INFECTION AND IMMUNITY, Oct. 1995, p. 4170–4173 Vol. 63, No. 10
0019-9567/95/$04.00+0

Influence of the *Bcg* Locus on Macrophage Response to the Dimorphic Fungus *Candida albicans*

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Received 16 February 1995/Returned for modification 5 April 1995/Accepted 26 June 1995

The *Bcg*/*Ity*/*Lsh* gene (candidate *Nramp*) controls natural resistance to several parasites, such as *Mycobacterium bovis*, *Leishmania donovani*, and *Salmonella typhimurium*. Using two macrophage (Mϕ) cell lines (B10R and B10S) derived from mouse strains congenic at *Bcg*, we found that Mϕs from resistant mice (B10R Mϕs) act more effectively against the two morphogenetic forms of the dimorphic fungus *Candida albicans* compared with Mϕs from susceptible mice (B10S Mϕs). Moreover, when assessed for tumor necrosis factor secretion in response to the hyphal form of *C. albicans*, B10R Mϕs are significantly more effective at expressing this secretory function than are B10S Mϕs, closely resembling the trend of response to lipopolysaccharide. Overall, these results provide insight into the influence of the *Bcg* locus on the Mϕ response to *C. albicans*.

The ability of the host to resist infection with a wide range of viral, bacterial, and parasitic pathogens is influenced by genetic factors (28–30). In mice, the innate resistance to infection with several mycobacterial species, such as *Mycobacterium lepraemurium*, *Mycobacterium bovis* (BCG), and *Mycobacterium intracellulare*, is under the control of a single, autosomal, dominant gene, located on chromosome 1, which exists in two allelic forms: Bcg^r (resistant) and Bcg^s (susceptible) (13). The *Bcg* locus is identical to those of two other genes, known as *Ity* (20) and *Lsh* (7), which control natural resistance to infection with other intracellular parasites, *Salmonella typhimurium* and *Leishmania donovani*, respectively (29). It is well established that the effector cell responsible for the phenotypic expression of the gene is the resident tissue macrophage (Mϕ), since early resistance to BCG has been shown to be bone marrow derived, silica sensitive, and radioresistant (14). The mechanism by which Bcg^r Mϕs exert enhanced cytocidal or cytostatic activity is not clear. However, they appear superior to Bcg^s Mϕs in the expression of surface markers (Ia and Acm-1 antigens) associated with activation (9), production of toxic oxygen and nitrogen radicals in response to a stimulus such as gamma interferon (IFN-γ) or BCG infection (2, 30), and in the rapid (within 30 min) upregulation of the early gene KC, which codes for a neutrophil-specific chemoattractant in response to mycobacterial lipoarabinomannan (23). Thus, it seems likely that the product of the *Bcg* gene is a molecule involved in Mϕ priming and activation.

Candida albicans is an opportunistic pathogen whose relevance has increased in recent years because of the augmented incidence of candidiasis in immunocompromised hosts (19). *C. albicans* can be considered to be a facultative intracellular pathogen, since it may survive within Mϕs and grow out of these cells by germination (32). Under both in vitro and in vivo circumstances, *C. albicans* is in fact known to undergo dimorphic transition from the yeast (Y-candida) to the hyphal (H-candida) form (19). Using a bone marrow-derived Mϕ cell line (ANA-1), we have previously demonstrated that H-candida provides a stimulatory signal to these cells (5). In particular, ANA-1 Mϕs respond to H-candida, but not to Y-candida, with increased tumor necrosis factor (TNF) mRNA and secretion of TNF alpha (TNF-α) protein (4, 5). To ascertain whether this phenomenon may be controlled by the *Bcg* gene, we used two previously established (21) Mϕ cell lines, hereafter referred to as B10S Mϕ and B10R Mϕ, being derived from C57BL/10A.Bcg^s (B10A.Bcg^s) and C57BL/10A.Bcg^r (B10A.Bcg^r) mice, respectively. Our results demonstrate that differences between the two Mϕ cell lines in terms of anti-*Candida* activity and TNF-α production in response to H-candida exist. Thus, the data suggest that the *Bcg* gene product may be involved in resistance against *C. albicans*, whose interaction in the host may be particularly complex because of its dimorphic properties.

B10S and B10R Mϕ cell lines were derived from the bone marrow of B10A.Bcg^s and B10A.Bcg^r mice, respectively, by infection with J2 retrovirus (21). The two cell lines were maintained in RPMI 1640 medium supplemented with glutamine (4 mM), gentamicin (50 μg/ml), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Lagan, United Kingdom), hereafter referred to as complete medium. These Mϕ cell lines faithfully resemble differentiated fresh bone marrow Mϕs in culture. No significant phenotypical and functional differences between Mϕ cell lines and their fresh bone marrow Mϕ counterparts have been observed (21).

C. albicans CA-6 was isolated from a clinical specimen and grown as previously described (5). To obtain H-candida, a pure yeast-form population was harvested, washed twice in saline, resuspended in complete medium, dispensed in culture plates, and incubated at 37°C. More than 98% of the microorganisms showed hyphal form after 3 h of incubation, as detailed elsewhere (26). The H-candida preparations were tested for endotoxin contamination, and only those preparations containing less than 0.05 ng of endotoxin per ml (as assessed by the *Limulus* amebocyte assay) were used.

For the evaluation of phagocytic activity, B10R or B10S Mϕs (10^6 cells per ml) were incubated with heat-inactivated Y-

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candida at an effector-to-target (E:T) ratio of 1:20 for 1 h at 37°C in 5% CO_2. Mϕs were then separated from nonphagocytized target cells by centrifugation on a fetal bovine serum gradient. Y-candida uptake was directly evaluated in Giemsa-stained preparations (27). A minimum of 200 Mϕs was scored, and any cell containing one or more Y-candida cells was counted as phagocytic. The phagocytosis index was calculated as follows: total number of phagocytized yeasts/total number of phagocytic cells.

Anti-*Candida* activity was evaluated both under basal conditions and in the presence of IFN-γ (obtained from Genentech, South San Francisco, Calif.) with or without lipopolysaccharide (LPS) from *Escherichia coli* (serotype O128:B12; Sigma Chemical Company, St. Louis, Mo.). B10R and B10S Mϕs were exposed or not for 18 h to IFN-γ (100 U/ml), with or without the addition of LPS (10 ng/ml), prior to assessment for anti-Y-candida or anti-H-candida activity. For the determination of anti-Y-candida activity, Mϕs (10^6 cells per ml) were plated (0.1 ml per well) in 96-well plates (Corning Glass Works, Corning, N.Y.) and infected with 0.1 ml of *C. albicans* (10^5 yeasts per ml). After 3 h of incubation at 37°C in 5% CO_2, the plates were vigorously shaken and Triton X-100 (0.1% final concentration) was added to the wells. Serial dilutions from each well were then made in distilled water and were plated (quadruplicate samples) on Sabouraud dextrose agar. The number of colony-forming units (CFU) was determined after 24 h of incubation at 37°C. Control cultures consisted of *C. albicans* incubated without effector cells. The results were expressed as a percentage of anti-Y-candida activity according to the formula [1 − (CFU of experimental group/CFU of control culture)] × 100. The determination of anti-H-candida activity was assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) colorimetric assay (15). Briefly, Y-candida microorganisms, resuspended in complete medium at the concentration of 4×10^5 yeasts per ml, were plated (0.1 ml per well) in 96-well flat-bottom microtiter plates (Corning Glass Works) and incubated at 37°C for 3 h to permit germination. Supernatants were then discarded, and 2×10^5 Mϕs per 0.1 ml were added. After an additional 3 h of incubation at 37°C in 5% CO_2, the supernatants were aspirated from the wells and 0.1 ml of 0.1% Triton X-100 in saline was added to lyse the Mϕs. After three washes with 0.2 ml of distilled water, 0.1 ml of RPMI 1640 medium containing 0.5 mg of MTT (Sigma Chemicals) per ml was added to the wells and the plates were incubated for an additional 4 h at 37°C. Then, the plates were centrifuged, the supernatants were discarded, and the pellets were resuspended in 0.1 ml of acidified isopropyl alcohol. The A_{540} and A_{690} per well were determined with an automated microplate reader (Titertek Multiscan; Flow Laboratories, McLean, Va.). A well containing only isopropanol was used as a blank. Control wells, containing H-candida but not Mϕs, were included in each experiment. Anti-H-candida activity was calculated by using the formula [1 − (OD of experimental wells/OD of control wells)] × 100, where OD is the optical density.

Cell-free supernatants were prepared for TNF activity evaluation, as described elsewhere (5). Briefly, B10R and B10S Mϕs were incubated with H- or Y-candida (E:T = 1:1) or with LPS (1 μg/ml) for 3 h at 37°C in 5% CO_2. Cell-free supernatants were then harvested and assayed for TNF activity. The quantitation of TNF activity was determined by a bioassay with L-929 cells as target cells, as previously described (24). Briefly, L-929 cells were seeded into 96-well flat-bottom plates (4×10^4 cells per well) and were incubated for 24 h at 37°C. The spent medium was then removed and replaced with test sample or standard TNF preparations containing actinomycin D (3 μg/ml). After 20 h of incubation, plates were stained with 0.5% crystal violet in 20% methanol for 15 min and were washed in tap water. After drying, the A_{450} was determined with a Titertek Multiscan plate reader. All determinations of TNF activity in test samples were compared with commercially available preparations of TNF with known titers; the results were expressed as units per milliliter. The significance of the data was evaluated by Student's *t* test.

TABLE 1. Comparison of phagocytic and anticandida activities by B10R and B10S Mϕs

Activity	E:T ratio	Mean % activity ± SEM for cell line[a]	
		B10R Mϕ	B10S Mϕ
Phagocytic[b]	1:20	60.2 ± 3.5 (4)	55.3 ± 4.1 (3)
	1:5	50.1 ± 2.3 (3)	48.4 ± 3.0 (3)
Anti-Y-candida[c]	10:1	40.1 ± 3.0	19.6 ± 2.8
	5:1	28.2 ± 2.7	10.5 ± 2.6
	1:1	16.2 ± 3.1	7.3 ± 1.9
Anti-H-candida[d]	5:1	33.2 ± 2.7	22.6 ± 3.4
	2:1	19.1 ± 3.5	12.7 ± 3.2
	1:1	10.9 ± 4.1	6.3 ± 2.7

[a] Data represent the means ± the standard errors of the means of six separate experiments. The value for each experiment is the mean of quadruplicate determinations.

[b] Mϕs were incubated with heat-inactivated Y-candida at the indicated E:T ratios for 30 min. The percentage of phagocytic cells was then evaluated microscopically. The phagocytosis index for each result is indicated in parenthesis.

[c] Mϕs were incubated with Y-candida cells at the indicated E:T ratios for 3 h. The CFU assay was performed as detailed in the text. The percentage of anti-Y-candida activity was evaluated according to the formula [1 − (CFU of experimental group/CFU of control culture)] × 100. For results with B10R Mϕs, $P < 0.01$ (relative to values for B10S Mϕs).

[d] Mϕs were incubated with H-candida cells at the indicated E:T ratios for 3 h. The MTT colorimetric assay was then performed as detailed in the text. The percentage of anti-H-candida activity was evaluated according to the formula [1 − (OD of experimental well/OD of control wells)] × 100. For results with B10R Mϕs, $P < 0.05$ (relative to values for B10S Mϕs).

All studies performed with BCG, *S. typhimurium*, or *L. donovani* as targets showed that the *Bcg*/*Ity*/*Lsh* locus did not influence phagocytosis of the pathogens, which was exerted to comparable extents by resistant and susceptible Mϕs (16, 21, 31). Similarly, we found no differences between the phagocytosis of Y-candida by B10R Mϕs and that by B10S Mϕs. In fact, as shown in Table 1, 60% of the B10R Mϕs were phagocytic, with a phagocytosis index of 4, while B10S Mϕs were 55% phagocytic, with a phagocytosis index of 3. We then examined the anti-*Candida* activities of B10R and B10S Mϕs. The results are shown in Table 1. Y-candida activity, evaluated as CFU inhibition, was 40% with B10R Mϕs and 20% ($P < 0.01$) when B10S Mϕs were employed. Significant differences were also observed in terms of anti-H-candida activity, as assessed by the MTT colorimetric assay. The levels of activity exerted by B10R and B10S Mϕs were 33.2% and 22.6% ($P < 0.05$), respectively (Table 1). A similar trend of results was consistently observed when different E:T ratios were employed (Table 1).

It is known that IFN-γ, alone or in combination with LPS, is capable of inducing or enhancing antimicrobial activity (3, 8). In order to ascertain the effects of such treatments on B10R and B10S Mϕ-mediated anti-*Candida* activities, Mϕs were stimulated with IFN-γ (100 U/ml), alone or in combination with LPS (10 ng/ml), for 18 h prior to evaluation of anti-*Candida* activity. We found that both treatments resulted in enhanced anti-Y-candida activities (Table 2). Treatment with

TABLE 2. Effects of various treatments on anticandida activities of B10R and B10S Mϕs

Cell line	Treatment[a]	Mean % activity ± SEM for[b]:	
		Anti-Y-candida[c]	Anti-H-candida[d]
B10R Mϕ	None	40.1 ± 3.0	33.2 ± 2.7
	IFN-γ	50.8 ± 2.5*	40.5 ± 3.3*
	IFN-γ + LPS	64.1 ± 4.5**	46.3 ± 4.0*
B10S Mϕ	None	19.6 ± 2.8	22.6 ± 3.4
	IFN-γ	26.2 ± 3.6*	31.3 ± 2.9*
	IFN-γ + LPS	32.4 ± 3.8**	36.5 ± 3.1**

[a] Mϕs were unstimulated or stimulated with IFN-γ (100 U/ml) with or without LPS (10 ng/ml) for 18 h and then were assessed for anti-Y-candida and anti-H-candida activities.

[b] Data represent the means ± the standard errors of the means of four separate experiments. The value for each experiment is the mean of quadruplicate determinations. *, $P < 0.05$ (for treated versus untreated Mϕs). **, $P < 0.01$ (for treated versus untreated Mϕs).

[c] Mϕs were incubated with Y-candida at an E:T ratio of 10:1 for 3 h. The CFU assay was performed as detailed in the text. The percentage of anti-Y-candida activity was evaluated according to the formula [1 − (CFU of experimental group/CFU of control culture)] × 100.

[d] Mϕs were incubated with H-candida at an E:T ratio of 5:1 for 3 h. The MTT assay was then performed as detailed in the text. The percentage of anti-H-candida activity was evaluated according to the formula [1 − (OD of experimental well/OD of control wells)] × 100.

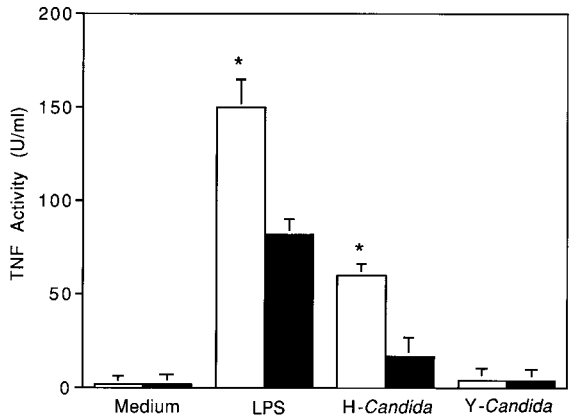

FIG. 1. TNF secretion by B10R and B10S Mϕs upon stimulation with H-candida, Y-candida or LPS. B10R (□) and B10S (■) Mϕ were incubated in the presence of Y-candida (E:T = 1:1), H-candida (E:T = 1:1) or LPS (1 μg/ml) for 3 h. Cell-free supernatants were harvested and assessed for TNF activity (see text). Data are the means ± the standard errors of the means (bars) of six separate experiments. *, $P < 0.01$ (for B10R versus B10S Mϕs).

IFN-γ plus LPS was more effective than that with IFN-γ alone for both B10R and B10S Mϕs. However, the difference in efficiencies observed with B10R and B10S Mϕs under basal conditions was retained upon stimulation, in that the anti-Y-candida activities of B10S Mϕs stimulated with IFN-γ plus LPS reached levels comparable to those of B10R Mϕ under basal conditions. Similar results were observed when anti-H-candida activities were evaluated (Table 2).

It has been previously demonstrated that microbial products elicit differential biomolecular responses in murine immune cells, depending upon their resistant or susceptible genotype. In particular, elevated levels of IFN-γ were produced by *Ity*^{r} spleen cells compared with *Ity*^{s} spleen cells, following in vitro stimulation with *S. typhimurium* (22). Moreover, a rapid up-regulation of KC gene expression was observed in *Bcg*^{r} Mϕs, but not in *Bcg*^{s} Mϕs, upon stimulation with mycobacterial lipoarabinomannan (23). On the bases of these reports and our previous demonstration that ANA-1 Mϕs are induced to secrete high levels of TNF-α in response to stimulation with H-candida but not with Y-candida (5), we analyzed the possible effect of the *Bcg* gene on the Mϕ secretory response to *C. albicans*. Hence, B10R and B10S Mϕs were exposed to H- and Y-candida for 3 h, at an E:T ratio of 1:1, and TNF-α levels were evaluated in cell-free supernatants. As depicted in Fig. 1, high levels (90 U/ml) of TNF-α were found in the supernatants of B10R Mϕs exposed to H-candida, while barely detectable levels of TNF-α were observed in the supernatants of B10S Mϕs. Y-candida alone was not able to stimulate Mϕs to produce TNF-α. When Mϕs were treated with LPS, higher TNF levels were found in B10R Mϕ supernatants than in B10S Mϕ supernatants.

The two B10S and B10R Mϕ cell lines, established from *Bcg*^{s} and *Bcg*^{r} mice, retain the functional differences linked to *Bcg* gene expression (21). Here, we show that B10R Mϕs, known to express higher levels of growth-inhibiting activity towards mycobacteria than do B10S Mϕs (21), are also capable of more effectively acting against *C. albicans*. Nevertheless, the two cell lines show phagocytic activities against *C. albicans* that are comparable to each other and similar to those observed when freshly isolated tissue Mϕs and other microbial targets are used (16, 31). The fact that functional differences between B10R and B10S Mϕs are observed whether phagocytable Y-candida or nonphagocytable H-candida microorganisms are employed as target cells suggests the involvement of the *Bcg* gene product in the fulfillment of both intracellular and extracellular anti-*Candida* activities by Mϕs. Treatment with IFN-γ alone or with LPS resulted in increased anti-*Candida* activity, although the efficacy of B10S Mϕs never reached that of B10R Mϕs. Furthermore, the *Bcg* locus appears to be involved in the control of Mϕ secretory functions. In fact, sustained TNF-α secretion was observed in B10R Mϕ, in response both to H-candida and to LPS, while B10S Mϕ showed a weak secretory response to such stimuli. This emphasizes the potential involvement of the *Bcg* locus in the pathogenesis of candidiasis. In fact, although unable to directly mediate toxic effects against *C. albicans* (reference 17 and data not shown), TNF plays a pivotal role in the outcome of candidiasis (17).

Intensive research has been carried out to identify the Bcg/Ity/Lsh gene product and to understand the mechanism(s) allowing its phenotypic expression. Vidal and coworkers (33) isolated a candidate gene, *Nramp* (natural resistance-associated Mϕ protein). On the basis of the presence of a small consensus motif showing identity with nitrate transporters, the authors hypothesize that *Nramp* functions as a nitrate and/or nitrite concentrator in phagolysosomes. However, this hypothesis does not explain the results documenting a role for *Nramp* in Mϕ priming and activation. The isolation and sequencing of the 5′ region of *Nramp* revealed a domain that contains three protein kinase C phosphorylation sites as well as a putative *Src* homology 3 (SH3) binding domain (1). Such a domain occurs as related sequences in tyrosine kinases (18) and is believed to mediate a protein-protein interaction obligatory for signal transduction (6), thus accounting for a wide range of *Nramp* functions, which include signal transduction and subsequent Mϕ priming and activation. Our data seem to support both the nitrate transporter and the transduction protein hypotheses. In fact, since the role played by nitric oxide in the killing of *C. albicans* is documented (10), the differences observed in anticandida activity may be attributed to a defect in the delivery of nitrates to the phagolysosomes, in the case of Y-candida killing, or to the extracellular environment, in the case of H-candida growth inhibition. Moreover, data on the effect of the

Bcg gene on TNF-α production are consistent with the hypothesis that *Nramp* regulates early events in priming and activation of Mϕs (23).

In addition to suggesting a role for *Nramp* in controlling the Mϕ response to *C. albicans*, our data may be useful in understanding the mechanisms through which H-candida stimulates TNF-α production. The demonstration that the myeloid-specific tyrosine kinase Hck is involved in signal transduction for TNF-α release in murine Mϕs (11), together with the knowledge that *Nramp* contains a tyrosine kinase binding domain (1), suggests that the TNF-α production observed in Mϕs in response to H-candida may be accomplished via tyrosine kinase.

Finally, Formica et al. (12) found that fibrinogen and fibronectin stimulate TNF-α production to a greater extent in Mϕs from the resistant strain than in Mϕs from the susceptible strain. Since fibrinogen and fibronectin interactions with Mϕs are via members of the β_1-β_2 integrin family (25), a hypothetical role for integrin involvement in H-candida interaction with Mϕs may be proposed.

In conclusion, our data provide the first evidence that the *Bcg* locus influences Mϕ response to the dimorphic pathogen *C. albicans*, offering a novel tool for future studies on H-candida and Mϕ interaction.

We thank Eileen Zannetti for editorial and secretarial assistance.

This study was supported by VIII Progetto AIDS (9305-44), Rome, Italy.

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