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H-2-Linked Control of In Vitro Gamma Interferon Production in Response to a 32-Kilodalton Antigen (P32) of *Mycobacterium bovis* Bacillus Calmette-Guérin

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A 32-kilodalton protein antigen (P32) was previously purified to homogeneity from culture filtrate of *Mycobacterium bovis* BCG (J. De Bruyn, K. Huygen, R. Bosmans, M. Fauville, R. Lippens, J. P. Van Vooren, P. Falmagne, H. G. Wiker, M. Harboe, and M. Turneer, Microb. Pathog. *2*:351–366, 1987). Spleen cells from BCG-sensitized mice produce significant amounts of gamma interferon (IFN-γ) in response to this P32 protein. The amount of secreted IFN-γ is influenced by mouse genotype, with C57BL/6 (*H-2^b^*), C57BL/10 (*H-2^b^*), and 129/Sv (*H-2^b^*) mice producing about four times more than BALB/c (*H-2^d^*), CBF$_1$ (*H-2^d/b^*), and DBA/2 (*H-2^d^*) mice do. Analysis of seven recombinant inbred strains derived from the BALB/c × C57BL/6 cross and of congenic mice differing in major histocompatibility complex-coding chromosome 17 fragments indicates a probable *H-2*-linked control of this IFN-γ induction, with *H-2^b^* cells producing high titers and *H-2^d^* cells producing low titers in response to the P32 antigen.

Interferons (IFN) are a group of antiviral proteins and glycoproteins induced in nucleated cells by numerous inducers such as viruses, synthetic polyribonucleotides, T-cell mitogens, and various microbial antigens (3, 28). In mice, the amount of IFN produced is controlled by the genotype, with C57BL/6 mice producing 5 to 10 times more IFN than BALB/c mice during a number of viral (9, 33), bacterial (11, 18), or protozoal (8, 23) infections. The regulatory genes causing this strain variation are completely unknown at present, but they appear to be different for each inducer examined.

In *Mycobacterium bovis* BCG-primed animals, low IFN-γ levels in BALB/c spleen cell cultures stimulated with tuberculin are apparently not caused by suppressor macrophages or suppressor T cells or by inactivation of secreted IFN (11). More probably, the low levels are due to direct differences in transcription at the mRNA level, as reported by Sadick et al. in another experimental model, i.e., murine cutaneous leishmaniasis (23).

In all prior studies on IFN-γ production in BCG-primed mice, purified protein derivative (PPD) has been used as the inducing antigen. Because PPD is a complex and varying mixture of several mycobacterial antigens, its use in the study of genetic variation of IFN production is obviously limited. We have recently reported on the purification of a 32-kilodalton protein (P32) from *M. bovis* BCG culture filtrate (7). P32 was identified as antigen 85A in the Closs reference system for mycobacteria; the antigen 85 complex is found exclusively in mycobacteria (4, 10). P32 is the first and major protein detectable in Sauton culture filtrate of actively growing BCG cultures and is also a well-represented component of BCG cells. Furthermore, P32 epitopes are present in PPD preparations (7). P32 antigen induces significant IFN-γ production in spleen cells from BCG-primed mice (7) and in peripheral blood leukocytes from tuberculous patients and healthy PPD skin test-positive controls (15). Moreover, positive anti-P32 immunoglobulin G antibodies could be detected in the serum of 56 of 102 tuberculous patients, but in only 1 of 72 BCG-vaccinated and 1 of 38 skin test-positive healthy subjects (30).

Here we show that the amount of IFN-γ produced in vitro by BCG-primed spleen cells challenged with P32 antigen is under genetic control, with C57BL/6 mice producing about four times more IFN-γ than BALB/c mice. Furthermore, we demonstrate a probable *H-2*-linked control of this variation in IFN-γ production.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, CBF$_1$, DBA/2, and BALB/c × C57BL/6 cross (C×B) line D, E, G, H, I, J, and K mice were bred in the animal facilities at the Pasteur Institute of Brabant, Brussels, Belgium. C57BL/10ScSnA and congenic BALB. B10/LiA (*H-2^b^*) and B10.D2/nSnA (*H-2^d^*) mice (17) were bred at the Netherlands Cancer Institute, Amsterdam. 129/Sv mice were obtained from J. Van Snick, Institute of Cellular Pathology, Brussels, Belgium. Only 3-month-old female mice were used.

Antigens. IFN-γ inducers used were PPD (bovine tuberculin, strain Vallée, Pasteur Institute of Brabant) and purified P32 prepared as described previously by successive hydrophobic chromatography on phenyl-Sepharose, ion exchange on DEAE-Sephacel, and molecular sieving on Sephadex G-100 of Sauton zinc-deficient culture filtrate of *M. bovis* BCG (7).

BCG priming. Mice were sensitized intravenously with 0.5 mg (± 2.5 × 10^6 CFU) of *M. bovis* BCG (strain GL2, grown as a surface pellicle in Sauton medium and prepared freshly every week at Pasteur Institute).

IFN production. Three weeks after BCG priming, mice were killed by cervical dislocation and their spleens were removed aseptically. Spleens from at least five mice per group were examined individually. Spleen cells were isolated by using a loosely fitting Dounce homogenizer, counted, adjusted at a concentration of 3 × 10^6 cells per ml,

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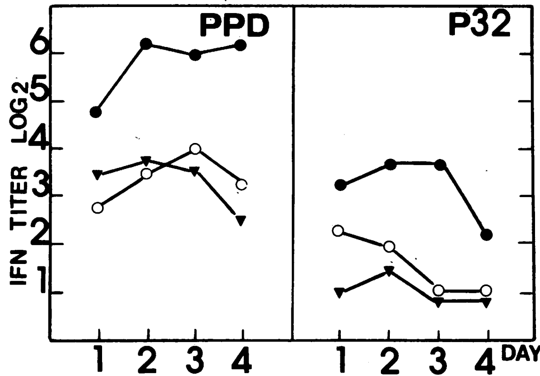

FIG. 1. Interferon production in 24-, 48-, 72-, and 96-h-culture supernatants of BCG-primed spleen cells stimulated with PPD or P32 (25 μg/ml). Symbols: ●, C57BL/6; ○, BALB/c; ▼, CBF_1.

and grown in round-bottomed microwell plates (Nunc) in RPMI 1640 medium supplemented with glutamine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum. A 20-μl volume of PPD or P32 dilutions was added to 180 μl of cell suspension, resulting in a final concentration of 25 μg/ml. Cells were cultured at 37°C in a humidified CO_2 incubator, and supernatants were harvested after 24, 48, 72, and 96 h. Supernatants from three separate wells were pooled.

IFN assay. Antiviral IFN-γ activity in the culture supernatants was measured by using a cytopathic effect reduction assay of vesicular stomatitis virus on mouse L929 fibroblasts. Briefly, serial twofold dilutions of 50 μl of IFN-containing supernatants were made in Dulbecco modified Eagle medium without serum in flat-bottomed microplates; plates were sterilized by UV irradiation, and 100 μl of L929 cells (5×10^4 cells in Dulbecco modified Eagle medium–7% fetal calf serum) was added per well. Following overnight incubation, cells were challenged with 50 μl of a vesicular stomatitis virus dilution at a multiplicity of infection of 1. The $\log_2$ titer was determined as the last dilution in which 50% of L929 cells were protected against the cytopathic effect of vesicular stomatitis virus. One $\log_2$ unit corresponds to 6 IU (Gg02-901-533 NIH, Bethesda, Md.).

Elimination of L3T4⁺ cells. Spleen cells were incubated for 2 h at 4°C in culture supernatant from the GK 1.5 hybridoma line, which produces anti-L3T4 antibodies (32). Cells were then treated with 5% fresh guinea pig complement at 37°C for 1.5 h, washed once in RPMI 1640 medium, and suspended to 3×10^6 cells per ml for culture.

RESULTS

Genotype variation in PPD- and P32-induced IFN-γ. The amount of IFN-γ produced by BCG-primed spleen cells in response to both PPD and P32 depended on the mouse strain (Fig. 1). BALB/c spleen cells produced about four times less IFN than C57BL/6 spleen cells. CBF_1 cells produced low IFN titers. Significant IFN levels were already obtained after 24 h of culture, and peak IFN levels were observed between 48 and 72 h. Genotype-linked differences were seen throughout the 4-day culture period and over a range of P32 concentrations from 1 to 50 μg/ml. At the lowest antigen concentrations, IFN levels were almost undetectable in low-responder mice. We therefore chose the dose of 25 μg/ml for further genetic studies, as it consistently induced IFN in all strain combinations.

TABLE 1. IFN-γ production by C57BL/6 spleen cells stimulated with PPD and P32

Treatment of C57BL/6 spleen cells	IFN-γ production (IU/ml) after stimulation with:	
	PPD	P32
No BCG priming	<3	<3
BCG priming 3 wk before assay	136	48
Complement only	114	28
Complement + anti-L3T4 monoclonal antibody[a]	5	<3
BCG priming 25 wk before assay	192	17

[a] No actual cell killing was observed in trypan blue exclusion assays, suggesting that GK 1.5 monoclonal antibody actually down-regulated L3T4 cells, as has been previously reported (22).

Characterization of P32-induced IFN. As reported previously (7), P32-induced antiviral activity could be completely neutralized by a polyclonal rabbit antibody directed against *Escherichia coli*-derived murine IFN-γ, indicating its genuine IFN-γ character. Control mice not sensitized with BCG produced no IFN in response to PPD or P32 (Table 1). BCG priming 3 or 25 weeks prior to the assay induced significant IFN levels. Treatment of BCG-primed C57BL/6 spleen cells with monoclonal anti-L3T4 antibody and complement completely abolished P32-induced IFN-γ production; PPD-induced IFN was reduced to about 5% of its original level.

P32- and PPD-induced IFN in various mouse strains. Table 2 shows mean IFN levels induced with PPD and P32 as measured in 48-h-culture supernatants. C57BL/6 mice produced significantly more IFN than BALB/c mice in response to PPD and P32. A cutoff value for low P32 response was determined to be 2.78 $\log_2$ units (mean BALB/c response plus 2 standard deviations). We classified CBF_1 and DBA/2 (*H-2*^{d}) as low producers and C57BL/6 and 129/Sv (*H-2*^{b}) as high producers. [P32 response of DBA/2 (*H-2*^{d}) cells was significantly lower than that of BALB/c cells.] All mice produced more IFN in response to PPD than to P32, but the ratio of PPD- to P32-induced IFN varied between 2.23 and 6.0. The highest ratios were generally found in low-responder strains.

P32- and PPD-induced IFN in C×B RI strains. IFN levels obtained in spleen-cell culture supernatant from seven recombinant inbred (RI) lines derived from the C×B cross are shown in Table 3. Two mouse lines (D and H) produced low levels of IFN-γ (not significantly different from BALB/c levels) in response to P32, whereas the other five lines (E, G, I, J, and K) produced large amounts of IFN-γ in response to P32 (comparable to C57BL/6 values). The strain distribution pattern (SDP) for P32-induced IFN is identical to the SDP for *H-2* in these lines, in the sense that D and H lines are *H-2*^{d} and the other five lines are *H-2*^{b}. PPD-induced IFN displayed more overlapping, and parental phenotypes were difficult to distinguish in some RI lines. The PPD pattern was similar to that of P32 in the D, E, G, and H lines. The J line behaved as a low responder to PPD, and the I and K lines showed intermediate responses. The ratio of PPD- to P32-induced IFN ranged between 1.02 and 4.47. Here again, the highest ratios were observed in those RI lines with low P32 response, i.e., D and H. In the J line, identical IFN levels were induced with PPD and P32, as if P32 were the only component in PPD capable of stimulating these mice to secrete IFN.

P32- and PPD-induced IFN in *H-2* congenic mice on BALB/c and C57BL/10 backgrounds. B10.D2 mice, with the *H-2*^{d}

TABLE 2. IFN-γ production by BCG-primed spleen cells in response to PPD and P32

Mouse strain	IFN-γ production in response to[a]:							
	PPD				P32			
	Mean ± SD	n	vs C[b]	vs B6[b]	Mean ± SD	n	vs C	vs B6
BALB/c (*H-2[d]*)	3.92 ± 1.75	6		<0.05	2.00 ± 0.39	5		<0.005
C57BL/6 (*H-2[b]*)	5.50 ± 0.61	6	0.05		4.00 ± 0.61	6	0.005	
CBF_1 (*H-2[d/b]*)	4.66 ± 0.89	8	NS	<0.05	2.12 ± 0.54	8	NS	0.005
DBA/2 (*H-2[d]*)	4.08 ± 0.41	6	NS	<0.005	1.51 ± 0.21	6	NS	0.005
129/Sv (*H-2[b]*)	5.60 ± 0.89	5	0.5	NS	4.45 ± 1.07	5	<0.005	NS
BALB.B10 (*H-2[b]*)	4.45 ± 0.11	5	NS	0.005[c]	3.65 ± 0.33	5	<0.005	NS[c]
B10.D2 (*H-2[d]*)	4.10 ± 0.78	5	NS[d]	0.005[c]	1.20 ± 0.33	5	NS[d]	<0.005[c]
C57BL/10 (*H-2[b]*)	5.50 ± 0.41	5	0.05	NS	3.83 ± 0.52	5	<0.005	NS

[a] Mean $\log_2$ IFN titers (± standard deviation). *n*, Number of animals tested. IFN was measured in 48-h-culture supernatants of BCG-primed spleen cells 3 weeks after BCG sensitization. Concentration of antigen, 25 μg/ml.
[b] Student's *t* test analysis, compared with parental BALB/c (C) and C57BL/6 (B6) values. Level of confidence, $P < 0.05$. NS, Not significant.
[c] Compared with C57BL/10 parental value.
[d] Compared with DBA/2 parental value.

major histocompatibility complex (MHC) chromosome fragment derived from DBA/2 on a B10 background, produced low IFN titers, identical to those for DBA/2. On the other hand, BALB.B10 mice, with the *H-2[b]* MHC chromosome fragment derived from C57BL/10 on a BALB/c background, produced high titers of IFN not significantly different from those for C57BL/10 (Table 2). IFN-γ titers induced with PPD were comparable to those for BALB/c in both congenic lines. Thus, also in these MHC-congenic pairs, the magnitude of the P32-induced IFN-γ response was found to be related to the expression of the *H-2* haplotype.

DISCUSSION

Spleen cells from BCG-primed mice were capable of producing substantial amounts of IFN-γ in response to a purified 32-kilodalton protein antigen from *M. bovis* BCG culture filtrate. This production depended on previous sensitization with BCG, which is in agreement with our recent clinical observation that tuberculin-negative healthy donors are incapable of specific lymphoproliferation or IFN-γ secretion in response to P32, whereas all tuberculin-positive subjects and about 60% of a group of patients with active tuberculosis do recognize the protein (15).

Furthermore, C57BL/6 splenocytes produced about four times more IFN-γ in response to P32 than BALB/c cells did. As we have previously reported, BCG-primed C57BL/6 cells produce more IFN-γ in response to PPD than BALB/c cells do (12). Studies on F_1, F_2, and backcross generation mice have indicated that one major autosomal locus is involved in the control of this PPD-induced IFN-γ activity, though the influence of additional minor loci could not be excluded (13). Further genetic analysis of the BCG-PPD IFN response turned out to be more difficult than expected at first. Studies on the seven RI lines derived from the BALB/c × C57BL/6 cross never revealed a clear-cut SDP (14; this report).

Using the P32 protein as the antigenic IFN-γ inducer, we could establish a clear SDP for the same seven RI lines: D and H were classified as BALB/c-type low responders, whereas the other five lines, i.e., E, G, I, J, and K, produced high levels of P32-induced IFN, comparable to C57BL/6 values. This response paralleled the pattern observed for PPD-induced IFN in four of seven RI lines. The SDP observed for P32-induced IFN followed the SDP described for the *H-2* locus (29): all *H-2[b]* haplotypes corresponded to high producers, and all *H-2[d]* haplotypes corresponded to low producers. Congenic pairs differing only at a chromosome 17 fragment containing the MHC locus were therefore studied, and they confirmed the *H-2*-linked control of P32-induced IFN, with BALB.B10 (*H-2[b]*) mice producing high IFN levels comparable to B10 values, and B10.D2 (*H-2[d]*) mice showing low titers of DBA/2-type magnitude.

Although we have not absolutely proven that *H-2* (and not a neighboring gene on the chromosome 17 fragment) is responsible for the observed strain difference in response to

TABLE 3. IFN-γ production by BCG-primed C×B RI mice in response to PPD and P32

C×B RI mouse line	IFN-γ production in response to[a]:							
	PPD				P32			
	Mean (± SD)	n	vs C[b]	vs B6[b]	Mean (± SD)	n	vs C	vs B6
D (*H-2[d]*)	4.83 ± 0.29	5	NS	<0.05	2.67 ± 0.76	5	NS	0.01
E (*H-2[b]*)	5.50 ± 0.20	6	<0.05	NS	4.19 ± 0.47	6	<0.005	NS
G (*H-2[b]*)	6.00 ± 0.35	5	<0.025	NS	4.65 ± 0.98	5	<0.005	NS
H (*H-2[d]*)	3.69 ± 0.55	6	NS	<0.005	1.87 ± 0.48	6	NS	<0.005
I (*H-2[b]*)	4.95 ± 0.80	5	NS	NS	3.75 ± 0.95	5	<0.005	NS
J (*H-2[b]*)	3.62 ± 0.58	6	NS	<0.005	3.67 ± 0.34	6	<0.005	NS
K (*H-2[b]*)	5.31 ± 0.31	5	NS	NS	3.87 ± 0.43	5	<0.005	NS

[a] Mean $\log_2$ IFN titers (± standard deviation). *n*, Number of animals tested. IFN was measured in 48-h-culture supernatants of BCG-primed spleen cells 3 weeks after BCG-sensitization. Concentration of antigen, 25 μg/ml.
[b] Student's *t* test analysis, compared with parental BALB/c (C) and C57BL/6 (B6) values. Level of confidence, $P < 0.05$. NS, Not significant.

P32, our results with *H-2* congenic mice, together with the SDP observed in the seven RI lines, make another explanation very unlikely. Examination of additional C×B RI strains, as well as linkage analysis on F_2 or backcross generation mice, will enable us to confirm the probable MHC linkage in a more definitive manner.

To our knowledge, this is the first description of a regulatory gene involved in antigenically induced IFN-γ production. In this respect it is interesting to note that susceptibility to mycobacterial infections and the immune reactions they evoke have been correlated to certain *H-2* and human leukocyte antigen (HLA) haplotypes. In mice, the *H-2*-linked gene(s) is known to control both granulomatous response to and host replication restriction of *Mycobacterium lepraemurium* (5). Moreover, Ivanyi and Sharp have recently demonstrated that murine antibody responses to defined mycobacterial protein epitopes are also under the control of *H-2* genes (16).

Control by *H-2* genes may be operating at the level of antigen presentation by macrophages to T cells, but obviously, more work is needed to prove that $H\text{-}2^b$ mice indeed produce more P32-induced IFN-γ than $H\text{-}2^d$ mice do as a result of a more immunodominant presentation of the relevant P32 epitopes on the $H\text{-}2^b$ macrophage.

For humans, HLA DR2-associated susceptibility to pulmonary tuberculosis has already been described (25), and in cases of leprosy, HLA-linked genes seem to control not susceptibility per se but the leprosy type. Thus, HLA DR3 is preferentially inherited by children with polar tuberculoid leprosy, whereas HLA MT1 is observed preferentially in children with lepromatous leprosy (31). Interestingly, lepromatous leprosy patients demonstrate low cellular immunity, as indicated by negative skin tests and low or absent lymphocyte proliferation and IFN-γ production in response to *Mycobacterium leprae* antigens (19).

Other genes have been implicated in protection against mycobacterial infections. In mice, the *bcg* gene located on chromosome 1 is known to affect early bacterial multiplication (26), and macrophages expressing he Bcg^r allele have a higher intracellular bactericidal potential than Bcg^s macrophages do (27). Whereas allelic differences in *bcg* genes reflect differences in innate susceptibility, variability at the *H-2* locus is more likely to interfere with the later stages of infection when acquired immunity plays the preponderant role. This has indeed been observed in the case of the protozoal infection caused by *Leishmania donovani*; innate susceptibility is under the control of the autosomal *lsh* gene on chromosome 1 (2), a gene which is probably identical to the above-mentioned *bcg* gene (26) and also to the *ity* gene involved in resistance to *Salmonella typhimurium* (21). The recovery pattern, on the other hand, of Lsh^s animals appears to be controlled by a gene(s) within or closely adjacent to the *H-2* complex (1).

As stated by Orme (20), it is still a matter of debate whether antigens relevant to vaccine development are to be looked for among constitutive proteins of mycobacteria or, instead, among secreted proteins produced only by living metabolizing organisms (as is the case for P32). Furthermore, a mycobacterial infection will expose the organism to a number of antigens, but not all of them will trigger a good IFN-γ response. Thus, the 64-kilodalton protein antigen of *M. bovis* BCG (6), known to be very immunodominant at the B-cell level, appears to be a less potent IFN-γ inducer than the 32-kilodalton protein (K. Huygen, manuscript in preparation).

Clearly, much work remains to be done to define the role (if any) of P32 immunity in protection against mycobacterial infections.

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