by MHC genes on a BALB/c background. A boost injection of CF in BCG-infected BALB.B10 mice (Fig. 4, lane A) led to the appearance of antibodies against the 35-kDa antigen. In sera from BALB.B10 mice, the 25-, 30-32-33-, and 40-kDa antigens were recognized to a lesser extent and antibodies against the 65-kDa HSP could not be detected after a CF boost.

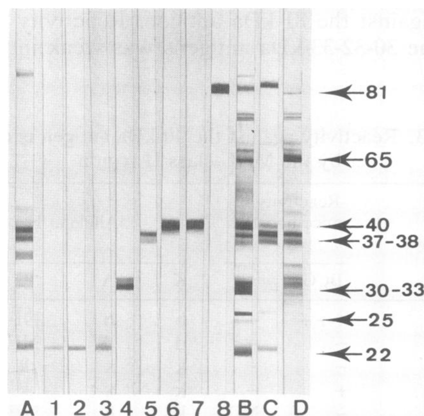

FIG. 4. Western blot analysis of serum antibodies and MAbs against BCG CF antigens. Lanes A and B contain serum from BALB.B10 mice infected with BCG and boosted with CF (lane A) or BCG (lane B). Lanes C and D contain serum from C57BL/6 (lane C) or BALB/c (lane D) mice infected with BCG and boosted with BCG. Other lanes: 1, VD12-1; 2, 9E5; 3, VIIIH1-1; 4, 8F7-6; 5, 2A1-2; 6, 2F8-3; 7, 2C1-5; 8, 4C9-1.

TABLE 4. Characterization of eight new MAbs directed against CF antigens and derived from BCG-infected C57BL/6 and BALB.B10 mice

MAb	Isotype	Strain of origin	Immunization	Estimated mol mass (kDa)
VD12-1	IgG1	C57BL/6	BCG + CF	22
9E5	IgG2A	BALB.B10	BCG + CF	22
VIII-H1-1	IgG1	C57BL/6	BCG + CF	22
8F7-6	IgG1	C57BL/6	BCG + BCG	30–33
2A1-2	IgG1	C57BL/6	BCG + BCG	37–38
2F8-3	IgG2A	BALB.B10	BCG + CF	40
2C1-5	IgG2A	BALB.B10	BCG + CF	40
4C9-1	IgG1	C57BL/6	BCG + BCG	81

Using spleen cells from C57BL/6 and BALB.B10 mice infected with live BCG and boosted with CF or live BCG, we derived eight new MAbs, with specificities that are characteristic of the H-2^b haplotype, directed against the 22-, 30-32-33-, 37-38-, 40-, and 81-kDa CF antigens. The immunization procedure and antigenic specificity are given in Table 4 and Fig. 4.

Identification of the 40-kDa antigen in CIE. As shown in Fig. 5, the use of MAbs 2F8-3 and 2C1-5 enabled us to identify the 40-kDa protein as antigen 88 in classical reference system for BCG antigens (9).

DISCUSSION

Using immunoblot analysis, we have examined the influence of genes of the MHC on the antibody response to *M. bovis* BCG CF antigens in mice infected with live BCG. Using B10 congenic mice with different H-2 haplotypes, B10 mice with recombinant H-2 haplotypes, and MHC class I and II mutant B6 mice, we have demonstrated that H-2-linked genes profoundly affect the antibody repertoire. Certain CF antigens, such as the 30-32-33-kDa fibronectin-binding antigen 85 and a 25-kDa antigen, were weakly recognized by sera from all mouse strains tested, whereas antibodies to other CF antigens were found in only some strains and were undetectable in others. Particularly, antibody responses against the 37-38-kDa doublet protein and the 40-kDa protein, i.e., the most immunodominant antigens for parental B10 and B6 mice, varied strongly among different MHC haplotypes. The 37-38-kDa antigen appeared as a doublet protein with a common B-cell epitope on the two components, since all sera and also the MAb 2A1-2 always reacted with the two components in CF. Reactivity against the 40-kDa protein was not linked to the response against the 37-38-kDa antigen, as clearly shown in B10.RIII, B10.S, B10.A(2R), and class II mutant bm12 mice. The highest reactivity against the 40-kDa protein was found in mice with f, p, and b haplotypes. Results with mice with recombinant or mutant MHC haplotypes indicate that the I-A region plays a crucial role in the recognition of this immunodominant antigen. Thus, mice with i5, bm1, and bm13 haplotypes, all expressing the b allele in the I-A region, reacted strongly against the 40-kDa protein, whereas the class II mutant bm12 mice recognized this antigen only weakly. B6 mutant mice with the bm12 haplotype have originated as the result of a gene conversion event in which a short stretch of nucleotides from the $A_\beta{}^b$ chain has been replaced by the corresponding

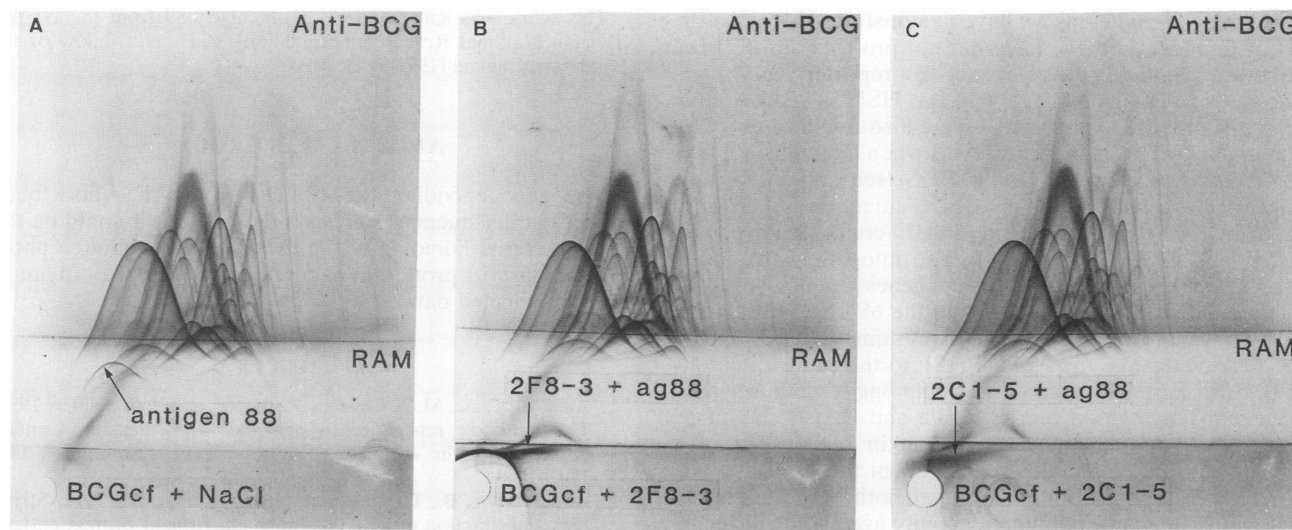

FIG. 5. Identification of the 40-kDa antigen as antigen 88 by CIE. (A) Control CIE pattern; (B) effect of MAb 2F8-3; (C) effect of MAb 2C1-5.

nucleotides of the E_{β}^{b} chain (which is not expressed in B6 cells). This has resulted in amino acid changes in three positions of the A_{β} chain, i.e., Ile-67→Phe, Arg-70→Gln, and Thr-71→Lys (21). This mutant bm12 sequence is known to be identical with the A_{β}^{k} sequence, and, interestingly, k haplotype B10.BR mice did not react against the 40-kDa antigen either. It is fascinating to observe that such subtle replacement of three amino acids is responsible for the complete abolition of an antibody response to the 40-kDa antigen, which is extremely immunodominant in the normal H-2^{b} haplotype. Mutant bm12 Ia+ cells have two to three times less surface Ia than B6 cells do, probably because the mutant β chain fails to associate as effectively with its α partner as the wild-type β chain does (30). Therefore, the defective bm12 response against the 40-kDa antigen probably results from a lack of association of the antigenic peptide with the mutant bm12 heterodimer, leading to inefficient antigen presentation. However, the active generation of suppressor T cells in bm12 mutant mice cannot be excluded either (13). Anyway, these results strongly favor the hypothesis that the bm12 site is a predominant site on Ia for T-cell recognition in B6 mice and, moreover, could indicate that the number of B- and/or T-cell epitopes on the 40-kDa protein is limited.

Using classical CIE with MAbs 2F8-3 and 2C1-5, we were able to identify the 40-kDa antigen as antigen 88 in the Closs et al. reference system for BCG antigens (9). These MAbs also identify a protein with similar molecular mass in CF from *M. tuberculosis*, *M. avium*, and *M. kansasii* (13a). Recently, a gene encoding a 40-kDa protein from *M. tuberculosis* CF was sequenced and identified as encoding an L-alanine dehydrogenase (2), using the HBT10 MAb derived from BALB.B10 mice hyperimmunized with *M. tuberculosis* CF (29). Independently, the gene coding for a 40-kDa NADP-dependent alcohol dehydrogenase from *M. bovis* BCG has recently been identified at the Institute Pasteur van Brabant (36). MAbs 2F8-3 and 2C1-5 do not show reactivity against either of these two enzymes (kindly given to us by A. Andersen and M. Stelandre), suggesting that we are dealing with a new antigen that awaits further identification. (Cloning of the corresponding gene is actually in progress.)

The 37-38-kDa doublet has not yet been definitively classified by CIE, but it is clear that this protein doublet is distinct from the well-known phosphate-binding 38-kDa lipoprotein, also called antigen 78 (21a). Moreover, MAb 2A1-2 does not react with the recombinant 38-kDa protein (30a), and no cross-reactivity can be observed in a latex agglutination assay (14) with the well-defined MAbs TB71 and TB72 (a kind gift from J. Ivanyi).

The two MAbs directed against the 40-kDa antigen were both found to be of the IgG2A isotype. Interestingly, switching to this isotype has been associated with active help from the gamma interferon-producing Th1 helper T-cell subset population (5, 17), a subset that we have found to be preferentially activated in BCG-infected mice with the H-2^{b} haplotype (23). Since the H-2^{b} haplotype is associated with a more efficient protective immunity against mycobacterial infection than the H-2^{d} (23) and H-2^{k} (7) haplotypes are, it can be speculated that recognition of the 40-kDa antigen by IgG2A antibodies may have a certain relevance to resistance to infection. The 40-kDa antigen is present not only in the CF but also in cytoplasmic extract, and it is probably also exposed on the bacterial cell surface, since BCG bacilli dotted on nitrocellulose membranes can capture MAbs 2F8-3 and 2C1-5 (13a). Antibodies against the 40-kDa protein could therefore play a role in opsonization of bacteria or in antibody-dependent cell-mediated cytotoxicity.

Genetic influences on the antibody response have also been found in mice infected intraperitoneally with live *M. tuberculosis* (6) and in mice immunized subcutaneously with *M. leprae* sonicate (1) or with the recombinant 65-kDa HSP from *M. bovis* BCG (8) or *M. leprae* (1). Comparison of our results with these previous studies must be made with great caution, since the immunization route may influence the antibody response observed. More importantly, sensitization with killed mycobacterial extract or with a purified protein does not necessarily lead to the same result as immunization with the intact bacillus during a live mycobacterial infection. Indeed, subcutaneous immunization with recombinant 65-kDa HSP in Freund incomplete adjuvant (FIA) resulted in high antibody levels in all H-2 congenic mice strains tested, among others, in B10, B10.BR, and

B10.M mice (8), whereas we have demonstrated here that on live BCG infection these mice did not produce anti-65-kDa antibodies. Similarly, Adeleye et al. reported very high antibody levels against the 65-kDa HSP in B10.A and B10.A(4R) mice (1), strains that were also found to produce very low anti-65-kDa antibody levels when infected with live *M. tuberculosis* (6) or *M. bovis* BCG (see above). In this study we have shown that live BCG infection will induce anti-65 kDa HSP antibodies only in B10-congenic mice with p, s, r, and g2 haplotypes. It is also interesting that on a BALB/c background, MHC-linked genes do not seem to have an effect on responsiveness to the 65-kDa HSP, since MHC-congenic BALB.B10 mice expressing the H-2^b haplotype reacted against the 65-kDa HSP to the same extent as the BALB/c parental strain did. This finding is reminiscent of our recent observation that BALB/c and BALB.B10 mice also produce more gamma interferon in response to the 65-kDa HSP than B6 mice do (23). From these results, we can confirm previous statements that both MHC and non-MHC genes influence immune reactivity against the intracellular 65-kDa HSP (1).

The exact mechanism by which MHC class II genes regulate the antibody production against the 40-kDa protein is not known. A number of genes related to antigen processing (coding for transporter- and proteasome-like proteins) and with a certain degree of polymorphism, have been mapped to this region (for a review, see reference 19). It is possible that these processing genes influence the variation in antibody repertoire, as we have reported here. Also, the expression of certain class II alleles may exert a direct immune response effect on the antibody repertoire, through variations in the efficiency of epitope presentation by the various haplotypes. The results obtained with B6.C-H-2^{bm12} mice do indicate that this is indeed the case. As demonstrated by the reactivity in B10.A(2R) and B10.MBR mice, the b or q allele in the D region was also associated with some degree of recognition of the 40-kDa protein in I-A^k mice, albeit to a lesser extent than the b allele in I-A region was. It is known that MHC class I restricted cytotoxic and suppressor $CD8^+$ T cells are capable of producing cytokines such as gamma interferon and interleukin-4, respectively (35), which may in turn influence antibody production either directly or through increases of class II MHC antigen expression on antigen-presenting macrophages or B cells.

Finally, although all mouse strains used in this study can be classified as susceptible, their development of protective immunity is clearly influenced by MHC (7, 23; unpublished data), and this may well determine the quantitative availability of mycobacterial antigens following boost injection with live BCG. In this respect it is interesting that antibody responses to the 40- and 65-kDa antigens (which are only weakly present in CF) were generally higher after a boost with live BCG than with CF, whereas responses against the 35-kDa antigen (present as a well-detected protein band in these CF) were higher after a boost with CF than with live BCG. On the other hand, reactivity against the major CF protein, the 30-32-33-kDa antigen, was similar for both types of boost. Clearly, more work is needed to elucidate the complex control mechanisms by which MHC influences the antibody repertoire against BCG CF antigens.

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ADDENDUM IN PROOF

The gene encoding the 40-kDa protein has now been cloned, and sequence analysis indicates that it could be the mycobacterial homolog of the *pstA* gene, encoding a phosphate transport protein in *Escherichia coli.* (M. Braibant et al., unpublished data).

REFERENCES

1. **Adeleye, T. A., M. J. Colston, R. Butler, and P. J. Jenner.** 1991. The antibody repertoire to proteins of *M. leprae*. Genetic influences at the antigen and epitope level. J. Immunol. **147:** 1947–1953.
2. **Andersen, A. B., P. Andersen, and L. Ljungqvist.** 1992. Structure and function of a 40,000-molecular-weight protein antigen of *Mycobacterium tuberculosis*. Infect. Immun. **60:**2317–2323.
3. **Andersen, P., D. Askgaard, L. Ljungqvist, J. Bennedsen, and I. Heron.** 1991. Proteins released from *Mycobacterium tuberculosis* during growth. Infect. Immun. **59:**1905–1910.
4. **Andersen, P., D. Askgaard, L. Ljungqvist, M. W. Bentzon, and I. Heron.** 1991. T-cell proliferative response to antigens secreted by *Mycobacterium tuberculosis*. Infect. Immun. **59:**1558–1563.
5. **Bossie, A., and E. S. Vitetta.** 1991. IFN-γ enhances secretion of IgG2A-committed LPS-stimulated murine B cells: implications for the role of IFN-γ in class switching. Cell. Immunol. **135:**95–104.
6. **Brett, S., and J. Ivanyi.** 1990. Genetic influences on the immune repertoire following tuberculosis infection in mice. Immunology **71:**113–119.
7. **Brett, S., J. M. Orrell, J. Swanson-Beck, and J. Ivanyi.** 1992. Influence of H-2 genes on growth of *M. tuberculosis* in the lungs of chronically infected mice. Immunology **76:**129–132.
8. **Brett, S. J., J. R. Lamb, J. H. Cox, J. B. Rothbard, A. Mehlert, and J. Ivanyi.** 1989. Differential pattern of T cell recognition of the 65 kDa mycobacterial antigen following immunization with the whole protein or peptides. Eur. J. Immunol. **19:**1303–1310.
9. **Closs, O., M. Harboe, N. H. Axelsen, K. Bunch-Christensen, and M. Magnusson.** 1980. The antigens of *M. bovis* BCG studied by crossed immunoelectrophoresis: a Reference System. Scand. J. Immunol. **12:**249–263.
10. **Cogniaux, J., N. De Schepper, M. L. Blondiau, B. Cornet, P. Horal, and A. Vahlne.** 1990. Characterization of monoclonal antibodies against the p17 core protein of the human immunodeficiency virus 1. J. Immunol. Methods **128:**165–175.
11. **Collins, F. M.** 1984. Protection against mycobacterial disease by means of live vaccines tested in experimental animals. Microbiol. Ser. **15:**787–839.
12. **Collins, F. M., J. R. Lamb, and D. B. Young.** 1988. Biological activity of protein antigens isolated from *Mycobacterium tuberculosis* culture filtrate. Infect. Immun. **56:**1260–1266.
13. **De Waal, L. P., J. De Hoop, M. J. Stulcart, H. Gleichman, R. W. Melvold, and C. J. M. Melief.** 1983. Nonresponsiveness to the male antigen H-Y in H-2 I-A mutant B6.C-H-2bm12 is not caused by defective antigen presentation. J. Immunol. **130:**655–660.
13a. **Drowart, A.** Am. Rev. Respir. Dis., in press.
14. **Drowart, A., C. L. Cambiaso, K. Huygen, E. Serruys, J.-C. Yernault, and J.-P. Van Vooren.** Detection of mycobacterial antigens present in short term culture media using particle counting immunoassay (PACIA). Am. Rev. Respir. Dis., in press.
15. **Ferreira, P., R. Soares, and M. Arala-Chaves.** 1991. Susceptibility to infection with *M. avium* is paradoxically correlated with increased synthesis of specific anti-bacterial antibodies. Int. Immunol. **3:**445–451.
16. **Fifis, T., C. Costopoulos, A. J. Radford, A. Bacic, and P. R.**

Wood. 1991. Purification and characterization of major antigens from a *Mycobacterium bovis* culture filtrate. Infect. Immun. **59**:800–807.

17. **Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, J. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann, and W. E. Paul.** 1990. Lymphokine control of in vivo immunoglobulin isotype selection. Annu. Rev. Immunol. **8**:303–333.
18. **Forget, A., J. C. Benoit, R. Turcotte, and N. Gusew-Chartrand.** 1976. Enhancement activity of anti-mycobacterial sera in experimental *Mycobacterium bovis* BCG infection in mice. Infect. Immun. **13**:1301–1306.
19. **Goldberg, A. L., and K. L. Rock.** 1992. Proteolysis, proteasomes and antigen presentation. Nature (London) **357**:375–379.
20. **Hansen, T. H., and D. H. Sachs.** 1989. The major histocompatibility complex, p. 446–487. *In* W. E. Paul (ed.), Fundamental immunology, 2nd ed. Raven Press, New York.
21. **Hansen, T. H., and H. Y. Tse.** 1987. Insights into immune-response gene function using an Ia mutant mouse strain. Crit. Rev. Immunol. **7**:169–191.

21a. **Harboe, M.** Unpublished data.

22. **Havlir, D. V., R. S. Wallis, W. H. Boom, T. M. Daniel, K. Chervenak, and J. J. Ellner.** 1991. Human immune response to *Mycobacterium tuberculosis* antigens. Infect. Immun. **59**:665–670.
23. **Huygen, K., D. Abramowicz, P. Vandenbussche, F. Jacobs, J. De Bruyn, A. Kentos, A. Drowart, J. P. Van Vooren, and M. Goldman.** 1992. Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice. Infect. Immun. **60**:2880–2886.
24. **Huygen, K., K. Palfliet, F. Jurion, J. Hilgers, R. ten Berg, J. P. Van Vooren, and J. De Bruyn.** 1988. H-2 linked control of in vitro interferon gamma production in response to a 32-kDa antigen (P32) of *Mycobacterium bovis* BCG. Infect. Immun. **56**:3196–3200.
25. **Huygen, K., K. Palfliet, F. Jurion, C. Lenoir, and J. P. Van Vooren.** 1990. Antibody repertoire against culture filtrate antigens in wild house mice infected with *M. bovis* BCG. Clin. Exp. Immunol. **82**:369–372.
26. **Huygen, K., L. Ljungqvist, R. ten Berg, and J. P. Van Vooren.** 1990. Repertoires of antibodies to culture filtrate antigens in different mouse strains infected with *Mycobacterium bovis* BCG. Infect. Immun. **58**:2192–2197.
27. **Huygen, K., J. P. Van Vooren, M. Turneer, R. Bosmans, P. Dierckx, and J. De Bruyn.** 1988. Specific lymphoproliferation, gamma interferon production, and serum immunoglobulin G directed against a purified 32 kDa mycobacterial antigen (P32) in patients with active tuberculosis. Scand. J. Immunol. **27**:187–194.
28. **Launois, P., K. Huygen, J. De Bruyn, M. N'Diaye, B. Diouf, L. Sarthou, J. Grimaud, and J. Millan.** 1991. T cell response to purified filtrate antigen 85 from *M. bovis* Bacillus Calmette-Guérin (BCG) in leprosy patients. Clin. Exp. Immunol. **86**:286–290.
29. **Ljungqvist, L., A. Worsaae, and I. Heron.** 1988. Antibody responses against *Mycobacterium tuberculosis* in 11 strains of inbred mice: novel monoclonal antibody specificities generated by fusions, using spleens from BALB.B10 and CBA/J mice. Infect. Immun. **56**:1994–1998.
30. **Lin, C. C. S., A. S. Rosenthal, H. C. Passmore, and T. H. Hansen.** 1981. Selective loss of antigen-specific Ir gene function in IA mutant B6.C-H-2bm12 is an antigen-specific Ir gene function in IA mutant B6.C-H-2bm12 is an antigen presenting cell defect. Proc. Natl. Acad. Sci. USA **78**:6406–6410.

30a. **Machtelinckx, L.** Personal commmunication.

31. **Mackaness, G. B.** 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. J. Exp. Med. **129**:973–992.
32. **Magliulo, E., V. De Feo, A. Stirpe, C. Riva, and D. Scevola.** 1973. Enhanced in vitro phagocytic power of macrophages from PPD-stimulated skin sites in human subjects hypersensitive to PPD. Clin. Exp. Immunol. **14**:371–376.
33. **Orme, I. M.** 1987. The kinetics of emergence and loss of mediator T lymphocytes acquired in response to infection with *M. tuberculosis*. J. Immunol. **138**:293–298.
34. **Orme, I. M., E. S. Miller, A. D. Roberts, S. K. Furney, J. P. Griffin, K. M. Dobos, D. Chi, B. Rivoire, and P. J. Brennan.** 1992. T lymphocytes mediating protection and cellular cytolysis during the course of *M. tuberculosis* infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. J. Immunol. **19**:189–196.
35. **Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin, and B. R. Bloom.** 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science **254**:279–282.
36. **Stélandre, M., Y. Bosseloir, J. De Bruyn, P. Maes, and J. Content.** 1992. Cloning and sequence analysis of the gene encoding an NADP-dependent alcohol dehydrogenase in *M. bovis* BCG. Gene **121**:79–86.
37. **Vordermeier, H. M., D. P. Harris, E. Roman, R. Lathigra, C. Moreno, and J. Ivanyi.** 1991. Identification of T cell stimulatory peptides from the 38 kD protein of *Mycobacterium tuberculosis*. J. Immunol. **147**:1023–1029.
38. **Worsaae, A., L. Ljungqvist, K. Haslov, I. Heron, and J. Bennedsen.** 1987. Allergenic and blastogenic reactivity of three antigens from *Mycobacterium tuberculosis* in sensitized guinea pigs. Infect. Immun. **55**:2922–2927.
39. **Young, D. A., L. Kent, A. Rees, J. Lamb, and J. Ivanyi.** 1986. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. **54**:177–183.