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Spleen Cell Cytokine Secretion in *Mycobacterium bovis* BCG-Infected Mice

KRIS HUYGEN,[1]* DANIEL ABRAMOWICZ,[2] PAUL VANDENBUSSCHE,[1] FRÉDERIQUE JACOBS,[2] JACQUELINE DE BRUYN,[1] ALAIN KENTOS,[2] ANNIE DROWART,[2] JEAN-PAUL VAN VOOREN,[2] AND MICHEL GOLDMAN[2]

Instituut Pasteur van Brabant, 642 Engelandstraat, 1180 Brussels,[1] and Hôpital Erasme (ULB), 1070 Brussels,[2] Belgium

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Three susceptible mouse strains, i.e., BALB/c (*H-2*^{d}), C57BL/6 (*H-2*^{b}), and major histocompatibility complex-congenic BALB.B10 (*H-2*^{b}), were infected intravenously with 4×10^6 CFU of live *Mycobacterium bovis* BCG and analyzed 4 weeks later for in vitro spleen cell cytokine secretion in response to purified protein derivative (PPD), BCG culture filtrate (CF), BCG cellular extract, total BCG, the purified extracellular 30-32-kDa antigen (the fibronectin-binding antigen 85), or the intracellular 65-kDa heat shock protein. C57BL/6 and BALB.B10 mice produced 5- to 10-fold more gamma interferon and interleukin-2 (IL-2) when stimulated with CF, PPD, and antigen 85 than BALB/c mice did. When stimulated with BCG extract and whole BCG, gamma interferon and IL-2 levels were generally lower and comparable in the three strains. IL-4 was detected in spleen cell culture supernatants from infected BALB/c mice but not from C57BL/6 or BALB.B10 mice. IL-5 could not be detected. C57BL/6 and BALB.B10 spleen cells also produced more tumor necrosis factor alpha and IL-6 after stimulation with PPD and CF than BALB/c cells did. Finally, BCG vaccination generated efficient protective immunity in C57BL/6 and BALB.B10 mice but not in BALB/c mice. These data suggest that secreted mycobacterial CF antigens selectively induce a strong TH_1 response in BCG-infected C57BL/6 and BALB.B10 mice, whereas in BALB/c mice this response is partly counterbalanced by TH_2 cells.

Protective immunity against mycobacterial infections is mediated by interactions between specifically sensitized T cells and activated macrophage-effector cells (32). These populations interact with each other by means of a complex network of lymphokines and monokines. The relative importance of each of these cytokines is, however, not completely understood for the moment and is somewhat controversial. Gamma interferon (IFN-γ), a potent activator of macrophages (31), is thought to play a crucial role in antimycobacterial protection, although some authors found that IFN-γ enhanced the replication of mycobacteria in human monocytes (10). Tumor necrosis factor alpha (TNF-α) has been reported to contribute to the deleterious immunopathological side effects of mycobacterial infections (38) but also favorably influences the development of protective granulomas (23) as well as the tuberculostatic properties of macrophages (12). Interleukin-4 (IL-4) is capable of controlling mycobacterial growth (8, 12), and, furthermore, this lymphokine induces cultured monocytes or macrophages to form giant multinucleated cells (25), typically associated with granuloma structures. Finally, IL-6 has been described to stimulate the tuberculostatic functions of murine bone-marrow-derived macrophages (11).

Mosmann et al. and Mosmann and Coffman have categorized murine helper T-cell clones into two distinct subsets by their lymphokine secretion pattern: TH_1 cells produce IL-2 and IFN-γ and mediate Jones-Mote-like DTH responses; TH_2 cells produce IL-4 and IL-5 and control immunoglobulin E (IgE) secretion of B cells (27, 28). *Leishmania major* infection in mice results in preferential activation of either TH_1 or TH_2 cells in different mouse strains, and this strongly influences the outcome of the infection. Thus, susceptible BALB/c mice mount a strong TH_2 type of response during infection with the protozoal parasite, whereas resistant C57BL/6 (B6) mice activate preferentially their TH_1 compartment (17).

Two articles have recently reported the activation of different T-helper subsets in tuberculoid and lepromatous leprosy in humans. First, mycobacterium-reactive T-cell clones derived from tuberculoid leprosy patients were found to exhibit a TH_1-like cytokine secretion pattern, with very high levels of IFN-γ and low levels of IL-4 and IL-5 (15). Second, Yamamura et al. (45) found that different cytokine mRNA profiles were present in leprosy skin lesions, depending on the severity of the disease. Thus, in paucibacillary lesions from tuberculoid leprosy, mRNAs coding for IL-2 and IFN-γ were most evident, whereas in multibacillary lesions from lepromatous leprosy, mRNA for IL-4 and IL-10 predominated (45).

We previously reported that mouse strains show a marked genetic variation in the amount of IFN-γ produced after intravenous infection with live *Mycobacterium bovis* BCG. Thus, BCG-infected BALB/c mice produce fourfold less IFN-γ when stimulated in vitro with purified protein derivative (PPD) or with the purified 32-kDa fibronectin-binding culture filtrate (CF) protein (also called P32 or antigen 85A) than B6 mice do (20). In this study, we have analyzed more extensively the cytokine secretion profile of BCG-infected BALB/c and B6 mice and of major histocompatibility complex (MHC)-congenic BALB.B10 (*H-2*^{b}) mice. We have found that intravenous infection with *M. bovis* BCG induces a vigorous TH_1 type response (IFN-γ and IL-2) in B6 and BALB.B10 spleen cells stimulated with CF antigens and PPD, whereas the TH_2 lymphokine IL-4 can be detected in BALB/c cultures only.

* Corresponding author.

MATERIALS AND METHODS

Mice. BALB/c, B6, and MHC-congenic BALB.B10 ($H\text{-}2^b$) mice were bred in the animal facilities of the Pasteur Institute of Brabant. Two-month-old animals were used for primary infection, and 4-month-old animals were used for challenge experiments.

BCG infection. Mice were inoculated intravenously in the tail vein with 0.5 mg (4×10^6 CFU) of freshly prepared *M. bovis* BCG GL2 (Pasteur Institute of Brabant) which had been grown for 2 weeks as a surface pellicle on Sauton culture medium. Cytokine production was assessed 4 weeks later. For the challenge infection, a second BCG inoculum (0.5 mg) was given 2 months after the first infection, and mice were sacrificed 2, 4, 6, and 8 weeks after the first or second infection. At least four animals were pooled in each experimental group.

Antigens. Live *M. bovis* BCG bacilli were prepared from a surface pellicle culture of the same *M. bovis* BCG GL2 used for infection. Concentrated CF antigens were obtained by ammonium sulfate precipitation of the culture supernatant of these BCG cultures (19). PPD was prepared at the Pasteur Institute of Brabant from 8-week-old CF of *M. bovis* Vallée. Soluble cellular extract (extract) was prepared from *M. bovis* BCG GL2 by disruption of the cells in a French press, followed by centrifugation at 10,000 × *g* for 30 min and 100,000 × *g* for 90 min at +4°C. The extracellular 30-32-kDa antigen (antigen 85) and the intracellular 65-kDa heat shock protein (65-kDa Hsp) were purified from *M. bovis* BCG as described previously (6, 7). Bacterial lipopolysaccharide (LPS) from *Escherichia coli* was prepared at the Pasteur Institute of Brabant by hot phenol-water extraction, as described by Westphal and Jann (43). All antigens were used at a final concentration of 25 μg/ml; LPS was used at a final concentration of 10 μg/ml. Endotoxin content was below 1 ng/ml in all antigen preparations as assessed by *Limulus* assay.

Cytokine production. Four weeks after primary infection, mice were killed by exsanguination and spleens were removed aseptically. Cells were isolated by use of a loosely fitting Dounce homogenizer, washed, adjusted to a concentration of 4×10^6 cells per ml, and grown in flat-bottom microwell plates (Nunc, Roskilde, Denmark), in RPMI 1640 medium, supplemented with glutamine, HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% heat-inactivated fetal calf serum. The various inducers were added in a volume of 20 μl to 180 μl of cell suspension. Cells were incubated at 37°C in a humidified CO_2 incubator, and supernatants were harvested after 24 h (IL-2 and TNF) and 72 h (IFN-γ, IL-4, IL-5, and IL-6). Time kinetics were identical for the three mouse strains used. Supernatants from three separate wells were pooled and stored frozen at −20°C until assay. Experiments were repeated three times, and the results of one experiment are shown.

IL-2 assay. A volume of 100 μl of culture supernatant was added to 100 μl of CTLL-2 cells (10^5/ml), and the mixture was incubated for 48 h (14). Tritiated thymidine (Amersham; specific activity, 8.3 Ci/ml) was added (0.4 μCi per well) during the last 17 h of culture. Cells were harvested by using a Titertek cell harvester (Skatron), and radioactivity recovered from the filters was counted in a Beckman LS230 liquid scintillation counter. Values (counts per minute) from triplicate cultures were converted into international units per milliliter by using a standard curve obtained with serial twofold dilutions of a reference IL-2 preparation (Janssen Biochimica, 500 IU/ml). Values lower than the mean + 3 standard deviations (SD) obtained in unstimulated control cultures were considered to be not significantly different.

IFN assay. Antiviral activity in culture supernatants was measured by using a cytopathic effect reduction assay of vesicular stomatitis virus on mouse L929 cells, as described before (20). Titers were calculated as mean $\log_2 \pm$ SD obtained from triplicate assays and converted into international units per milliliter (Gg02-901-533; National Institutes of Health, Bethesda, Md.).

IL-4 assay. The IL-4 content of supernatants was evaluated by the ability of the supernatants to increase the expression of class II MHC antigens on fresh splenic B cells as described before (1). Briefly, serial dilutions of culture supernatants (beginning at 75% [vol/vol]) were added to 5×10^5 BALB/c B cells (anti-Thy-1 and complement-treated spleen cells) in a final volume of 200 μl in microwell plates. In addition, anti-IFN-γ antibodies were added to the cultures to avoid possible inhibitory influences of IFN-γ on IL-4-induced B-cell Ia expression (26). After 36 h of culture, B cells from duplicate cultures were harvested and stained for class II (I_E) MHC antigen expression with the fluoresceinated 14-4-4 monoclonal antibody (MAb) (35). Mean fluorescence intensities were determined by using a fluorescence-activated cell sorter (FACStar-plus; Becton Dickinson). A titration curve was constructed with recombinant murine IL-4 (from methotrexate-amplified CHO cells, kindly provided by W. Fiers and P. Vandenabeele, Laboratory of Molecular Biology, Rijksuniversiteit te Gent, Ghent, Belgium) ranging from 0.01 to 10 U/ml, and the IL-4 content of supernatants was expressed in units per milliliter. The anti-IL-4 MAb 11B11 always inhibited the increase in class II MHC expression by more than 90%, indicating that only IL-4 was responsible for this activity.

IL-5 assay. IL-5 activity was measured in an enzyme-linked immunosorbent assay (ELISA), using the TRFK5 and TRFK4 anti-IL-5 MAbs, generously provided by R. Coffman (38). Briefly, ELISA plates were coated overnight with TRFK5 (1:2,000), washed, and blocked with bovine serum albumin. Next, culture supernatant was added overnight, and, after washing, the second MAb (TRFK4 [IgG2a]) was added. Finally, the enzymatic reaction was developed by using a peroxidase-conjugated rat anti-mouse IgG2a, OPD (chromogen), and H_2O_2.

IL-6 assay. IL-6 activity was assessed in a colorimetric assay by measuring hexose-aminidase levels of 7TD-1 mouse-mouse hybridoma cell cultures grown in the absence or presence of serial fivefold dilutions of culture supernatants (21). Each sample was tested in triplicate. IL-6 titers are expressed in mean laboratory units per milliliter. One unit corresponds to about 1 pg of IL-6. Values lower than the mean + 3 SD obtained in unstimulated control cultures were considered to be not significantly different.

TNF assay. Mouse TNF-α activity was determined by using a Factor-test mouse TNF-α ELISA kit (Genzyme, catalog no. 1509-00). Results are expressed as mean picograms per milliliter from duplicate assays.

Enumeration of BCG CFU in the spleen. Spleen cells from primary-infected and BCG-vaccinated mice were prepared after 2, 4, 6, and 8 weeks of infection, and serial threefold dilutions in phosphate-buffered saline (PBS) without antibiotics were plated on Middlebrook 7H10 Bacto Agar (Difco). Appropriate dilutions were tested in quadruplicate. Petri dishes were sealed in plastic bags and incubated at 37°C, and colonies were counted visually after 18 days of incubation.

TABLE 1. IL-2 production in spleen cell cultures from BCG-infected mice

Antigen[a]	IL-2 titer (IU/ml ± SD)[b]		
	BALB/c	B6	BALB.B10
None	0.36 ± 0.11	1.38 ± 0.26	0.10 ± 0.02
PPD	1.30 ± 0.07	6.30 ± 0.58	5.34 ± 0.54
CF	1.76 ± 0.36	13.70 ± 0.63	7.64 ± 0.62
BCG extract	2.50 ± 0.22	7.78 ± 0.36	4.78 ± 0.93
Whole BCG	2.22 ± 0.23	4.50 ± 0.28	2.62 ± 0.31
30-32-kDa protein	0.76 ± 0.14	8.84 ± 0.52	1.92 ± 0.22
65-kDa Hsp	1.60 ± 0.12	1.72 ± 0.13 (NS)	0.32 ± 0.12
LPS	0.34 ± 0.03 (NS)[c]	0.88 ± 0.22 (NS)	0.14 ± 0.08 (NS)

[a] Antigens were used at a final concentration of 25 μg/ml; LPS was used at 10 μg/ml.
[b] IL-2 titers in 24-h spleen cell culture supernatants, calculated from triplicate cultures of CTLL-2 cells grown for 48 h in 1/2 dilution of samples.
[c] NS, not significantly different from control values in unstimulated cultures.

TABLE 3. IL-4 production in spleen cell cultures from BCG-infected mice

Antigen (concn)	IL-4 titer (U/ml)[a]		
	BALB/c	B6	BALB.B10
None	0.10	0.02	0.03
PPD (25 μg/ml)	0.21	0.02	0.04
CF (25 μg/ml)	0.35	0.03	0.04
BCG extract (25 μg/ml)	0.23	0.02	0.03
Whole BCG (25 μg/ml)	0.16	0.04	0.04

[a] IL-4 titers in 72-h spleen cell culture supernatant.

RESULTS

IL-2 production in spleen cell cultures from BCG-infected mice. In vitro IL-2 production was evaluated 4 weeks after infection with *M. bovis* BCG. Preliminary experiments showed that IL-2 production peaked at 24 h and rapidly declined afterwards. Spontaneous IL-2 production was highest in B6 mice. BALB/c cells produced low levels of IL-2 in response to all antigens; B6 and BALB.B10 cells produced considerably more IL-2, especially in response to secreted CF antigens and PPD (Table 1). Thus, IL-2 production in response to PPD, CF, and the 30-32-kDa antigen was 5- to 10-fold higher in B6 than in BALB/c mice. Responses to extract and whole BCG were only three- and twofold higher, respectively, in B6 mice than in BALB/c mice. BALB.B10 cells produced four times more IL-2 than BALB/c cells in response to PPD and CF and two times more in response to BCG extract and the 30-32-kDa protein. Taking into account the amount of spontaneously produced IL-2 in B6 cultures, the 65-kDa Hsp induced the highest IL-2 titers in BALB/c cells. The addition of the anti-IL-2 MAb S4B6 (rat Ig2a anti-IL-2 [28]) completely abrogated the growth activity observed on CTLL-2 cells, indicating that genuine IL-2 (and not IL-4) was measured (data not shown). Further evidence was provided by the fact that CTLL-2 cell growth could not be neutralized with the anti-IL-4 MAb 11B11. Since IL-6 has been reported to enhance cytotoxic T lymphocyte (CTL) responses (40) and in view of the substantial IL-6 levels found in these culture supernatants (see below), we also examined the effect of *E. coli*-derived recombinant human IL-6 (a gift of W. Seebald, Würzburg, Germany) on CTLL-2 proliferation. IL-6 titers for doses up to 7.5×10^5 U/ml did not stimulate growth of CTLL-2 cells (data not shown).

IFN-γ production in spleen cell cultures. We have previously reported that spleen cells from BCG-infected BALB/c mice produce four times less IFN-γ in response to PPD and the purified 32-kDa antigen (antigen 85A) than spleen cells from B6 or BALB.B10 mice (20). As shown in Table 2, B6 and BALB.B10 spleen cells also produced more IFN-γ than BALB/c cells did in response to whole CF. Lower IFN-γ titers were found after stimulation with BCG extract or whole BCG, and the titers were more or less similar for the three strains. In contrast, the 65-kDa Hsp induced higher IFN-γ titers in spleen cells from BALB/c and BALB.B10 mice than in those from B6 mice. The IFN detected in antigen-stimulated culture supernatants was characterized as genuine IFN-γ because 90 to 95% of its antiviral activity could be neutralized by using the potent MAb F3 directed specifically against murine IFN-γ (18). Furthermore, supernatants from unstimulated BALB.B10 spleen cells contained substantial amounts of spontaneously produced IFN, which was characterized as 100% acid-stable IFN-α/β (data not shown).

IL-4 production in spleen cell cultures. It was first verified that the various antigenic preparations did not interfere as such with the IL-4 assay (data not shown). Preliminary experiments indicated that IL-4 activity peaked in spleen cell cultures after 72 h. Unstimulated BALB/c spleen cells produced significant amounts of IL-4, and antigenic stimulation increased these levels. IL-4 titers were highest in response to whole CF (Table 3). In contrast, no IL-4 activity was found in spleen cell supernatants from B6 or BALB.B10 mice. The activity measured in the supernatants was genuine IL-4 because the enhancing effect on Ia expression could be completely abrogated with MAb 11B11 (rat IgG1 anti-IL-4).

TABLE 2. IFN-γ production in spleen cell cultures from BCG-infected mice

Antigen (concn)	IFN-γ level in mice[a]					
	BALB/c		B6		BALB.B10	
	Titer	IU	Titer	IU	Titer	IU
None	<1	<6	<1	<6	2.62 ± 0.17	18
PPD (25 μg/ml)	3.16 ± 0.56	27	4.95 ± 0.38	93	4.58 ± 0.52	72
CF (25 μg/ml)	3.25 ± 0.66	28	5.45 ± 0.75	131	4.83 ± 0.38	85
BCG extract (25 μg/ml)	2.96 ± 0.58	23	3.25 ± 0.25	28	3.58 ± 0.29	36
Whole BCG (25 μg/ml)	2.41 ± 0.14	16	3.44 ± 0.55	32	3.04 ± 0.76	25
30-32-kDa protein (25 μg/ml)	1.79 ± 0.40	10	3.53 ± 0.58	35	3.62 ± 0.69	37
65-kDa Hsp (25 μg/ml)	3.00 ± 0.35	24	1.62 ± 0.17	9	3.47 ± 0.30	33
LPS (10 μg/ml)	<1	<6	<1	<6	<1	<6

[a] Titers are expressed as mean $\log_2$ ± SD of quadruplicate assays in 72-h culture supernatants from mice infected 4 weeks previously with BCG.

TABLE 4. IL-6 production in spleen cell cultures from BCG-infected mice

Antigen[a]	Mean IL-6 titer (U/ml)[b]		
	BALB/c	B6	BALB.B10
None	656 ± 34	204 ± 21	596 ± 28
PPD	2,298 ± 213	3,459 ± 789	7,280 ± 318
CF	2,551 ± 237	7,539 ± 531	12,245 ± 1,012
BCG extract	5,698 ± 465	6,192 ± 782	16,666 ± 1,423
Whole BCG	11,827 ± 1,174	2,151 ± 171	12,181 ± 987
30-32-kDa protein	534 ± 43 (NS)[c]	901 ± 87	2,638 ± 111
65-kDa Hsp	578 ± 170 (NS)	224 ± 32 (NS)	1,135 ± 153
LPS	1,260 ± 98	916 ± 78	1,584 ± 125

[a] Antigens were used at a final concentration of 25 μg/ml; LPS was used at 10 μg/ml.
[b] IL-6 titers (±SD) in 72-h spleen cell culture supernatant.
[c] NS, not significantly different from control values in unstimulated cultures.

IL-5 production in spleen cell cultures. By using an immunoenzymatic assay with the TRFK4 and TRFK5 anti-IL-5 MAbs (38), IL-5 could not be detected in any of the culture supernatants tested 4 weeks after primary BCG infection.

IL-6 production in spleen cell cultures. Significant levels of IL-6 were detected in spleen cell culture supernatants from BCG-infected mice (Table 4). The growth activity was characterized as genuine IL-6 because it could be neutralized completely by the anti-IL-6 MAb 6B4 (42) (a kind gift of J. Van Snick). We previously reported that non-T cells are the main IL-6-producing cells in BCG-infected mice (21). All three strains produced substantial amounts of IL-6. In BALB/c cells, maximal production was induced with whole BCG and BCG extract, whereas little IL-6 was induced with PPD and CF. In B6 and BALB.B10 cultures, on the other hand, PPD and CF were potent IL-6 inducers. Interestingly, stimulation with the 30-32-kDa antigen also resulted in increased IL-6 levels in B6 and in BALB.B10 cells. LPS induced comparable IL-6 titers in the three mouse strains.

TNF-α production in spleen cell cultures. Elevated levels of TNF-α were found in spleen cell culture supernatants from BCG-infected mice. The highest TNF-α levels were obtained after stimulation with whole BCG or BCG extract, and these levels were comparable in all three strains. On the other hand, TNF-α production in response to CF and PPD was two- to threefold higher in B6 and BALB.B10 cells than in BALB/c cells (Table 5). Biological assays on TNF-sensitive L929 indicator cells failed to detect TNF activity in these supernatants, suggesting the presence of TNF inhibitors (40a).

Bacterial proliferation in the spleen of BCG-infected mice. Bacterial proliferation, calculated from the number of BCG CFU found in the spleen 2, 4, 6, and 8 weeks after a primary infection with BCG, was comparable in the three mouse strains (Fig. 1a). A marked difference was observed, however, in the development of protective immune memory during primary infection. In BCG-vaccinated B6 and BALB.B10 mice, a second BCG inoculum was eliminated more rapidly than the first inoculum, reflected by a 5- to 10-fold lower number of BCG CFU in the spleens of vaccinated mice compared with that in the spleens of primary-infected mice (Fig. 1b). BCG vaccination in BALB/c mice was not effective, as CFU values in primary-infected and rechallenged mice were comparable. In some experiments, CFU values were even higher in vaccinated than in primary-infected BALB/c mice.

TABLE 5. TNF-α production in spleen cell cultures from BCG-infected mice

Antigen (concn)	TNF-α (pg/ml)[a]		
	BALB/c	B6	BALB.B10
None	152 ± 12	180 ± 14	76 ± 8
PPD (25 μg/ml)	330 ± 34	794 ± 69	1,015 ± 95
CF (25 μg/ml)	460 ± 47	860 ± 95	1,115 ± 120
BCG extract (25 μg/ml)	745 ± 52	754 ± 68	888 ± 74
Whole BCG (25 μg/ml)	3,200 ± 250	2,700 ± 240	3,200 ± 310

[a] TNF-α levels (±SD) in 24-h culture supernatants.

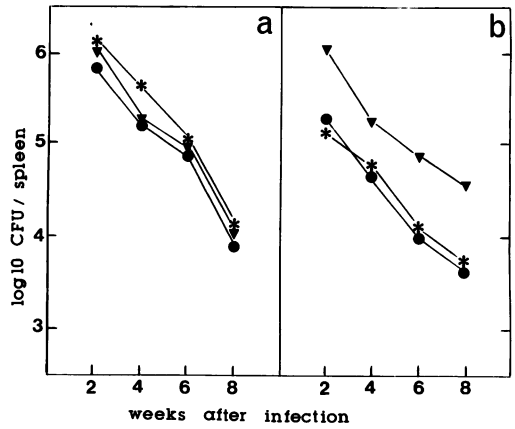

FIG. 1. Number of BCG CFU recovered from the spleen of primary-infected (a) or rechallenged (b) BALB/c (▼), B6 (●), or BALB.B10 (*) mice, as measured 2, 4, 6, and 8 weeks after BCG infection. Results are expressed as $\log_{10}$ CFU per spleen (mean of at least four mice per group). l

DISCUSSION

Acquired protective immunity against mycobacteria is the result of interactions between specifically sensitized T cells and macrophages harboring the infectious organism. Little is known about the precise antigens involved in protective immunity against these pathogens, but living organisms have been reported to be more effective in the generation of specific acquired resistance than dead mycobacterial preparations (33). An increasing number of authors have therefore argued that secreted antigens, present in large amounts in mycobacterial CFs, rather than intracellular components may be essential for the induction of protective immunity (2, 16, 34).

In this article, we have analyzed cytokine production in response to secreted CF antigens and to whole BCG bacilli and BCG extract by spleen cells from three susceptible mouse strains infected intravenously with *M. bovis* BCG. B6 and BALB.B10 mice (both *H-2*^{b}) were found to produce 5- to 10-fold more IL-2 and IFN-γ in response to PPD, CF, and the extracellular 30-32-kDa antigen (also called fibronectin-binding antigen 85, which is the major protein in early BCG CFs) than BALB/c mice (*H-2*^{d}). On the other hand, BCG-infected BALB/c spleen cells demonstrated substantial IL-4 production, maximal in response to CF antigens, whereas B6 or BALB.B10 cells did not produce any IL-4. IL-2 and IFN-γ production in response to BCG extract and to whole

BCG was of lower magnitude and more or less comparable for the three strains. IL-2 and IFN-γ production in response to the 65-kDa Hsp was higher in BALB/c than in B6 mice. These data suggest that secreted mycobacterial CF antigens selectively induce a strong TH_1 response in BCG-infected mice of the *H-2*^{b} haplotype, whereas in BALB/c mice with the *H-2*^{d} haplotype, TH_1 cells are mainly reactive against intracellular components and, moreover, a substantial TH_2 cell activation can be observed.

Previous papers by Boom et al. and Roch and Bach on cytokine production in BCG-infected mice have reported on the failure to detect IL-4 activity in BALB/c mice (3, 36). These data are not necessarily in contradiction with our findings, as both authors examined cytokine secretion of lymph node cells, which did not produce IL-4 in our experiments either (data not shown). Second, the infection route was different (subcutaneous versus intravenous in our study). On the other hand, our data do extend the initial report of Brett and Ivanyi, showing that mice intraperitoneally infected with *Mycobacterium tuberculosis* demonstrate splenic IL-4 mRNA, especially on a BALB/c background (4).

The exact genetic mechanisms that determine the preferential activation of this CF-reactive TH_1 subset in *H-2*^{b} mice are not yet defined, but *H-2*-linked genes are well known to influence the immune response towards mycobacterial infection. Indeed, the granulomatous reaction to *Mycobacterium lepraemurium* (5), antibody formation after hyperimmunization with *M. tuberculosis* extract (22) or CF (44), and antibody production as well as IFN-γ secretion after infection with live BCG (19, 20) are all influenced to some extent by genes from the MHC complex. It is tempting to speculate that differences in MHC antigen expression at the surface of the accessory cell are reflected by differences in antigen-presenting capacity and monokine secretion, which subsequently might result in differential T-helper-cell subset activation. Interestingly, the levels of the monokines IL-6 and TNF-α in PPD- and CF-stimulated cultures were also higher in mice with the *H-2*^{b} haplotype. Along this line, MHC control of T-helper-cell subset activation has been observed in a completely different experimental system, i.e., in mice immunized with type IV human collagen (30).

These results are reminiscent of another infectious model, i.e., murine leishmaniasis, in which a clear dichotomy of T-cell response has been observed in BALB/c and B6 mice infected with the protozoal parasite *L. major* (17, 24). Some fundamental differences between the two experimental systems must, however, be emphasized. Whereas the susceptibility to cutaneous leishmaniasis is clearly different for susceptible BALB/c and resistant B6 mice, BALB/c and B6 mice have similar susceptibilities to *M. bovis* BCG infection, both expressing the susceptible allele of the *bcg* gene (39). Bacterial multiplication of the BCG vaccine is observed during the first weeks of infection, and later BCG is gradually eliminated, in both strains, by acquired cell-mediated immune effector mechanisms. In this article, we have described, however, striking differences in the development of long-term protective memory in these BCG-vaccinated mouse strains: B6 and BALB.B10 mice are capable of a rapid clearance of a second challenge inoculum of BCG 2 months after primary infection, whereas BALB/c mice eliminate this second inoculum at a rate no higher than the rate at which they eliminate the first infection.

Recently, Yamamura et al. analyzed cytokine profiles in leprosy skin biopsy specimens by polymerase chain reaction amplification of extracted mRNA (45). In tuberculoid leprosy lesions, mRNAs for IL-2 and IFN-γ were most evident, whereas in lepromatous leprosy lesions, mRNAs for IL-4 and IL-5 predominated. Interestingly, tuberculoid lesions also contained more TNF-α and IL-6 mRNA and lepromatous lesions contained more IL-10. BCG-infected BALB/c mice, therefore, appear to have a spleen cell cytokine secretion pattern similar to that of lepromatous leprosy patients, whereas B6 and BALB.B10 cytokine profiles resemble those found in tuberculoid leprosy patients. Interestingly, in leprosy, human leukocyte antigen-linked genes have been reported to control not the susceptibility per se but the type of leprosy (41).

Optimal proliferation of TH_1 and TH_2 subsets is thought to depend on distinct antigen-presenting cells and their respective costimulatory factors (13). Moreover, amplification of the respective T-helper subsets is controlled by counterregulatory cytokines, TH_2 proliferation being directly inhibited by the TH_1 lymphokine IFN-γ and cytokine secretion of TH_1 cells being inhibited by the cytokine IL-10, probably via inhibition of monokine (IL-1, IL-6, TNF-α) secretion and class II MHC expression on antigen-presenting cells (9, 29). We hypothesize that at the onset of BCG infection, extracellular CF components will elicit elevated production of IL-6 and TNF-α in *H-2*^{b} mice, which in turn will lead to TH_1 activation, IFN-γ secretion, increased IL-6 and TNF production by IFN-γ-activated macrophages, and subsequent inhibition of IL-4 production by TH_2 cells. In *H-2*^{d} mice, on the other hand, CF antigens will stimulate antigen-presenting cells to a lesser extent, leading to lower IL-2 and IFN-γ levels and resulting in substantial IL-4 production. Furthermore, high TH_1 activity against CF antigens seems to correlate with protective immunity. Analysis of the cytokine secretion early during BCG infection could give us more insight into the mechanisms by which MHC genes exert this regulatory control.

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