Nitrite production by macrophages derived from BCG-resistant and -susceptible congenic mouse strains in response to IFN-γ and infection with BCG

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SUMMARY

Reactive nitrogen intermediates (RNI) have been implicated in the interferon-γ (IFN-γ)-induced anti-microbial action of macrophages against a wide variety of pathogens. We have been studying the production of NO_2^- by macrophage lines derived from the bone marrow of either B10.A (*Bcg^s*) strain mice (B10S cell lines), or their congenic BCG-resistant partners of the B10A.*Bcg^r* (*Bcg^r*) strain (B10R cell lines). We have discovered that there is a significant difference in the production of NO_2^- of B10S compared with B10R macrophages in response to IFN-γ. By 48 hr following treatment with 10 U/ml IFN-γ, B10R macrophages had produced an approximately threefold higher level of NO_2^- than B10S macrophages. Similar results were obtained when experiments were performed with total splenic cells harvested from the spleens of B10.A.*Bcg^r* and B10.A strain mice. The bacteriostatic activity, as assessed by the [^{3}H]uracil incorporation by *Mycobacterium bovis* BCG, was higher in B10R macrophages compared to B10S macrophages. The bacteriostatic activity of B10R and B10S macrophages correlated with the amount of nitric oxide produced by the macrophages. The anti-mycobacterial activity was inhibited by N^{8}MMLA, a specific inhibitor of nitrite and nitrate synthesis from L-arginine. Addition of L-arginine to IFN-γ-stimulated macrophages in the presence of N^{8}MMLA restored nitrite production and bacteriostatic activity of macrophages. Northern blot analysis of macrophage nitric oxide synthase (iNOS) revealed that the difference in NO_2^- production by IFN-γ-treated B10S and B10R lines was reflective of the difference in iNOS mRNA expression.

INTRODUCTION

Macrophages are the natural habitat for proliferating pathogenic mycobacteria such as *Mycobacterium tuberculosis*, *M. leprae* and *M. bovis*. Macrophages also constitute the first and essential line of defence against *Mycobacteria* and other intracellular parasites, including *Leishmania* and *Salmonella*.

Studies performed in rabbit, guinea-pig and murine models have revealed a genetically determined differential ability to control the proliferation of *Mycobacteria* within host macrophages.[1] The development of recombinant inbred strains of

Abbreviations: BCG, bacillus Calmette–Guérin; CFU, colony-forming unit; CM, complete medium; CsCl, caesium chloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; N^{8}MMLA, N^8-monomethyl-L-arginine; PMA, phorbol myristate acetate; TNF, tumour necrosis factor.

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mice from bacillus Calmette-Guérin (BCG)-susceptible and BCG-resistant parental strains confirmed an inherited pattern of resistance and susceptibility to the multiplication of *M. bovis* BCG in the spleens and livers of infected hosts.[1,2]

Parallel studies of the host response to *S. typhimurium*[3] and *L. donovani*[4] revealed a pattern of genetically controlled resistance similar to that observed in the case of BCG. Further study revealed the existence of a common locus that confers resistance to these intracellular parasites, mapped to the centromeric region of murine chromosome 1 (*Bcg/Ity/Lsh* gene)[2,4] to a region found to be synthenic with human chromosome 2q35.[5]

In vitro studies have shown that in splenic and resident peritoneal macrophages derived from *Bcg^r* and *Bcg^s* recombinant inbred and congenic strains of mice, there is a direct correlation between the in vivo resistance and susceptibility to *M. bovis* and the capacity of the macrophages to control mycobacterial multiplication.[6,7]

Macrophages derived from mice of *Bcg^r* mouse strains have been shown to secrete higher levels of reactive oxygen intermediates (ROI) than those harvested from *Bcg^s*-susceptible strains. Both class II major histocompatibility

complex (MHC) antigens and I-Aβ mRNA expression are elevated in *Bcg^r* strain macrophages when stimulated with phorbol myristate acetate (PMA) and/or interferon-γ (IFN-γ).[8,9] The precise molecular mechanism(s) by which the as yet unidentified *Bcg* gene product is able to determine the fate of *Mycobacteria*, *Salmonella* or *Leishmania* remains obscure.

A novel biochemical pathway of activation of IFN-γ-treated macrophages has been described.[10] In this pathway, the macrophage utilizes L-arginine in order to produce L-citrulline and intermediary products such as nitric oxide (NO$^.$).[11] Current investigations indicate that NO$^.$ or reactive nitrogen intermediates (RNI) are closely associated with the ability of mouse macrophages to inhibit the proliferation of *L. major*,[12] *L. enrietti*,[13] *L. donovani*,[14] *M. bovis*,[15] *M. tuberculosis*,[16] and other intracellular micro-organisms.[17] Recently, a candidate for the putative *Bcg* gene (denominated *Nramp1*) was cloned by Vidal *et al.*[18] The *Nramp* gene was found to be expressed exclusively in macrophage populations from reticuloendothelial organs and macrophage lines. Interestingly, a structural similarity was found between *Nramp* and the membrane transporter CrnA, responsible for nitrate import in the eukaryote *Aspergillus nidulans*.[18]

To facilitate and standardize our studies of macrophage functions regulated by the expression of the *Bcg* gene, we have established macrophage lines from strains of mice congenic at the *Bcg* locus as described previously in detail,[9] and designated B10R and B10S macrophage lines, respectively.

In this report, we describe studies of inducible nitric oxide synthase (iNOS) and nitrite production in response to *M. bovis* BCG and IFN-γ stimulation by B10R and B10S macrophage lines and by splenic cells of B10.A (*Bcg^s*) and B10.A.*Bcg^r* congenic mice. We found that macrophage lines produced low levels of NO$_2^-$ in the presence of live *M. bovis* BCG but its production was increased dramatically when the cells were stimulated with IFN-γ. When cell suspensions prepared from spleens harvested from congenic *Bcg^r* and *Bcg^s* strain mice were used, a significant difference in nitrite production was found when the cells were stimulated with IFN-γ. The elevated production of nitrite by B10R macrophages was consistent with the enhanced level of iNOS expression observed in response to IFN-γ.

MATERIALS AND METHODS

Reagents

N^g-monomethyl-L-arginine (N^{g}MMLA), acetate salt, was obtained from Calbiochem-Behring Corp. (La Jolla, CA); Neuman and Tytell serumless medium was obtained from Gibco (Grand Island, NY); penicillin–streptomycin was obtained from ICN Biochemicals (Costa Mesa, CA); fetal bovine serum was obtained from Hyclone (Logan, UT); and a Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Richmond, CA). Bovine serum albumin, sodium nitrite, d3-phosphoric acid, L-glutamine, saponin, sulphanilamide and *N*-(1-naphtyl) ethylenediamine were all purchased from Sigma (St Louis, MO). [5,6-^{3}H]Uracil (specific activity 34 Ci/mmol) was purchased from New England Nuclear (Boston, MA); guanidine isothiocyanate, CsCl and agarose, from Gibco-BRL (Gaithesburg, MD), Dubos oleic agar base from Difco (Detroit, MD); formaldehyde from Fisher Scientific (Fair Lawn, NJ). Recombinant IFN-γ was

purchased from Amgen Biologicals (Thousand Oaks, CA); the specific activity was $>4 \times 10^6$ U/mg of protein.

Mice

Mice, male and female, at 9–12 weeks of age, were used for the experiments. B10.A strain mice were purchased from the NCI (Frederick, MD). B10.A.*Bcg^r* mice were bred in the Montreal General Hospital Research Institute Animal Facility, Montreal, Canada under specific pathogen-free (SPF) conditions.

Macrophage cell lines

Macrophage lines were derived from the bone marrow of B10.A and B10.A.*Bcg^r* strain mice, congenic at the *Bcg* locus, as previously published.[9] The cultured cells were used in experiments when they approached confluence. Viability, determined by trypan blue exclusion, was greater than 90%.

Splenic cells

To obtain splenic cells, the spleens were ground through mesh, the red blood cells lysed with 0·85% ammonium chloride, and the suspension washed twice in phosphate-buffered saline (PBS) without calcium and magnesium.

Quantification of nitrite production by macrophages

The estimation of NO$_2^-$ in supernatants of stimulated and non-stimulated macrophages was performed by colorimetric spectrophotometry at 543 nm using the Griess reagent.[19] Background values were subtracted from those obtained for all experimental samples. Results are expressed as the nitrite production (mmol)/total protein content of macrophages estimated by the Bradford protein assay (Bio-Rad, Ontario, Canada).

Infection and/or stimulation of macrophage lines and mouse cells

To study the production of nitrite, 1×10^6 macrophages were seeded in 1-ml volumes of Neuman and Tytell medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin (complete medium; CM) in 24-well flat-bottomed plates. After 2 hr of adherence, macrophage lines were infected with *M. bovis* BCG (Montreal strain; IAF Biovac Inc., Laval, Quebec, Canada) for 48 hr using a 5:1 ratio of bacteria to macrophages (without washing). Cells treated with IFN-γ were administered 10 U/ml of the cytokine for the same period of time. We also measured the nitrite production in macrophages seeded into 96-well flat-bottomed microtitre plates (Nunclon, Nunc, Roskilde, Denmark). In this case, macrophages were initially infected with BCG for 4 hr using a bacteria to macrophage ratio of 20:1, then they were washed six times. The macrophages were stimulated with IFN-γ and/or plated in the presence of N^{g}MMLA to a final volume of 200 μl. After 4 hr, a period of time sufficient to allow phagocytosis of the bacteria, the cells were washed extensively (six times) with 200-μl volumes of warm CM to eliminate extracellular bacilli, and IFN-γ or N^{g}MMLA was replaced to the original concentration. To ascertain the presence of extracellular bacilli into the washed wells, the number of colony-forming units (CFU) of each individual culture was determined by culturing serial dilutions in Dubos solid medium. Less than 0·01% of the initial input of bacteria remained after the washing step. Following the stipulated

period of time, the medium was collected and replaced with 200 μl of CM containing 0·1% saponin (final concentration) and 1 μCi of [³H]uracil. After 16 hr, cells were harvested using a semi-automatic cell harvester and the radioactivity was estimated by liquid scintillation using a β-counter.

To assess the ability of mouse cells to produce nitrite in response to immunomodulators, splenic cells, were cultured for 48 hr at 37°, 5% CO_2, in the absence or presence of different concentrations of IFN-γ. Nitrite present in supernatants was quantified as above.

Evaluation of L-arginine addition on the ability of macrophages to control intracellular growth of BCG

1 × 10⁵ macrophages was seeded in 200 μl of medium containing 10% fetal bovine serum but without antibiotic. Macrophage infection, lysis and quantification of the incorporation of [³H]uracil after 48 hr were done as previously described. IFN-γ, 10 U/ml, N⁸MMLA, 200 μM, and increasing concentrations of L-arginine (0·1–10 mM) were added to the wells (final volume, 200 μl). Results are expressed as the percentage of bacteriostatic activity and are compared to the amount of nitrite present in the wells. The bacteriostatic activity of the macrophages was calculated as follows:

%bacteriostasis =

$$\left(\frac{1 - \text{c.p.m.M}\phi + \text{bacteria} + \text{modulator}}{\text{c.p.m.M}\phi + \text{bacteria}} \right) \times 100$$

where the modulator is N⁸MMLA, IFN-γ, or L-arginine, depending on the particular experiment.

Four replicas were done for each treatment. Results represent the average of three independent experiments. The Neuman and Tytell medium used for cell culture contains 200 μM of L-arginine.

Northern blot analysis

A 2·3 kb fragment of the putative macrophage nitric oxide synthase gene[20] was subcloned in pCL-BS3 plasmid conferring Ampʳ (kindly provided by Dr S. Snyder, Johns Hopkins University, Baltimore, MD). To perform Northern analysis of iNOS, 1 × 10⁷ macrophages was seeded in 100-mm plastic Petri dishes and stimulated with IFN-γ for the selected periods of time. Cells were subsequently lysed with the guanidium isothiocyanate solutions and total cellular RNA was prepared as described elsewhere.[21] Briefly, the RNA was isolated by centrifugation on CsCl cushions and separated on 1·2% agarose gels containing 2·2 M formaldehyde. 1 × 10⁶ c.p.m./ml of the NOS probe labelled by random priming was added to the hybridization buffer. The GADPH probe was generated by polymerase chain reaction (PCR) using the following oligo-nucleotide primers: sense primer, 5′ CCC TTC ATT GAC CTC AAC TAC ATG G 3′, anti-sense primer, 5′ AGT CTT CTG GGT GGC AGT GAT GG 3′. After PCR amplification, the 456 bp PCR product was isolated and used as a probe in the Northern analysis. The expression of GAPDH mRNA, which is constitutively expressed and not modulated by IFN-γ in macrophages, was used as an internal control to ensure that the same amount of RNA per lane was loaded. The RNA was transferred to Nytran nylon membranes (Schleicher and Schuell, Keene, NH), and hybridized with probes which were labelled to high specific activity (> 10⁸ c.p.m./mg) by the

random priming technique using the Random Priming System (BRL, Gaithersburg, MD).

Effect of acidified NO_2^- on M.bovis BCG

1 × 10⁶ CFU of *M. bovis* BCG Montreal was seeded into flat-bottomed microtitre plates and incubated at 37° for 48 hr in 200 μl of CM medium (without antibiotics) containing different concentrations of sodium nitrite (range 0·1–10 mM) titrated to pH 5·0. Bacteria were pulsed with 1·0 μCi of [³H]uracil for 16 hr. The antimycobacterial effect of NO_2^- was determined by the decrease of [³H]uracil incorporation in NO_2^--containing samples compared with controls. The incorporation of the radioactivity was counted in a β-counter.

Statistical analysis

Non-parametrical statistical analysis was used. The significance of the difference between groups was determined using the Mann–Whithney two sample test. *P* values were calculated using the one-tailed or the two-tailed *t*-test.

RESULTS

Production of NO_2^- by B10S and B10R macrophage lines treated with BCG or IFN-γ

We have established macrophage lines from mouse strains congenic at the *Bcg* locus, differing in their susceptibility to infection with *M. bovis* BCG. As previously described,[9] these lines preserve the difference in the ability to control mycobacterial cell growth observed *in vivo* in macrophages derived from the B10.A (*Bcgˢ*) and B10.A.*Bcgʳ* murine strains. Our initial aim was to measure the production of nitrite by both types of macrophages in response to either *M.bovis* BCG or IFN-γ. Twelve different macrophage lines, six B10R and six B10S, were used throughout this study.

To determine whether or not the B10R and B10S macrophage lines differed in their capacity to produce NO_2^- in response to IFN-γ and BCG, we studied the NO_2^-
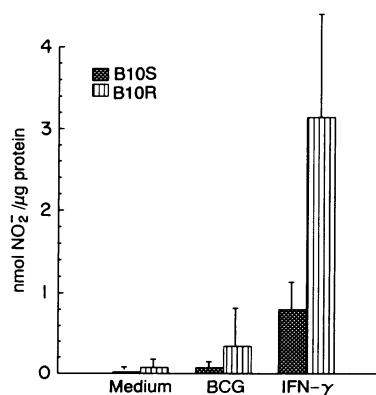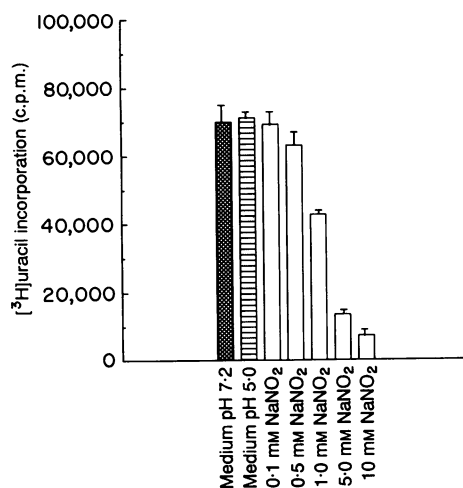

Figure 1. NO_2^- production by B10R and B10S macrophage lines in response to *M. bovis* BCG and IFN-γ. The basal production of NO_2^- as well as their response to BCG and IFN-γ after 48 hr of stimulation is depicted. Values represent a minimum of three and a maximum of six experiments performed in each macrophage line. The *P* values were 0·065 for basal production, 0·0931 for BCG stimulation, and 0·0022 for IFN-γ stimulation.

production by the macrophages infected for 48 hr with *M. bovis* BCG, or stimulated with 10 U/ml of IFN-γ for the same period of time. As shown in Fig. 1, stimulation of the macrophages with IFN-γ resulted in a potent induction of NO_2^- production in both B10S and B10R macrophages compared to non-stimulated controls. Comparison of the nitrite production by B10R and B10S macrophages in response to IFN-γ indicated that B10R produced a significantly higher amount of nitrite, approximately 3.9 times more than B10S ($P = 0.0022$). Infection with BCG induced a modest increase in NO_2^- production over non-stimulated controls both in B10S and B10R macrophages (3.8-fold increase in B10S versus 4.5-fold increase in B10R). Although B10R macrophages produced approximately 4.6 times more NO_2^- compared with B10S, this difference was not significant statistically ($P = 0.0931$).

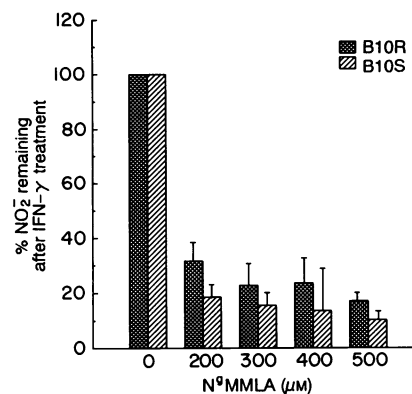## NO· inhibits the proliferation of *M. bovis* BCG

Since the macrophage lines produced NO_2^- in response to BCG infection and IFN-γ stimulation, our next step was to determine whether or not nitric oxide, the postulated effector molecule in the L-arginine pathway of macrophage activation, could curtail the proliferation of *Mycobacteria*. We therefore treated *M. bovis* BCG bacilli with $NaNO_2$, which has been reported to induce nitric oxide in an acid pH environment.[13,16]

As shown in Fig. 2, by 48 hr after treatment there was a significant decrease in the incorporation of uracil into BCG, in a concentration-dependent manner. Maximal inhibition of uracil incorporation (89%) was achieved at a concentration of 10 mM $NaNO_2$. The reduction in the proliferation of BCG was not due to the acidic conditions necessary to generate nitric oxide, since the uracil incorporation by BCG cultured in acidic medium alone was similar to the proliferation of BCG cultured at physiological pH (pH 7.2).

Figure 3. Specific inhibition of NO_2^- production in B10R and B10S macrophages by N^gMMLA. Macrophages were treated for 48 hr with 10 U/ml of IFN-γ in the presence or absence of various concentrations of N^gMMLA. Data represent analysis of triplicate samples. SD was <10%.

Inhibition of macrophage IFN-γ-induced nitrite production by N^g-MMLA

N^gMMLA has been described as a specific competitive inhibitor of the L-arginine-mediated pathway of nitrite production.[11,12] To determine whether the macrophage NO_2^- production in response to BCG/IFN-γ was derived from the L-arginine pathway, different concentrations of the inhibitor, ranging from 200 to 500 μM, were added to wells containing macrophages treated with IFN-γ. As shown in Fig. 3, N^gMMLA was able to reduce dramatically, but not

Table 1. Effect of N^g-MMA on the nitric oxide production and bacteriostatic activity of BCG-infected macrophages stimulated with IFN-γ

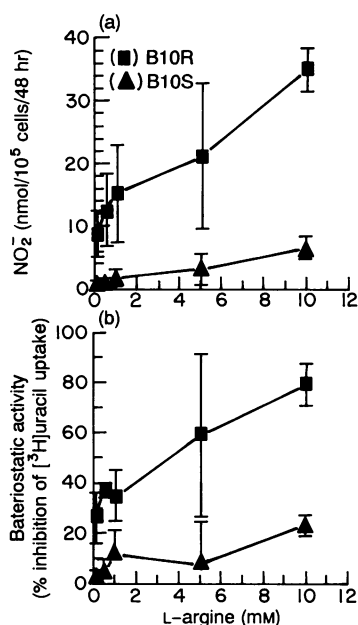| Cell treatment*	N^gMMLA added†	NO$_2^-$ production (nmol/48 hr)‡		Bacteriostatic activity (%)§	
		B10R	B10S	B10R	B10S
—	—	9·5 (7·6)¶	0·4 (0·5)	—	—
	+	2·1 (1·8)	0·4 (0·4)	9·2 (39·9)	−24·1 (−14·2)
IFN-γ	—	43·1 (17·2)	19·3 (17·8)	62·1 (17·6)	27·9 (20)
	+	19·0 (13·9)	5·0 (5·8)	39·9 (22·7)	8·0 (17·1)

*1 × 10⁵ macrophages were infected with 2 × 10⁶ BCG for 4 hr. Extracellular bacilli were eliminated as described in the Materials and Methods. IFN-γ (10 U/ml) and N^gMMLA were added simultaneously.
†N^gMMLA, 500 μM.
‡§Nitrite production and bacteriostatic activity were quantified as described in the Materials and Methods. Data represent mean of three independent experiments. Values are from four to six replicas per treatment.
¶(SD).

Figure 2. Inhibition of the proliferation of *M. bovis* BCG by $NaNO_2$. 1 × 10⁶ CFU of BCG was seeded in 200 μl of CM medium in 96-well plates in the absence or presence of different concentrations of $NaNO_2$. After 48 hr, 1 μCi/well of [³H]uracil was added to the cultures and the isotope incorporation was measured during the last 16 hr of the culture period. Hexaplicate samples were used for each treatment. SD was <15%.

Figure 4. L-arginine addition to culture wells increased nitrite production and bacteriostatic activity by macrophages. 1×10^5 macrophages were infected with 2×10^6 CFU of *M. bovis* BCG for 4 hr, washed extensively and then simultaneously treated with IFN-γ (10 U/ml) and N^gMMLA (200 μM). Increasing concentrations of L-arginine were added to selected wells. Nitrite production and bacteriostatic activity by the macrophages were quantified after 48 hr of treatment. (a) NO_2^- production; (b) bacteriostatic activity. Data represent the average of three independent experiments. Values represent the average of four to six replicas. The differences in both NO_2^- production and bacteriostatic activity between B10R and B10S macrophages were statistically significant for all doses of L-arginine tested ($0.002 < P < 0.05$).

completely, the production of NO_2^- by the macrophage lines. Maximal inhibition was observed at 500 μM N^gMMLA (82·9 versus 89·7% inhibition of NO_2^- production by B10R and B10S lines, respectively).

B10R macrophages are more efficient in controlling BCG proliferation than B10S macrophages; effect of N^g-MMLA and L-arginine addition

The next series of experiments was designed to verify the role of nitric oxide in the differential ability of B10R and B10S macrophages to control the intracellular proliferation of *M. bovis* BCG. We addressed this question by the use of the competitive inhibitor, N^g-MMLA. The results shown in Table 1 indicate that in the presence of IFN-γ, the bacteriostatic activity of B10R macrophages was approximately 2·2 times higher than the bacteriostatic activity of B10S macrophages (62·1% versus 27·9%, respectively). The bacteriostatic activity positively correlated with the concentration of nitrite present in wells 48 hr after macrophages were stimulated with IFN-γ. B10R macrophages secreted 43·1 nmoles NO_2^- while B10S macrophages produced 19·3 nmoles NO_2^-. We observed that the treatment of B10R macrophages with N^gMMLA resulted in a slight increase in the percentage of bacteriostasis

rather than a decrease as seen in the case of B10S macrophages. However, due to the high SD, this difference was not statistically significant ($P > 0.05$). The presence of N^gMMLA inhibited the bacteriostatic activity of both B10R and B10S macrophages (35·7% and 71·3%, respectively), although the inhibitory activity was more pronounced in B10S macrophages. Furthermore, the ability of B10R and B10S macrophages to inhibit the [^{3}H]uracil uptake by *M. bovis* BCG directly correlated with the amount of nitric oxide produced by the stimulated cells. Nevertheless, a complete abrogation of the mycobacteriostatic activity by the macrophages was not observed during the period of time studied.

To further verify that the bacteriostatic capacity of the macrophages was related to nitric oxide production, increasing concentrations of L-arginine were added to BCG-infected macrophages stimulated with IFN-γ cultured in the presence of N^gMMLA. As shown in Fig. 4, the increase in the amount of L-arginine added to the culture medium was paralleled by a linear increase in the amount of NO_2^- secreted (Fig. 4a) as well as by an increase in the bacteriostatic activity (Fig. 4b) of both B10R and B10S macrophages. Both the highest production of nitric oxide and bacteriostatic activity were reached with 10 mM L-arginine.

Overall these in vitro results indicate that there is a close association between nitrite secretion and bacteriostatic ability of macrophages towards *M. bovis*. They also show that the superior ability of B10R macrophages to control mycobacterial growth may be related to the competence of B10R macrophages to produce nitric oxide.

Superior ability of tissue macrophages obtained from *Bcg^r* strain mice to produce NO_2^- in response to IFN-γ

Previous studies have indicated that macrophages harvested from BCG-resistant animals are more efficiently activated in response to IFN-γ compared to macrophages obtained from BCG-susceptible mice.[6,7,9] It has also been shown that *M. bovis* BCG replicates preferentially in the spleen and liver of BCG-susceptible strains.[1,2]

Our next objective was to compare the ability of splenic cells, derived from B10.A.*Bcg^r* and B10.A(*Bcg^s*) mice, to produce NO_2^- in response to stimulation by IFN-γ. As shown

**Table 2.** Nitrite production by splenic cells stimulated with IFN-γ*

	NO_2^- (nmol/5 $\times$ 10^6 cells/48 hr†)					
	Exp. 1		Exp. 2		Exp. 3	
	B10R‡	B10S	B10R	B10S	B10R	B10S
Medium	1·97	1·27	0·88	1·0	0·85	0·85
IFN-γ 50 U/ml	4·41	1·47	7·42	5·18	5·28	3·71
IFN-γ 100 U/ml	2·76	1·36	8·14	5·40	ND	ND

* 5 $\times$ 10^6 unseparated splenic cells/ml were cultured for 48 hr in the absence or presence of IFN-γ.

† Nitrite was quantified as indicated in the Materials and Methods.
‡ B10R = B10.A.*Bcg^r*; B10S = B10.A.
ND, not done.
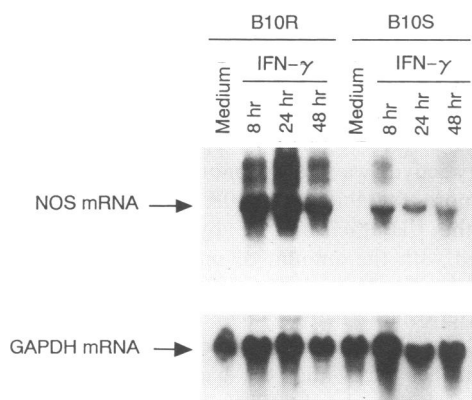
Figure 5. Macrophage nitric oxide synthase mRNA expression by B10R and B10S macrophage lines. Ten micrograms of total RNA purified from IFN-γ-stimulated macrophage lines was hybridized with a ^{32}P-cDNA probe for the macrophage iNOS as described in the Materials and Methods.

in Table 2, unseparated splenic cells derived from mice of the BCG-resistant congenic strain (B10A.*Bcg^r*) secreted approximately 1·6-fold more NO_2^- in response to either 50 U and 100 U IFN-γ than cells derived from the BCG-susceptible strain.

B10R macrophages express higher iNOS mRNA levels than B10S macrophages in response to IFN-γ

To test whether or not the differential production of NO_2^- by B10S and B10R macrophages was a consequence of differential mRNA expression of nitric oxide synthase, we performed Northern blot analysis using a probe specific for macrophage nitric oxide synthase. Kinetic analysis revealed that the expression of iNOS reached a maximum between 8 hr and 12 hr (data not shown) and then reached a plateau level by 24 hr. The levels of nitric oxide synthase expression were higher for all doses used and at all time-points tested in the B10R macrophages as compared to their B10S counterparts, representative results are presented in Fig. 5.

DISCUSSION

Macrophages play an important role in controlling the proliferation of intracellular micro-organisms. This function is of paramount importance during the onset of the immune response, when macrophages, alone or in collaboration with natural killer (NK) cells, are able to inhibit the proliferation of and can kill pathogenic intracellular micro-organisms at an early stage of the infection.[22]

It has been established, using distinct inbred and congenic mouse strains, that there is a genetic component in the ability of the host to resist mycobacterial infections, expressed at the level of the macrophage. This differential ability of host macrophages to control the growth of mycobacteria was shown to be regulated by the expression of a gene mapped to the centromeric portion of murine chromosome 1, and designated *Bcg*.[1,2] Interestingly, the *Bcg* locus has been mapped at or near the loci conferring resistance or susceptibility to both *L. donovani* (*Lsh* gene) and *S.typhimurium* (*Ity* gene).[4]

Little is known about the molecular mechanisms which govern the ability of macrophages to control the intracellular

growth of parasites. Among the mechanisms proposed are microbial deprivation of L-tryptophan,[23] IFN-γ-induced reduction in monocyte transferrin receptors,[24] and the liberation of ROI including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) by the macrophages.[25,26]

Recently, reactive nitrogen intermediates (RNI), produced by macrophages and other cell types in response to IFN-γ, or IFN-γ plus tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), have been shown to play an important role in the control of the intracellular growth of micro-organisms. Intracellular parasites such as *Leishmania*, *Trypanosoma*, *Plasmodium*, *Toxoplasma*, as well as bacteria including *Mycobacteria* and *Francischella*, have been reported to be inhibited or killed by RNI.[12–16,27] NO·-generated damage at the level of DNA in *S. typhimurium* has also been reported.[28]

The results of the present study suggest that the production of RNI are maybe under the control of the *Bcg* gene. Specifically, our work has addressed two questions. (1) Do macrophages expressing the *Bcg^r* or the *Bcg^s* alleles differ in their production of RNI? (2) Are RNI involved in the differential ability of macrophages derived from BCG-resistant and -susceptible strains of mice to control mycobacterial growth?

Our data have shown that macrophages expressing the *Bcg^r* allele produce more NO_2^- than those expressing *Bcg^s*. Using macrophage lines derived from mice congenic at the *Bcg* locus, we found significant difference in the capacity of B10R and B10S lines to produce NO_2^- in response to IFN-γ. Furthermore, splenic adherent cells of B10A.*Bcg^r* mice stimulated with IFN-γ secreted more nitrite in comparison to B10.A cells.

To date, there have been two reports comparing the ability of macrophages derived from resistant and susceptible hosts (*Bcg^r*/*Lsh^r*) to produce NO_2^-. Appelberg and Sarmento,[29] using inbred (C57Bl/6, DBA/2, C3H/He, CD1) and *Bcg* congenic mouse strains (CD2, BALB/c), measured the production of NO_2^- by *M. avium*-elicited peritoneal macrophages, 15 and 30 d after infection, in the presence or absence of lipopolysaccharide (LPS). They found that there was no direct correlation between resistance and susceptibility to *M. avium* and the capacity to produce NO_2^- in response to LPS. Furthermore, in the case of mouse macrophages, it is more relevant to study splenic adherent cells instead of peritoneal macrophages since *in vivo* experiments have demonstrated that the former macrophage population is able to express the resistant or susceptible phenotype at the level of more effective bacterial killing.[1,2]

Recently, a study conducted by Roach *et al.*[14] indicated that bone marrow macrophages derived from B10.A.*Lsh^r* strain mice were more potent leishmanicidal effector cells than their B10.A (*Lsh^s*) counterparts, and that there was a direct correlation between leishmanicidal activity and nitrite production. Interestingly, elicited peritoneal macrophages from the congenic mice did not display such a correlation, suggesting that different macrophage subpopulations may differ in the expression of the *Lsh* gene. Thus, our results using bone marrow-derived macrophage lines are consistent with the study of Roach *et al.*[14]

A candidate gene for the *Bcg* gene (*Nramp*) was recently cloned by Vidal *et al.*[18] *Nramp* encodes an integral membrane protein that has structural homology with known prokaryotic

and eukaryotic transport systems. Thus, *Nramp* has a structural similarity with a membrane transporter of *A. nidulans*, CrnA, implicated in the transport of nitrogen compounds. The authors speculate that *Nramp* functions as a concentrator of nitrite/nitrate in phagolysosomes, and it is possible that alterations in this putative transport system would affect the capacity of *Bcg^s* macrophages to control intracellular replication of antigenically unrelated ingested microbial targets. It was shown by Vidal *et al.*[18] that susceptibility to infection (*Bcg^s*) in 13 *Bcg^r* and *Bcg^s* strains tested was associated with a non-conservative gly-105 to Asp-105 substitution within the predicted transmembrane domain of *Nramp*.

De Chastellier *et al.*,[30] studying the intracellular growth of *M. avium* in BALB/c (*Bcg^s*) and its congenic resistant, C.D2 (*Bcg^r*), found that the percentage of phagosome–lysosome fusion was twice as high in *Bcg^r* macrophages, and the percentage of intact viable bacteria residing in acid phosphatase-negative phagosomes was twice as low in *Bcg^r* macrophages as in their counterparts. This result suggests that at least two different, maybe linked, mechanisms of antibacterial activity could be responsible for the resistant and susceptible phenotype of anti-bacterial activity by macrophages.

We clearly observed an innate difference in the ability of B10R and B10S macrophages to curtail the growth of BCG using infected macrophages in the presence or absence of IFN-γ and N^{g}MMLA. Specifically, the bacteriostatic capacity of B10R macrophages was higher than the bacteriostatic activity of B10S macrophages. The production of nitric oxide by the IFN-γ-treated macrophages could at least contribute to their ability to inhibit the [^{3}H]uracil uptake by mycobacteria. Firstly, the bacteriostatic activity of both B10R and B10S macrophages was inhibited by N^{g}MMLA; secondly, the addition of L-arginine was able to re-establish, in a dose-dependent manner, the ability of B10R and B10S macrophages to inhibit the tritiated uracil uptake by BCG. Thirdly, there was a positive correlation between NO$_2^-$ produced by the macrophages in response to IFN-γ and the bacteriostatic activity of the macrophages.

Interestingly, it has been shown in SCID mice, that IFN-γ, probably derived from NK cells, is also present following infection with *L. monocytogenes* or BCG,[22] indicating that this lymphokine may allow the host to mount a non-specific anti-microbial response within a short period of time after infection. We have observed that IFN-γ mRNA is expressed in cultured spleen cells within 24 hr following *in vitro* infection with BCG,[31] and thus the differential responsiveness of macrophages from susceptible compared to resistant mice to IFN-γ could play a pivotal role in the outcome of the infection.

In conclusion, this study has shown that dramatic differences in NO$_2^-$ production seen between macrophages expressing alternative alleles of the *Bcg* gene could contribute to the inhibition of mycobacterial proliferation in resistant animals.

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