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PHENOTYPIC EXPRESSION OF *Bcg* GENE IN MACROPHAGES: REGULATION OF MHC CLASS II EXPRESSION AND NITRIC OXIDE PRODUCTION

by

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SHORTENED VERSION OF THESIS TITLE

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SHORTENED TITLE: MHC CLASS II AND NITRIC OXIDE REGULATION BY BCG^r AND Bcg^s MACROPHAGES.

to my father who died not knowing what science was,
to my mother who believes that science will make a Doctor,
to my wife, who believes in science but does not know what it is,
and to my daughter who does not know nor believe in science

ABSTRACT

Defense against invading pathogens is mediated by both natural and acquired immunity and different protective mechanisms are stimulated depending on the nature of the pathogen. Genetic factors are implicated in resistance and susceptibility to infection. In the mouse, the trait of innate resistance or susceptibility to infection with several species of *Mycobacteria*, including *M. bovis BCG, M. lepraemurium, M. intracellulare* and *M. smegmatis*, is controlled by the expression of the single gene on chromosome 1, designated *Bcg*. The *Bcg* gene is present in two allelic forms in inbred mouse strains: the dominant resistant allele (Bcg^r) and the recessive susceptibility allele (Bcg^s).

The phenotypic effects of Bcg gene are seen in the macrophage. Macrophages derived from the resistant strains of mice appear to be more efficient than Bcg^s macrophages in expressing surface markers associated with the activated state, as well as in producing nitric radicals in response to IFN-γ or to infection with *Mycobacteria.*

In this thesis, macrophage cell lines derived from the bone marrow of B10.A (B10S macrophages) and B10A.Bcg^r (B10R macrophages) were used to study functional parameters associated with Bcg gene activity. 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 or their congenic BCG-resistant partners of the B10A.Bcg^r (Bcg^r) strain. We have discovered that there is a significant difference in the production of NO_2^- of B10S as compared with B10R macrophages in response to IFN-γ. By 48 hours following treatment with 10 U/ml IFN-γ, B10R macrophages produce approximately three fold higher level of NO_2^- than B10S macrophages. Similar results were obtained when experiments were performed with splenic cells harvested from B10.A.Bcg^r and B10.A strain mice. The bacteriostatic activity against *M. 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 antimycobacterial activity was inhibited by N^gMMLA, 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^{g}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.

The *Bcg* gene differentially affects the ability of BCG-resistant and -susceptible strains of mice to express important macrophage genes including Major Histocompatibility Complex (MHC) class II genes. We have analyzed the molecular mechanism involved in IFN-γ-induced MHC class II expression using B10R and B10S macrophages. We have found that differences at the level of I-A_β gene transcription, and I-A_β and I-A_α mRNA stability may be responsible for observed differences between steady-state levels of I-A_β mRNA in B10R and B10S macrophages and consequently in the Ia surface protein expression. Furthermore, we have studied the binding of proteins prepared from nuclear extracts of non-stimulated and IFN-γ-stimulated B10R and B10S macrophages to the S, X and Y cis-acting elements of the I-A_β promoter. Differences observed in protein binding to the X box may explain the difference in transcription activation of the I-A_β gene. In addition, we found that B10R macrophages transfected with an *Nramp-1* antisense cDNA containing a ribozyme construct expressed lower amounts of Ia antigen compared to mock-transfected macrophages in response to IFN-γ. Overall, these findings strongly suggest involvement of the *Nramp-1/Bcg* gene in the control of Ia antigen expression in macrophages.

Finally, I have developed a new PCR-based method that allow an assessment of the mycobacterial content of infected macrophages. This method allows an assessment of the level of *M. bovis BCG* infection from a variety of sources, including peritoneal macrophages and macrophage lines, within a few hours, making it an assay of choice for rapid determination of the level of mycobacterial growth in infected cells, in experimental models of mycobacterial infection.

Overall, we hope these results help to understand the basis of *M. bovis BCG*-induced protection against *M. tuberculosis* and to develop effective treatments of tuberculosis and leprosy.

RÉSUMÉ

La résistance de l'hôte contre les agents pathogènes est médiée par l'immunité naturelle et l'immunité acquise. Les mécanismes de protection impliqués varient selon la nature du pathogène. Chez la souris, la résistance naturelle à des infections par des mycobactéries telles que *M. bovis BCG*, *M. lepraemurium*, *M. intracellulare* et *M. smegmatis* est controllée par le gène *Bcg* situé sur le chromosome 1. Ce gène s'exprime au niveau des macrophages et se présente sous 2 formes allèliques: l'allèle dominante de résistance (Bcg^r) et l'allèle de sensibilité (Bcg^s). L'expression des marqueurs de surface associés à l'activation cellulaire est plus élevée dans les macrophages dérivés de souris de lignées resistantes que dans les macrophages de souris sensibles. Les macrophages qui expriment l'allèle de résistance au locus Bcg ont également une capacité accrue à produire de l'oxyde nitrique lorsqu'ils sont infectés par des mycobactéries ou stimulés avec de l'IFN-γ. Dans cette thèse, la production d'oxyde nitrique et l'activité antimycobactérienne ont été étudiées dans des lignées de macrophages dérivés de la moelle osseuse de souris B10A.*Bcg*^{r} (macrophages B10S) et de leur congéniques B10A.*Bcg*^{r} (macrophages B10R). Après 48 heures en présence de 10 U/ml d'IFN-γ, les macrophages B10R produisent approximativement 3 fois plus de NO_2^- que les macrophages B10S. Des résultats similaires ont été obtenus en utilisant des cellules spléniques de souris B10.A.Bcg^r et de souris B10.A. De plus, nous avons observé que l'activité bactériostatique vis-à-vis *M. bovis* (BCG) était plus élevée dans les macrophages B10R comparativement aux macrophages B10S. L'activité bactériostatique des macrophages B10R et B10S a été associée au niveau d'oxyde nitrique produit. L'activité antimycobactérienne a été inhibée par le N$^{\omega}$MMLA, un inhibiteur spécifique de la synthèse de nitrites et de nitrates à partir de L-arginine. L'addition de L-arginine à des macrophages cultivés en présence d'IFN-γ et de N$^{\omega}$MMLA restore leur capacité à produire de l'oxyde nitrique ainsi que leur activité bactériostatique. Une analyse de type Northern a révélée que la différence au niveau de la production de l'oxyde nitrique entre

les macrophages B10S et B10R était étroitement liée au niveau d'expression de l'ARN messager de la forme inductible de la synthase de l'oxyde nitrique (iNOS).

Le gène Bcg module également la capacité des souris BCG sensibles et BCG résistantes à exprimer les gènes du complexe majeur d'histocompatibilité (CMH) de classe II. Dans cette thèse, j'ai étudié les mécanismes moléculaires impliqués dans l'expression des molécules de classe II du CMH en réponse à l'IFN-γ, en utilisant des macrophages dérivés de souris résistantes ou sensibles aux infections mycobacteriennes (B10R ou B10S respectivement). J'ai observé des différences au niveau de la transcription du gène I-A_β et de la stabilité des ARNm I-A_β et 1-A_α entre les macrophages B10R et B10S. Ces observations pourraient être à l'orgine des différences entre les macrophages B10R et B10S au niveau de l'expression de l'ARNm I-A_β et, conséquement, dans l'expression des protéines de surface Ia. J'ai également étudié les protéines préparées à partir d'extraits nucléaires de macrophages B10R et B10S pour leur capacité à se fixer aux élements S, X et Y du promoteur I-A_β. Les différences observées dans la fixation au fragment X pourraient expliquer la différence dans la transcription des gène I-A_β. De plus, nous avons observé que le niveau d'expression de l'antigène Ia en réponse à IFN-γ est plus faible dans les macrophages B10R transfectés avec l'ADNc *Nramp-1* antisens contenant un "ribozyme", comparativement aux macrophages transfectés contrôles. Dans l'ensemble, ces observations suggèrent fortement que le gène *Nramp-1/Bcg* est impliqué dans la régulation de l'expression de l'antigène Ia dans les macrophages. Finalement, j'ai developpé une nouvelle méthode basée sur l'amplification par PCR permettant de mesurer la charge mycobactérienne dans les macrophages infectés. Cette méthode de choix permet de mesurer en quelques heures le niveau de croissance de *M. bovis BCG* dans des macrophages de sources différentes, notamment les macrophages péritoneaux et les lignées de macrophages.

Nous espérons que ces résultats aideront a comprendre les mécanismes impliqués dans la protection contre *M. tuberculosis* et à développer des traitements efficaces contre la tuberculose et la lèpre.

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Abbreviations

AFB	acid fast bacilli
α	alpha
APC	antigen-presenting cells
asp	asparagine
ATPase	adenine triphosphatase
BCG	Bacillus Calmette-Guerin
bp	base pairs
β	beta
BRM	biologic response modifiers
BSA	bovine serum albumin
°C	centigrade degrees
cDNA	complementary deoxyribonucleic acid
CGD	chronic granulomatous disease
CFU	colony forming unit
Ci	Curie
CM	complete medium
cNOS	constitutive nitric oxide synthase
cpm	counts per minute
CsCl	cesium chloride
CSF	colony-stimulating factor
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
ds	double stranded
DTT	dithiotreithol
e-ALAS	erythroid 5-amino levulinic acid synthase

EDTA	ethylenediamine tetraacetic acid
EMSA	electrophoretic mobility shift assay
FBS	fetal bovine serum
Fc	fraction crystallizable
FACS	fluorescense-activated cell sorter
fg	femtogram
fig	figure
FITC	fluorescein isothiocyanate
γ	gamma
g	gravity
GAPDH	glyceraldehyde phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
gly	glycine
H	histocompatibility
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigens
H_2O_2	hydrogen peroxide
hr	hour
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IgG	gamma immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IRE	iron response element
IRP	iron response protein
kb	kilobase
kD	kilodalton
LPS	lipopolysaccharide

M	molar
m	milli
ma	membrane-associated
MgCl2	magnesium chloride
MHC	major histocompatibility complex
μg	microgram
μl	microliter
mg	milligram
μM	micromolar
ml	milliliter
mm	millimeter
mmol	millimole
MLR	mixed leukocyte reaction
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
$NaNO_2$	sodium nitritre
ng	nanogram
N^gMMLA	N^g-monomethyl L-arginine
NK	natural killer
nm	nanometer
NO	nitric oxide
NOS	nitric oxide synthase
NO_2^-	nitrite
NTCM	Neuman and Tytell complete medium
O_2^-	superoxide anion
·OH	hydroxyl radical

$^1O_2$	singlet oxygen
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol myristate acetate
PPD	purified protein derivative
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
s	seconds
SAC	spleen adherent cells
SCID	severe combined immunodeficiency
SDP	strain distribution pattern
SDS	sodium dudecyl sulfate
sIa	surface Ia
sp.act.	specific activity
SPF	specific pathogen-free
TBE	tris-borate-EDTA
TE	Tris-EDTA
TDO	tryptophan 2,3-dioxygenase
TfR	transferrin receptor
TGF	transforming growth factor
TK	tyrosine kinase
TNF	tumor necrosis factor
U	unit
V	volts

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Preface

In accordance with the "Guidelines concerning thesis preparation" of the Faculty of Graduate Studies and Research, manuscripts of papers which have been published or which have been submitted for publication have been incorporated in the thesis. This format for the thesis preparation has been approved by the Division of Experimental Medicine, Department of Medicine. The following is quoted directly from the Guidelines:

> "Candidates have the option, subject to the approval of their Deparment of including as a part of their thesis, copies of the text of a paper(s) submitted for publication, or a clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collections of manuscripts published or to be published. The thesis must include, as a separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of study, when this review is appropriate, and (5) a final overall conclusion and/or summary".
>
> "Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g., in appendices) to allow clear and precise judgement to be made of importance and originality of the research reported in this thesis".

Each of the manuscripts included in this thesis (chapters 2, 3, 4, and the appendix) has its own Summary, Introduction, Materials and Methods, Results, Discussion and Bibliography. Connecting texts for each of these chapters are included as Prefaces, located at the beginning of each chapter. The general abstract, general introduction (chapter 1), general discussion (chapter 5) and appendix, relate to the combined work presented in this thesis. References appear at the end of each chapter.

The work described in this thesis was performed by the author under the supervision and guidance of Dr. Danuta Radzioch at the Centre for the Study of Host Resistance, Montreal General Hospital. The author received technical assistance and guidance provided by Mrs. Marie Boulé, senior technician in Dr. Radzioch's laboratory. The author and Drs. D. Radzioch and E. Skamene are coauthors of all manuscripts presented. The papers appearing in chapters 2, 3, and appendix have been published*. Dr. Igor Kramnik is coauthor in papers 3 and 4. Dr. Kramnik participated actively in the experiments using splenic cells (chapter 3) and flow cytofluorometric analysis (chapter 4). The paper in chapter 4 has been submitted for publication. My contribution to the paper described in the appendix (paper 1) was restricted to the c-fms and I-A_s mRNA expression in macrophage lines, and to the assessment of phagocytosis by the macrophage lines.

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CHAPTER I

GENERAL INTRODUCTION

TUBERCULOSIS, *Bcg* GENE AND THE CONTROL OF MYCOBACTERIAL INTRACELLULAR GROWTH IN MACROPHAGES.

The evolution of multicellularity is based on the ability of individual cells to collaborate, recognize themselves and function as an integrated organism. Therefore, multicellular organisms developed the capacity to maintain this "aggregated individuality" by recognizing and adapting appropriately to intracellular and extracellular changes.

Intracellular microorganisms have also evolved to recognize, penetrate, function and reproduce inside their host cells. Both the host cell and the invading microorganism have been adapting their responses to increase their chances of survival and reproduction. The host who fails to adapt properly to this evolutionary "compromise" will suffer from pathology of disease. The invading microorganism that does not undergo proper adaptation will suffer erradication and will not have a chance to survive.

Mycobacterial infection is one of the examples of adaptation to intracellular survival inside a restricted set of eukaryotic tissues. *Mycobacterium tuberculosis* complex that includes: *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium africanum*, have successfully adapted to thrive inside cells of the mononuclear phagocytic system (MPS) of higher vertebrates including man.

Tuberculosis: a world-wide problem

Mycobacterium tuberculosis is the causative agent of tuberculosis in human populations. It has been estimated that one quarter of the world's population is infected with this bacteria (Wallis and Ellner, 1994), and by the year 2000, an estimated 10 million people will newly acquire the active disease annually, of which one-third will die (Ehlers et al., 1994). The increase in the incidence of tuberculosis world-wide has been

associated with the increasing prevalence of the human immunodeficency virus (HIV) infection and with the emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* (Orme et al., 1993). No evidence for increased mutation rates in *M. tuberculosis*, nor increased virulence explain the recent fatal outbreaks of the disease (Weiss, 1992).

Two main strategies have been developed to control the growth of mycobacterial infections in humans. The first one, chemotherapy, was successfully administered in the mid-1940s (Grosset, 1989). The emergence of multi-drug resistant strains has weakened the efficiency of chemotherapy to control and eradicate mycobacterial infections. The second strategy, used at the population level, is vaccination with attenuated strains of *Mycobacterium bovis* BCG. The protective efficacy against tuberculosis has ranged from 0 to 80% in different populations (Fine, 1989). Several reasons have been proposed to explain these divergent results, such as, differences among vaccines, methodological differences, differences in *Mycobacterium tuberculosis* strains, nutritional or genetic differences in the populations under study, etc. (Fine, 1989). Overall, inadequate knowledge of the basic mechanisms involved in the initial control of proliferation and killing of mycobacteria by the MPS, hampers the efforts to establish long-lasting immunity to mycobacterial antigens in humans.

Animal models to study mycobacterial infection and multiplication

Given the obvious difficulties in studying the molecular and cellular mechanisms of antimycobacterial immunity among humans, the use of experimental animal systems has proven to be of critical importance. The influence of environmental factors in animal models can be controlled and the contribution of environmental and genetic factors can be easily separated.

Several animal models of disease which mimic particular aspects of human infections have been used. Rabbits and guinea pigs show cavitary lesions and caseous necrosis similar to human disease, whereas the mouse model has been used to investigate genetic resistance/susceptibility to the early phase of mycobacterial infections (Schurr et al., 1991), delayed type hypersensitivity (DTH) and granuloma formation (Ehlers et al., 1994).

Resistance/susceptibility to mycobacterial infection

The existence of inherited factors that contribute to the susceptibility to mycobacterial infections has been postulated for decades. Studies concerning racial differences in resistance and susceptibility to tuberculosis indicate that Black-Americans are more susceptible to initial invasion by *Mycobacterium tuberculosis*, while Ashkenazi Jew seem to be resistant to tuberculosis (O'Brien, 1991). Genes linked and unlinked to Major Histocompatibility Class II antigens have been associated with resistance and susceptibility to mycobacterial infection and proliferation inside the host (Skamene, 1989; de Vries, 1989; Schurr et al., 1991; Stead, 1992). *In vitro* studies on monocyte-derived macrophages of human origin have shown that macrophages from whites permit less rapid replication of *M. tuberculosis* than those from blacks (Crowle and Elkins, 1990). McPeek et al. (1992) have found that monocytes show a pattern of HLA-DR expression consistent with relative resistance to *M. tuberculosis* in 70% of whites but in only 30% of American blacks. So far, no single human gene was unequivocally shown to be associated with direct resistance or susceptibility to mycobacterial infections.

The phenomenon of inherited natural resistance and susceptibility to intracellular multiplication of mycobacteria was observed in rabbits early in this century (Lurie and Dannenberg, 1965; Lurie et al., 1952). The trait of resistance to tuberculosis was shown by Mendelian analysis to be dominant, multiple, and additive over that of susceptibility (Lurie et al., 1952). The studies in other animal models (such as the "Biozzi" mice) have also pointed to a genetic regulation of resistance and susceptibility to mycobacterial

infections. In this particular model, mice bred for low antibody (L) responsiveness to sheep erythrocytes were able to control proliferation of *Mycobacterium bovis* BCG in the spleens more efficiently than mice bred for high (H) antibody responsiveness (Gheorghiou et al., 1985).

Three parasites: one gene

Genetic resistance and susceptibility to intravenous injection of low doses (10^3 to 10^5 colony forming unit, CFU) of *M. bovis* BCG was observed among different inbred mouse strains by Forget et al. (1981). The mouse strains could be separated into two distinct, non-overlapping groups with respect to the growth of bacteria in the spleen and the liver. Furthermore, the genetic control of the susceptibility or resistance of mice to infection with low doses of BCG was not linked to the MHC H-2 complex (Forget et al., 1981; Gros et al., 1981). With the use of classical genetic analysis, Gros et al. (1981) determined that resistance to BCG was controlled by a single, dominant, autosomal gene, that was designated the *Bcg* gene. The product of the *Bcg* gene was found to influence the early phase of the *in vivo* response to infection with BCG (Gros et al., 1981).

The *Bcg* gene was mapped on mouse chromosome 1, in exactly the same locus previously found to confer resistance and susceptibility to *Salmonella typhimurium* (*Ity* gene) (Plant and Glynn, 1979) and *Leishmania donovani* (*Lsh* gene) (Bradley et al., 1979). A full concordance of the strain distribution pattern (SDP) of resistant and susceptible alleles of *Bcg*, *Ity*, and *Lsh* among the recombinant inbred strains gave additional support to the conclusion that the genetic control of resistance to *M. bovis* BCG, *S. typhimurium*, and *L. donovani* was exerted by a single gene with pleiotropic effects (Skamene et al., 1982). Additional studies indicated that the growth of BCG substrains and atypical mycobacteria such as *M. kansasii* and *M. fortuitum* (Denis et al., 1986), *M. intracellulare* (Denis et al., 1986; Goto et al., 1989), *M. lepraemurium*

(Brown et al., 1982; Skamene et al., 1984), and *M. smegmatis* (Denis et al., 1990) were also under the control of the *Bcg* gene.

Evidence that the macrophage is the cell type expressing the *Bcg/Lsh/Ity* gene.

Immunological studies demonstrated that mice genetically resistant to BCG infection were able to prevent bacterial multiplication without the need for an acquired immune response. This conclusion was based on the evidence that DTH against mycobacterial antigens (PPD), granuloma formation in spleen and liver, and resistance to challenge with homologous (BCG) and heterologous (*Listeria monocytogenes*) bacteria, were greatly inferior in animals carrying the Bcg^r allele (Pelletier et al., 1982). The kinetic studies of BCG infection in congenitally athymic mice that carried the "nude" mutation on Bcg^r (AKR/J) or Bcg^s (BALB/c) background showed that the functional absence of T lymphocytes did not influence the expression of the *Bcg* gene (Gros et al., 1983). Furthermore, *in vivo* depletion of T cells or B cells (Skamene, 1989) did not affect the resistant phenotype in BCG-infected mice. On the other hand, treatment of BCG-resistant mice with silica, a chemical agent preferentially taken up by macrophages, was able to reverse the resistant phenotype (Gros et al., 1983). Experiments conducted by O'Brien et al. (1979) using Ity^r mice infected with *S. typhimurium*, led to similar conclusions. Since *M. bovis*, *L. donovani*, and *S. typhimurium* are intracellular parasites it is possible that the *Bcg* gene controls the efficiency of macrophages in combating the infection (Skamene et al., 1982).

Additional evidence pointing to the macrophage as the cell type involved in the phenotypic expression of the *Bcg/Lsh/Ity* gene was obtained from *in vitro* experiments. Stach and colleagues (1984), used resident peritoneal macrophages explanted from inbred and congenic mouse strains to demonstrate that the multiplication of *M. bovis*, (assessed by the bacterial ^{3}H-uracil uptake), was faster in Bcg^r macrophages compared to Bcg^s macrophages. Splenic and peritoneal macrophages of resistant mice infected with *S.*

typhimurium (Lissner et al., 1983), and Kupffer cells infected with *L. donovani* (Crocker et al., Blackwell et al., 1984; 1989; Olivier et al., 1989; Roach et al., 1991) were also shown to control the growth of these intracellular pathogens more efficiently than macrophages derived from susceptible mice.

Effect of *Bcg/Lsh/Ity* gene on microbial multiplication: microbiostatic and/or microbicidal?

It is not clear how the *Bcg/Lsh/Ity* gene influences the microbistatic and microbicidal activity of macrophages. The mechanism through which this gene(s) executes its actions has not yet been explored. Early reports (Swanson and O'Brien, 1983) suggested that the *Ity* gene regulates the ability of mice to control the proliferation of intracellular bacteria. It was found that bacterial numbers in the spleen and the liver of Ity^s mice were significantly greater than in Ity^r mice 24 hours following the infection. The extent of phagocytosis of *S. typhimurium* in reticuloendothelial organs was similar regardless of the *Ity* genotype. Furthermore, phagocytosis of the bacteria was followed by a 30-60% decline in the number of viable bacteria, that was attributed to the bactericidal activity of liver and splenic macrophages. The efficiency of this early phase killing was not under *Ity* control since it did not differ between Ity^r and Ity^s mouse strains.

In order to understand whether the effect of the *Ity* locus was due to differential killing of virulent *S. typhimurium* or differential growth rates of *S. typhimurium*, Benjamin et al. (1990) infected *Ity*-congenic mice with a stationary phase *S. typhimurium* containing a single copy of the plasmid pHSG422 that exhibits defective replication at body temperature. Since the numbers of plasmid-containing *Salmonella* are only slightly affected by bacterial division, decline in the numbers of plasmid-containing *Salmonella* indicated its killing. An 18-fold greater growth of *Salmonella* was found in Ity^s mice than in Ity^r mice from 4 to 44 hours postinfection. Therefore, it was concluded that the major

effect of the *Ity* locus on the resistance to *Salmonella*, was the regulation of its growth in the liver and spleen.

Pleiotropic effects of the *Bcg/Lsh/Ity* gene

One of the critical questions concerning the ability of macrophages expressing the *Bcg/Lsh/Ity* gene is the mode of action of this gene(s). Studies aimed at comparing functional and phenotypic parameters of activation in Bcg^r and Bcg^s macrophages detected various examples of differentially regulated metabolic pathways which are thought to represent pleiotropic expression of the resistance locus. Macrophages from resistant strains (Bcg^r) were superior producers of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) compared to macrophages from susceptible strains (Bcg^s), following either infection with BCG or treatment with interferon (IFN-γ) (Denis et al., 1988a; Blackwell et al., 1988). Denis et al. (1988b) showed that splenic macrophages isolated from uninfected BALB/c.Bcg^r congenic mice contained a higher percentage of Ia-positive cells than splenic macrophage populations obtained from BALB/c mice. Differences in Ia expression have also been reported by Kaye et al. (1988), and Johnson et al. (1985). Another example of plasma membrane bound molecules which are regulated differentially by Bcg^r and Bcg^s macrophages is the AcM.1 marker of macrophage activation (Taniyama et al., 1983). Macrophages from resistant mice infected with BCG, or activated with IFN-γ and lipopolysaccharide (LPS) significantly upregulated the expression of AcM.1 while macrophages from Bcg^s mice could not be stimulated to display changes in their level of AcM.1 expression under the same conditions (Buschman et al., 1989).

Some other pleiotropic effects of the *Bcg/Lsh/Ity* gene have also been reported. When bone marrow-derived macrophages from Lsh^r and Lsh^s congenic strains were activated with IFN-γ and LPS, a dose-dependent difference in the amount of tumor necrosis factor (TNF-α) released over 24h was observed. The magnitude of this response was enhanced in macrophages preinfected with *L. donovani* amastigotes, suggesting that

the parasite itself may act as a trigger for the production and/or release of TNF-α (Blackwell et al., 1989). In a more recent study, macrophages from *Lsh*^{r} mice were shown to produce more TNF-α as compared to *Lsh*^{s} macrophages when they were cultured in matrixes of extracellular matrix proteins, such as fibrinogen and fibronectin (Formica et al., 1994). The addition of IFN-γ to the cultured macrophages, or infection with *L. donovani*, increased the production of TNF-α even further.

The fact that differences in TNF-α release could be measured at the protein level within 4h of addition of priming/activating signals suggested that differences in the expression of early response genes, such as *c-fos*, *c-myc*, *JE* and *KC*, might also play an important role (Blackwell et al., 1991). Clear differences in the mRNA expression for *KC* were indeed observed in resident peritoneal macrophages stimulated with LPS or IFN-γ plus LPS. *Lsh*^{r} macrophages expressed more KC mRNA than *Lsh*^{s} macrophages. Similar differences in KC mRNA expression, but not in JE mRNA expression, in response to lipoarabinomannan from avirulent *M. tuberculosis* (araLAM) were also reported (Roach et al., 1994). The possibility that TNF-α may play an important role in genetically-determined resistance and susceptibility to intracellular infections controlled by the *Bcg/Lsh/Ity* gene was stressed by the results reported by Mastroeni et al. (1993). The authors tested effect of the late administration of anti-TNF-α antibodies on the course of virulent *Salmonella typhimurium* infection. Administration of anti-TNF-α antiserum to resistant mice prevented the suppression of exponential bacterial growth in the MPS. These data indicate that TNF-α is essential for the control of virulent *Salmonella* in mice (Mastroeni et al., 1993). Similar observations, stressing the role of TNF-α and control of growth of *S. typhimurium in vivo* have also been reported (Tite et al., 1991; Nauciel and Espinasse-Maes, 1992). Roach and colleagues showed the difference in nitric oxide (NO) production in response to IFN-γ and LPS by bone marrow-derived macrophages derived from *Lsh*^{r} and *Lsh*^{s} congenic mice (Roach et al., 1991). In this study, NO production by *Lsh*^{r} macrophages was significantly higher than

the production in *Lsh*^{r} macrophages. In addition, it seemed to be correlated with leishmanicidal activity and TNF-α production by the macrophages.

Additional pleiotropic effects have been reported in the *S. typhimurium* model. Japanese investigators (Kita et al., 1992) examined the initial inflammatory response following *Salmonella* infection in both *Ity*^{r} and *Ity*^{s} mice and studied the kinetics of production of several cytokines, including IFN-γ in explanted cells. Only splenocytes from *Ity*^{r} were able to produce IFN-γ upon stimulation with *Salmonella* antigens. Ramarathinam et al. (1993) reported similar results when using splenocytes from *Ity*^{r} and *Ity*^{s} inbred and congenic mouse strains.

Finally, differences involving differential responsiveness to granulocyte-macrophage colony stimulating factor (GM-CSF) (Morrisy and Charrier, 1990), myelopoietic responsiveness (Peterson et al., 1992), membrane-associated interleukin 1 (maIL-1) (Kita et al., 1992), interleukin 1ß (IL-1ß) mRNA (Blackwell et al., 1991), and phagosome-lysosome fusion (de Chastellier et al., 1993) have also been reported to differ between *Bcg/Lsh/Ity* resistant and susceptible animals or cells derived from them. Taken together, these experiments suggest that the pleiotropic effects induced by the *Bcg* gene in murine macrophages may be closely associated with the genetic regulation of macrophage priming for activation (Schurr et al., 1991).

Nramp-1, a candidate gene for *Bcg/Lsh/Ity* gene

Recently, a candidate gene for the *Bcg/Lsh/Ity* gene was cloned (Vidal et al., 1993; Barton et al., 1994), and its product is called Natural Resistance Associated Macrophage Protein (Nramp). Northern blotting analysis indicated that *Nramp-1* is expressed in reticuloendothelial organs such as the spleen and liver, splenic macrophages and macrophage cell lines but not in other tissues or cell lineages (Vidal et al., 1993).

Nramp-1 encodes a polypeptide with no apparent sequence similarity to any previously identified protein. Hydropathy analysis of the predicted amino acid sequence identifies 10-12 strongly hydrophobic domains that are similar to the membrane-spanning regions of polytopic membrane proteins. The *Nramp-1* gene product also contains two potential N-linked glycosylation sites as well as two potential sites for protein kinase C phosphorylation (Vidal et al., 1993). Subsequently, Barton et al. (1994) described an additional exon of *Nramp-1*, located 5' of the sequence published by Vidal et al. (1993). This newly described domain is rich in proline, serine, and basic amino acids, includes three protein kinase C phosphorylation sites and a putative Src homology 3 binding domain. Nucleotide sequence analysis of the *Nramp-1* cDNA showed that in 27 inbred mouse strains of either *Bcg*^{r} and *Bcg*^{s} phenotypes, the susceptibility trait was associated with a nonconservative glycine to aspartic acid amino acid substitution within predicted transmembrane domain 2 of the protein (Vidal et al., 1993).

The Nramp protein contains a conserved sequence motif known as the "binding-protein-dependent transport system inner membrane component signature", identified in prokaryotic and eukaryotic transport proteins. This motif is present in the permease for the nitrate uptake encoded by the *CrnA* gene of the eukaryote *Aspergillus nidulans* (Vidal et al., 1993). The structural similarity between *Nramp-1* and *CrnA* prompted speculation that *Nramp-1* could function as a nitrite/nitrate concentrator at the phagolysosome, and that alterations in this putative transport system would affect the capacity of *Bcg*^{r} macrophages to control intracellular replication of antigenically unrelated microbial targets (Vidal et al., 1993).

Based on the presence of SH3 binding domains in the amino terminal of Nramp, Barton and colleagues proposed that the SH3 domain of *Nramp-1* could interact with members of the Src family of membrane-associated tyrosine kinases, including early response genes such as *Hck* and *Fgr* (Barton et al., 1994).

A syntenic region of the mouse chromosome 1 containing the *Bcg* gene has been mapped on the human chromosome 2q35 (Schurr et al., 1990). Recently, Cellier and colleagues cloned the human homologue of the mouse *Nramp-1* gene. Mouse and human *Nramp-1* display a 93% overall sequence homology. Sequence analysis indicates that human *Nramp-1* encodes a polypeptide with 10-12 transmembrane domains, two N-linked glycosylation sites and an evolutionary conserved consensus transporter motif. Northern blot analysis indicated that human *Nramp-1* mRNA was highly expressed in peripheral blood leukocytes, and in the lung and spleen. Macrophages were identified as cells expressing human *Nramp-1* mRNA (Cellier et al., 1994).

MECHANISMS OF MACROPHAGE CONTROL FOR MICROBISTATIC/MICROBICIDAL ACTIVITIES.

At the cellular level, macrophages play an important role in killing and/or inhibiting proliferation of many microorganisms. Since our understanding of the microbial proliferation inside cells of the MPS depends on the read-out techniques developed to detect them, a brief section is devoted to widely used methods of detection of mycobacterial infection and multiplication. This section also contains a brief description of postulated antimicrobial mechanisms, with emphasis on those affecting survival of microorganisms controlled by the *Bcg/Lsh/Ity* gene.

Methods to assess mycobacterial infection and multiplication in macrophages.

Several *in vitro* methods are currently being used to detect the presence of intracellular mycobacteria and to quantify their growth within macrophages (Crowle et al., 1981; Rook and Rainbow, 1981). Light microscopy following acid fast staining is a widely used method to estimate the percentage of infected macrophages in a given population, but the estimation of the number of acid-fast bacilli in infected macrophages (AFB) is rather subjective. The ^{3}H-uracil incorporation method developed by Rook and

Rainbow (1981), is based on the fact that around 80% of all radioactive uracil fed into mycobacterial cultures is incorporated into RNA, while the remainder labels the DNA fraction (Somogyi and Foldes, 1983). Although quantitative, this method does not account for either the metabolically inactive (dormant) bacilli or the dead microorganisms. The enumeration of mycobacterial CFU in infected cells and tissues is most reliable but requires at least two weeks and special care to avoid bacterial clumping during seeding (Collins, 1990). Finally, the amplification of mycobacterial DNA from cells and tissues harboring *Mycobacteria* can be examined by the use of the Polymerase Chain Reaction (Williams et al., 1990; Eisenach et al., 1990). So far, this method has been used mainly for diagnostic purposes (Brisson-Noel et al., 1990).

Reactive nitrogen intermediates (RNI)

Until 1987, the only inducible biochemical mechanism that could explain activated macrophage cytotoxicity was synthesis of reactive oxygen intermediates (ROI) by NADPH oxidase. The fact that antioxidant molecules did not inhibit expression of activated macrophage cytotoxicity in many experimental systems (Kaufmann et al., 1990), and mammalian macrophages with a genetic deficiency of the NADPH oxidase-mediated respiratory burst were still capable of expressing cytotoxicity for tumor cells as well as for intracellular protozoa such as *Leishmania* and *Toxoplasma* (Pearson et al., 1983; Sibley et al., 1985), suggested that other cytotoxic mechanisms of major importance must also exist.

In 1987, it was reported that the expression of cytokine-inducible cytotoxicity by activated macrophages was L-arginine-dependent (Hibbs et al., 1987a; Hibbs et al., 1987b). In this system, L-arginine is oxidized by an inducible NADPH-dependent enzyme, nitric oxide synthase (NOS) (Bredt et al., 1991; Xie et al., 1992; Lyons et al., 1992; Lowenstein et al., 1992; Charles et al., 1993) to yield L-citrulline, nitrite and nitrate, along with highly RNI (Hibbs et al., 1987; Marletta et al., 1988). NO mediates L-arginine dependent tumor cytotoxicity during co-culture with interferon-γ and LPS

activated macrophages, possibly via nitrosylation reactions which remove labile iron atoms from Fe-S prosthetic groups of aconitase and complexes I and II of the mitochondrial electron transport (Drapier et al., 1986; Drapier et al., 1988; Granger et al., 1988), as well as inhibition of the non-heme iron-containing enzyme ribonucleotide reductase (Lepoivre et al., 1990; Lepoivre et al., 1992), a key enzyme in DNA synthesis.

Inorganic nitrogen oxides have also been implicated in the microbiostatic effect of the activated macrophages against the fungal pathogen *Cryptococcus neoformans* (Granger et al., 1988). More recently, it has also been shown that NO plays a role in the cytostatic or cytocidal activities of macrophages against obligate and facultative intracellular, as well as extracellular pathogens including *Mycobacterium tuberculosis* (Denis, 1991; Chan et al., 1992), *Mycobacterium bovis* (Flesch and Kaufmann, 1991), *Mycobacterium avium* (Denis, 1991), *Mycobacterium leprae* (Adams et al., 1991), *Leishmania donovani* (Roach et al., 1991), *Leishmania major* (Liew et al., 1991; Green et al., 1990a; Green et al., 1990b; Evans et al., 1993), *Leishmania enrietii* (Mauel et al., 1991), *Listeria monocytogenes* (Beckerman et al., 1993), *Ehrlichia risticii* (Park and Rikihisa, 1992), *Francisella tularensis* (Fortier et al., 1992; Green et al., 1993), plasmodia (Nussler et al., 1991; Mellouk et al., 1991, Matthew et al., 1994), *Toxoplasma gondii* (Adams et al., 1990), *Trypanosoma cruzi* (Munoz-Fernandez et al., 1992), *Cryptococcus neoformans* (Granger et al., 1990), *Chlamydia trachomatis* (Mayer et al., 1993), and to schisostomula of the helminth *Schistosoma mansoni* (James et al., 1989).

NO is the labile product of the metabolic activity of two different isoenzymes: constitutive nitric oxide synthase (cNOS) and inducible nitric oxide synthase (iNOS). They are encoded by two different genes mapped on different chromosomes (Nathan, 1994). Even though they are partially homologous, they differ in many respects including tissue distribution, cofactor requirements, biological activity and mode of regulation (for a review, see Nathan, 1992; Lowenstein et al., 1994). The inducible form of NOS is

present in macrophages, polymorphonuclear neutrophils and endothelial cells. IFN-γ is the major physiological inducer of iNOS. Augmentation of iNOS mRNA and protein levels also occurs in response to combinations of IFN-γ and LPS, IL-1, TNF-α , or infectious agents such as mycobacteria or leishmania.

The mechanism responsible for the microbicidal/microbistatic effects of NO is not fully understood (for review, see Green et al., 1993). DNA damage was reported to occur after treatment of *Salmonella typhimurium* with NO-releasing compounds (Wink et al., 1991). Furthermore, it has been reported that exposure of *Clostridium botulinum* to acidified NO_2^- results in the formation of nitrosyl-Fe complexes (Nathan and Hibbs, 1991). NO gas or NO generated by activated macrophages interacts both with ferredoxin from *Clostridium pasteurianum* (Stuehr et al., 1989) and an iron-associated subunit of *Escherichia coli* ribonucleotide reductase. Cowden and Clark (1991) found that oxides of nitrogen diffuse into *Plasmodium falciparum*-infected erythrocytes, couple with cysteine or glutathione, and form intracellularly toxic nitrosothiol groups that are 1000 times more effective in killing *P. falciparum in vitro* than NO and sodium nitrate or nitrite alone. Alternatively, disruption of pH homeostasis by NO may contribute to the demise of certain intracellular pathogens like *Leishmania* by inhibiting its H^+-ATPase (Mauel et al., 1991).

Iron deprivation

The low concentration of free iron in body fluids creates bacteriostatic conditions for many microorganisms and is therefore an important defense mechanism against invading bacteria. Bacteria need iron for growth and thus have adapted to the limited iron availability in mammalian hosts by evolving diverse mechanisms for the assimilation of iron sufficient for growth (reviewed in Wooldridge and Williams, 1993). Cellular depletion of this metal suppresses DNA synthesis as well as the functioning of aerobic respiratory enzymes (Weinberg, 1992).

Iron is delivered to most mammalian cells via endocytosis of diferric-transferrin bound to the transferrin receptor (TfR). In the endosome, iron is released from transferrin and transferred to the cytosol. Once in the cytoplasm, iron is used either for metabolic processes or sequestered in ferritin (reviewed in Thorstensen and Romslo, 1990). Mobilization of iron from both transferrin and ferritin is dependent upon an acidic environment such as that found in endocytic vesicles and lysosomes. Iron-transferrin complex releases ferric ions to the intermediate iron pool only after the endocytic vesicle is acidified. Ferritin is thought to recycle iron to the intermediate iron pool by entering lysosomes and then undergoing pH-dependent proteolysis (Byrd and Horwitz, 1991).

Bacteria have developed several mechanisms to scavenge iron from their eukaryotic hosts. Siderophore-mediated iron uptake involves the synthesis of low molecular weight iron chelators called siderophores which compete with the host iron-binding glycoproteins ferritin and transferrin for iron. An alternative way to achieve sufficient iron-uptake level can occur via outer membrane protein receptors that recognize the complex of transferrin or ferritin with iron (reviewed in Otto et al., 1993).

The relationships between iron availability and microbistatic/microbicidal function of macrophages have been studied in models of infection including *M. tuberculosis* (Hart et al., 1982; Crowle and May., 1989), *M. avium* (Douvas et al., 1993), *L. monocytogenes* (Alford et al., 1991), *L. pneumophila* (Byrd and Horwitz, 1991), *Plasmodium* (Krogstad et al., 1985), and *Histoplasma* (Lane et al., 1993; Newman et al., 1994), and *Paracoccidioides* (Cano et al., 1994). Results from the previous studies support the conclusion that interference with intracellular iron metabolism dramatically affects the capacity of macrophages to control proliferation of intracellular microorganisms. Thus, the decrease in the intracellular concentration of iron, increases the ability of human (Byrd and Horwitz, 1991; Douvas et al., 1993; Newman et al., 1994) and mouse macrophages (Alford et al., 1991; Lane et al., 1993) to kill or arrest the growth of several intracellular microorganisms. Alford and colleagues suggested that macrophages

must have enough available intracellular iron to perform listericidal function, but too much iron can favor growth of the bacterium (Alford et al., 1991).

Cellular iron homeostasis is also very important in the post-transcriptional regulation of gene expression. A cis-acting RNA motif, the so-called iron responsive element (IRE), and a trans-acting cytoplasmic protein, known as iron regulatory protein (IRP) cooperate to coordinate the iron-dependent expression on the intracellular storage protein ferritin, of the TfR, and of erythroid 5-amino levulinic acid synthase (e-ALAS), the first enzyme in the heme biosynthetic pathway. Iron deprivation stimulates IRE-binding by IRP, thereby repressing ferritin and e-ALAS mRNA translation and protecting TfR mRNA from degradation, resulting in the increased transferrin receptor expression (reviewed in Klausner et al., 1993).

Recently, it has been shown that NO produced by cytokine-stimulated macrophages is involved in iron release (Drapier and Hibbs, 1988), modulation of iron-dependent enzyme activities (Hibbs et al., 1987; Drapier et al., 1988; Lepoivre et al., 1990), mobilization of iron from ferritin (Reif and Simmons, 1990) and formation of coordination complexes with iron-carrying macromolecules (Lancaster and Hibbs, 1990; Pellat et al., 1990). More recent evidence indicates that endogenously produced NO may modulate the post-transcriptional regulation of genes involved in iron homeostasis (ferritin, transferrin, IRP), supporting the hypothesis that [Fe-S] cluster of IRF mediates iron-dependent regulation (Drapier et al., 1993; Weiss et al., 1993). Furthermore, Weiss et al. (1994) observed that mRNA levels for NOS were significantly increased by treatment with desferrioxamine and reduced by Fe(3+). This increase in the steady-state levels of NOS mRNA was due to increased transcription rates of NOS mRNA. Therefore, parasites consuming iron would contribute to increased formation of NO and therefore to enhanced cytotoxicity toward themselves (Weiss et al., 1994).

Phagosome-lysosome fusion

Ingestion of a pathogen by cells of the MPS sets off a series of events including phagosome fusion to lysosomes. Lysosomes fuse with the pathogen-containing phagosome and release their acid hydrolases that are able to damage the ingested parasites. Intracellular parasites, however, have developed several strategies to avoid fatal consequences of phagosome-lysosome fusion. For example, *S. typhimurium* and *L. donovani* are able to survive and multiply inside the phagolysosome. Mechanisms such as presence of enzyme-resistant cell surfaces, secretion of enzyme inhibitors or secretion of molecules that increase intraphagolysosomal pH (ammonia), have been proposed to explain the survival of these parasites (reviewed in Moulder, 1985).

In the case of *Mycobacteria*, several strategies that might explain the survival of the bacteria in host cells have been observed. The prevention of phagosome-lysosome fusion has been proposed as the main survival mechanism of *M. tuberculosis*. Mycobacterial products have been proposed to interact with lysosomal membranes rendering them nonfusible. However, this is not the only explanation for survival, since some mycobacterial species such as *M. lepraemurium* as well as *M. tuberculosis*, survive in phagosomes that have fused with lysosomes, and *M. leprae* was shown to be capable of escaping from the phagosome into the cytoplasm (Moulder, 1985).

The importance of the phagosome-lysosome fusion in the control of mycobacterial growth inside macrophages has been stressed by experiments in which agents suspected to promote phagosome-lysosome fusion were utilized. Flesch and Kaufmann (1988) observed that chloroquine induced a dose-dependent growth inhibition of *M. bovis* in unstimulated bone marrow macrophages, and speculated that phagosome-lysosome fusion was involved. More recently, De Chastellier et al. (1993) studied the implications of phagosome-lysosome fusion in restriction of *M. avium* growth in bone marrow macrophages from BCG-resistant and -susceptible congenic mice. The authors found that

the percentage of phagosome-lysosome fusions was twice as high in *Bcg*^{r} compared to *Bcg*^{s} macrophages. In addition, the percentage of intact viable bacteria residing in acid phosphatase-negative phagosomes was about twice as low in *Bcg*^{r} as in their *Bcg*^{s} counterparts. An enhanced phagolysosome fusion in *Bcg*^{r} macrophages was proposed as the explanation for these findings (De Chastellier et al., 1993).

Since proper function of hydrolytic enzymes present in lysosomes depends on acidic pH, control of the phagolysosome pH might play an important role in the antimicrobial effect. The vesicles containing mycobacteria (phagolysosomes), were believed to be acidic vesicles (with a pH of approximately 5). However, Crowle et al. (1991) presented evidence indicating that this belief was probably correct only for dead or impaired *M. tuberculosis* and *M. avium*. Appelberg and Orme (1993) observed that the addition of weak acid to culture medium containing IFN-γ-stimulated and *M. avium*-infected mouse macrophages enhanced the bacteriostatic effect of IFN-γ, while the presence of weak base counteracted this effect. The authors hypothesized that the mycobacteriostatic effect of IFN-γ was mediated through the acidification of the infected phagosome, perhaps through activation of proton pumps in the phagosomal membrane (Appelberg and Orme, 1993). A direct assessment of intracellular acidity indicated that the vesicles in cultured human mononuclear phagocytes which contained living and healthy *M. tuberculosis* and *M. avium* were most likely the phagosomes that were not acidic (Crowle et al., 1993).

Reactive Oxygen Intermediates (ROI)

During phagocytosis, or upon appropriate stimulation, cells of the MPS produce substantial amounts of O_2^- and H_2O_2. These substances may subsequently interact whith each other and produce hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$). The increased production of ROI is coupled to a large increase in oxygen consumption, and in the activity of the hexose monophosphate shunt pathway (Badwey and Karnovsky, 1980;

Nathan, 1991). Most data suggesting that *Mycobacteria* are sensitive to the action of ROI come from indirect evidence showing that: 1) macrophages produce ROI during the phagocytosis of *Mycobacteria*, 2) macrophages activated by cytokines, bacterial products, or pharmacological agents, eg. phorbol myristate acetate (PMA), produce substantial quantities of ROI, 3) in cell free systems, ROI are able to kill *Mycobacteria* and, 4) under certain circumstances, scavengers of ROI partially inhibit the ability of activated macrophages to kill *Mycobacteria*.

Evidence accumulated during recent years, however, suggests that ROI do not play a prominent role in the control of intracellular proliferation of microorganisms. Macrophages derived from patients with chronic granulomatous disease (CGD) have a genetic defect of NAPDH oxidase (a key enzyme involved in production of ROI), and are able to kill intracellular microorganisms including *Mycobacteria*. Furthermore, use of scavengers of toxic oxygen metabolites (superoxide dismutase, catalase, histidine, diazabicyclooctane) failed to influence the capacity of IFN-γ-activated bone marrow macrophages to inhibit the growth of *M. bovis* (Flesch and Kaufmann, 1988). Douvas et al. (1986) also showed that human monocyte-derived adherent cells, during a period of increased mycobactericidal activity, do not have increased amounts of reactive oxygen species. In addition, Rook et al. (1986) also found that IFN-γ failed to stimulate human macrophages to suppress or kill *M. tuberculosis* even though the macrophages had an enhanced capacity to produce ROI as demonstrated by the ability to reduce nitroblue tetrazolium when stimulated by phorbol myristate acetate (PMA). Similarly, O'Brien et al. (1991) found that guinea-pig alveolar macrophages were able to kill *M. tuberculosis*, but the killing was independent of susceptibility to hydrogen peroxide or triggering of the respiratory burst.

Tryptophan deprivation

Results published in 1984 (Pfefferkorn et al., 1984) indicated that human fibroblasts infected with *Toxoplasma gondii* and stimulated with IFN-γ, were able to kill the protozoan parasite. They showed that the minimal concentration of IFN-γ that blocked the growth of *T. gondii* was consistently two fold higher in Dulbecco's modified MEM than in Eagle's minimal medium. An analysis of these media indicated that tryptophan was the factor responsible for this difference (tryptophan concentration was higher in the former). Consistent with these observations, Pfefferkorn and colleagues (1986) have found that the increase in the concentration of tryptophan in the medium diminished the antitoxoplasma effect of IFN-γ. The microbiostatic activity of human bladder carcinoma cells against *Chlamydia psittaci* induced by IFN-γ could be entirely reversed by the addition of exogenous tryptophan (Byrne et al., 1986). Similar antimicrobial mechanism seemed to be involved in IFN-γ-stimulated macrophages infected with *Leishmania donovani* (Murray et al., 1989) and *Legionella pneumophila* (Gebran et al., 1994).

Tryptophan is an essential amino acid and is the least abundant of the amino acids required for mammalian cellular integrity. Two mammalian enzymes catabolize L-tryptophan: indoleamine 2,3-dioxygenase (IDO), and hepatic tryptophan 2,3-dioxygenase (TDO). IDO catalyzes the initial pyrrole ring oxidative cleavage step in the degradation of L-tryptophan to produce N-formylkinurenine and kynurenine. IDO differs from TDO in its broader substrate specificity, broader tissue distribution, and nonconstitutive nature as well as its inducibility by cytokines such as IFN-γ (Carlin et al., 1987). IDO can be induced by cancer, viral infection, bacterial LPS, IFN and interleukin 2 (IL-2) (reviewed by Carlin et al., 1989). Increased urinary excretion of tryptophan metabolites has been reported to occur in several diseases including tuberculosis (Werner et al., 1987).

Human peripheral blood mononuclear cells (PBMC) and macrophages, as well as murine macrophages are able to synthesize IDO in response to a variety of stimuli

including IFNs (Byrne et al., 1986; Carlin et al., 1987; Murray et al., 1989; Gebran et al., 1994; Thomas et al., 1994). Furthermore, increases in IDO activity, and subsequent catabolism of tryptophan, have been correlated with the ability of human and murine macrophages to control proliferation of some intracellular microorganisms *in vitro* (Murray et al., 1989; Schmitz et al., 1989; Gebran et al., 1994).

REGULATION OF MHC CLASS II ANTIGENS IN *Bcg*^{r} AND *Bcg*^{s} MACROPHAGES

The class II genes of the major histocompatibility complex (MHC) encode highly polymorphic, cell surface glycoproteins (called Ia antigens). These molecules play a central role in the immune response by forming a fundamental part of the ligand for the antigen-specific T cell receptor. The Ia antigen-T cell receptor interaction is required for both the development of the T-cell repertoire in the thymus and the presentation of antigenic peptides to helper T cells in the periphery. Proper function of the Ia antigens depends not only on the polymorphic nature of their structures and their ability to bind the antigenic peptide but also on the regulated expression of these proteins on the surface of cells interacting with the appropriate T lymphocyte.

In the mouse, there are two isotypic forms of Ia: I-A and I-E. Each is a heterodimer composed of an α chain (33 to 34 kD) and a ß chain (28 kD) associated noncovalently on the cell surface (reviewed in Mengle-Gaw and McDevitt, 1985). The four murine class II genes are coordinately regulated and display a complex pattern of expression.

Regulation of MHC class II expression

Class II gene expression in the cells of B lineage is developmentally regulated. Immature pre-B cells are class II negative (Kincade et al., 1981), while mature B cells express class II antigens constitutively (Mond et al., 1981; Greenstein et al., 1981). Terminally differentiated plasma cells lose their capacity to express class II antigens (Halper et al., 1978). While class II molecules are expressed constitutively in B cells, they can be induced in macrophages by several stimuli. Both macrophage-like cell lines and tissue macrophages can be induced to express class II antigens by IFN-γ (King and Jones, 1983; Zlotnik et al., 1983; Stuart et al., 1988), but other cytokines have also been reported to modulate the expression of Ia antigens, including TNF-α, GM-CSF and IL-4 (Glimcher and Kara, 1992).

The regulation of surface class II expression is achieved through the combination of transcriptional and post-transcriptional effects. Transcriptional effects are mediated by factors that interact in a sequence-specific manner with elements found in the gene promoter, or within introns. Additional enhancer elements, located both in the introns and at the positions some distance upstream of the start of transcription, have been described for the class II genes (Glimcher and Kara, 1992; Ting and Baldwin, 1993; Sullivan et al., 1987). Most work on the class II cis-acting elements has involved the proximal promoter region, encompassing approximately 200 bp upstream of the transcription start site.

Initial comparisons of the sequences of the human and murine class II genes revealed two highly conserved sequence elements in the region 40 to 150 nucleotides 5' of the start site of transcription. These elements have been called the X and Y boxes, and several studies have shown them to be important for both the constitutive and induced expression of class II genes in the human and murine cells (Dorn et al., 1987; Thanos et al., 1988; Yang et al., 1990). Some other sequence motifs have also been considered important for class II gene expression. Experiments involving deletions at the 5' end of

the X box have revealed that a stretch of 30-40 bp, the Z/W area, including the 7 bp S box, play an important role in the class II regulation (Thanos et al., 1988; Basta et al., 1987; Dedrick and Jones, 1990).

The *Bcg* gene and the influence on MHC class II expression in macrophages

One of the most important observed pleiotropic effects of the *Bcg/Lsh/Ity* gene refers to its ability to upregulate MHC class II expression in macrophages. The first indication that macrophages from resistant and susceptible mice may differ in their Ia expression was reported by Johnson and Zwilling (1985). They reported that Ia antigen was stably expressed by macrophages from BCG-resistant mice but was expressed transiently by macrophages from BCG-susceptible mice (Johnson and Zwilling, 1985). The difference in I-A expression was linked to the *Bcg* gene by showing that I-A could be induced to be differentially expressed by macrophages from BALB/c (*Bcg*^{s}) and its congenic resistant counterpart BALB/c.*Bcg*^{r} (C.D2, *Bcg*^{r}) (Zwilling et al., 1987). Later, experiments conducted by Denis et al. (1988a) showed that the percentage of Ia$^+$ macrophages was significantly higher in the spleen of noninfected C.D2 mice when compared to BALB/c mice. Additional studies have shown that the frequency of Ia$^+$ macrophages was markedly increased in *Bcg*^{r}, but not in *Bcg*^{s} mice following BCG infection (Schurr et al., 1989). Similar differences in Ia expression were reported by Kaye et al. (1988). They reported that splenic adherent cells (SAC) obtained from *Lsh*^{r} mice infected for 7 days with *L. donovani* expressed more I-A antigen than *Lsh*^{s} mice. Furthermore, peritoneal macrophages from *Lsh*^{r} mice infected with amastigotes of *L. donovani*, and stimulated with IFN-γ, expressed more I-A antigen than peritoneal macrophages from *Lsh*^{s} mice treated in an identical manner (Kaye et al., 1988). The differences in MHC class II expression observed in different population of macrophages obtained from BCG-resistant and -susceptible animals may be partially explained by differences at the macrophage I-A mRNA level as published by Radzioch et al. (1991).

The reported differences in the amount of class II antigens expressed on the surface of *Bcg*^{r} and *Bcg*^{s} macrophages have been used to explain some other functional characteristics that differ between *Bcg/Lsh/Ity* resistant and susceptible animals. Thus, Denis et al. (1988b) reported that *Bcg*^{r} macrophages supported the mixed leukocyte reaction (MLR) across the H-2 barrier much better than the *Bcg*^{s} macrophages, and Kaye et al. (1988) reported that *L. donovani*-infected *Lsh*^{r} mice showed a rapid increase in accessory cell activity, allowing for a greater subsequent T-cell expansion as compared to *Lsh*^{s} mice. In addition, differences in the antigen presentation ability of macrophages obtained from BCG-resistant and -susceptible animals (Denis et al., 1988a), and *L. donovani*-resistant and -susceptible animals (Kaye et al., 1988) have been published. However, some other investigators reported no differences in antigen presentation by BCG-resistant and -susceptible mice (Hilburger and Zwilling, 1994).

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OBJECTIVES

Macrophages constitute an essential line of defense against intracellular microorganisms including Mycobacteria.

The major objective of the study was to define the molecular mechanism underlying the phenotypic differences observed between macrophages derived from mice resistant (B10A.*Bcg*^{r}) and susceptible (B10.A) to infection with *Mycobacterium bovis* BCG.

Specific aims:

Using bone marrow-derived macrophage lines generated from B10.A (*Bcg*^{s}; B10S cell lines) and its congenic resistant counterpart B10A.*Bcg*^{r} strain mice (*Bcg*^{r}; B10R cell lines), the following specific aims were address:

1. study the molecular basis responsible for differential nitric oxide production in response to infection with *Mycobacterium bovis* BCG and stimulation with IFN-γ, in the context of its biological importance for antimycobacterial activity.

2. molecular mechanisms underlying the difference in MHC class II antigen expression between macrophages derived from mice susceptible and mice resistant to infection with *M. bovis* BCG.

3. one of the important aims was also to develop a fast, simple and reliable method to estimate mycobacterial infection and multiplication in macrophages.

CHAPTER II

ASSESSMENT OF MYCOBACTERIAL INFECTION AND MULTIPLICATION IN MACROPHAGES BY PCR.

PREFACE

Reliable *in vitro* methods for the detection of mycobacterial growth within macrophages are essential for the objective determination of the resistant or susceptible phenotype dictated by the *Bcg* host resistant gene. Realization of the shortcomings of the currently available assays, namely the lack of objectivity and the lengthy readout time, prompted us to exploit the polymerase chain reaction (PCR) methodology. This methodology allows the quantitation of mycobacteria-specific DNA sequences within infected macrophages. Twenty-base oligonucleotide primers that recognize a 123 bp repeated sequence present in *M. bovis* and *M. tuberculosis* DNA have been used to describe the conditions of the PCR. This time-efficient technique yielded highly reproducible results which correlate well with those obtained by two independent, currently available methods. The assay has proven to be useful for the rapid determination of the level of mycobacterial growth in infected cells, in the routinely studied experimental models of infection with *Mycobacteria*.

SUMMARY

The Polymerase Chain Reaction (PCR) has been successfully used for the diagnosis of mycobacterial diseases. The amplification of mycobacterial DNA has been performed using 20-bp oligonucleotide primers that recognize a 123 bp repeated sequence

present in *M. bovis* and *M. tuberculosis* DNA. Here we describe in detail the conditions of PCR reaction which allow a quantitative assessment of mycobacterial content in infected macrophages. The technique shows good correlation with the widely used Colony Forming Unit (CFU) and [^{3}H]uracil incorporation methods. It is highly reproducible, effective and time-efficient. Our method allows a quantitative assessment of the level of *M. bovis* BCG infection in macrophages from a variety of sources including peritoneal macrophages and macrophage lines, within few hours.

INTRODUCTION

Mycobacteria are facultative intracellular parasites which replicate inside cells of the mononuclear phagocyte system. Failure to control the intracellular growth of different species of mycobacteria by monocytes and macrophages leads to cell destruction and disease in several hosts including man, cattle, rabbits, guinea pigs and mice (16,18).

Several *in vitro* methods have been developed to detect the presence of intracellular mycobacteria and to quantitate their growth within macrophages (3,13). Although these procedures are informative to some extent, there are some limitations associated with each method. Firstly, light microscopy and an acid-fast stain may be used to assess the percentage of infected macrophages in a given population, but the estimation of the number of acid-fast bacilli (AFB) per infected macrophage is rather subjective. Secondly, the enumeration of mycobacterial CFU in infected tissues is most reliable, but requires at least two weeks and special care to avoid bacterial clumping during seeding (4). Thirdly, the widely used measurement of [^{3}H]uracil incorporation by metabolically active bacteria (13), although quantitative, does not account for either the metabolically inactive (dormant) bacilli or the dead microorganisms, precluding estimation of the total load of intracellular bacteria under specific experimental conditions.

Recently, amplification of mycobacterial DNA by the Polymerase Chain Reaction method (PCR) was successfully used for diagnosis of *M. leprae* (17) and *M. tuberculosis* (1,6) in clinical samples. Using primers specific for mycobacterial DNA sequences, we describe the conditions which allow precise quantitative assessment of BCG content in infected macrophages. Furthermore, we have compared the results obtained using this method with widely used CFU and [^{3}H]uracil incorporation methods.

Overall, the quantitative PCR method provides a reliable, simple, reproducible and a rapid tool for the assessment of mycobacterial content in infected macrophages.

MATERIALS AND METHODS

Bacteria.

Mycobacterium bovis BCG substrain Montreal was used. The stock culture was cultivated twice for 7 days in Dubos-Tween-Albumin liquid medium, and the last culture was filtered through a 5 μm membrane (Gelman, Ann Arbor, MI) to eliminate bacterial clumps. The resulting bacterial suspension was centrifuged at 10,000 x g at 4°C for 20 min and, the cell pellet was resuspended in Neuman-Tytell medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) without antibiotics (Neuman-Tytell Complete Medium, NTCM).

Macrophages and macrophage lines.

To obtain resident peritoneal macrophages, mice were killed by CO_2 inhalation and the peritoneal cavity was flushed with 10 ml of ice-cold NTCM. The resulting cell suspension was centrifuged at 800 x g for 10 min at 4°C and resuspended in NTCM. The percentage of macrophages in cell suspensions was estimated by performing a differential count on cytocentrifuged cells following Diff-Quik staining (AHS del Caribe Inc., Puerto Rico), and the cells were subsequently seeded at $1x10^6$ macrophages/well

in 24-well cultures plates (Falcon, Lincoln Park, NJ). After overnight incubation at 37°C, 5% CO_2, cells were washed with warm NTCM to remove non-adherent cells.

Macrophage lines B10R and B10S derived from congenic B10A.*Bcg*^{r} and B10A.*Bcg*^{s} murine strains, respectively, were established as described in detail (12). Cells were grown in Dulbecco's modified medium (ICN Biomedicals Inc., Costa Mesa, CA) supplemented with 10% FBS, 2 mM L-glutamine (ICN Biomedicals Inc., Costa Mesa, CA) and antibiotics. Cell lines were used when they became confluent.

Purification of genomic DNA from BCG

The bacterial suspension was centrifuged at 10,000 x g for 20 min at 4°C and resuspended in TE buffer, pH 8.0 (Tris.HCl 10mM, EDTA 1mM). Bacteria were disrupted by bead-beating for 5 min in a Mini Beadbeater (Biospec Products, Bartlesville, OK) using 1 ml of 0.1mm zirconium beads (Biospec Products, Bartlesville, OK) pre-equilibrated in TE, pH 8.0. DNA was extracted with 2 volumes of phenol:chloroform:isoamyl alcohol (25:24:1) twice and 3 times with chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 3 vols 100% ethanol, 200 μl of 3M NaAcetate and 10 μg of yeast tRNA at -20°C. After overnight incubation, samples were centrifuged at 25,000 x g for 20 min at 4°C in an Eppendorf centrifuge, washed two times with 70% ethanol and dried by speed vacuum centrifugation. DNA was resuspended in TE, pH 8.0 and the concentration was estimated by spectrophotometry at 260 nm.

Amplification of mycobacterial DNA by PCR

Amplification reactions were performed using Taq polymerase derived from *T.aquaticus* and other reagents as directed by manufacturer's instructions (GeneAmp kit, Perkin-Elmer Cetus, Norwalk, CT). The primers used were specific for *M. bovis* and *M. tuberculosis* as previously described (6). The reaction was performed using an automated thermal cycler (DNA Thermal Cycler, Perkin-Elmer Cetus, Norwalk, CT). Amplification reactions were performed as described (6). Briefly, samples were

denatured at 94°C for 5 min, and 25 amplification cycles were performed. Each cycle consisted of denaturation at 94°C for 2 min, annealing of primers at 68°C for 2 min, and primer extension at 72°C for 2 min. The extension was increased by 5 s with each subsequent cycle. In some experiments, the annealing time was modified. The amplification product was analyzed by electrophoresis on 1% agarose gels containing ethidium bromide.

Infection of macrophage monolayers.

Macrophage lines and resident peritoneal macrophages were used. Cells were seeded into 24 well culture plates, incubated overnight and infected at a bacteria:macrophage ratio of 1:1, 5:1, or 10:1 with *M. bovis* BCG for different periods of time. At the end of infection period, cells were washed extensively with warm NTCM and then lysed with 1% NP-40 in TE, pH 8.0 or 0.25% saponin in NTCM.

When experiments using the [^{3}H]uracil incorporation technique or the CFU determination were done, cells were seeded at 1x10^5/well in 96-well flat bottom microtiter plates (Nunclon, Nunc, Denmark). After lysis, 1 μCi/well of [^{3}H]uracil (sp. act. 27 Ci/mmol, New England Nuclear) was added to each well for 18h. Cell lysates were collected in fiberglass membranes (Skatron, Sterling, VA) using a semiautomatic cell harvester (Skatron, Lier, Norway) and the radioactivity incorporated in the individual wells was quantitated by scintillation counting using a ß-counter. For the CFU assay, several dilutions prepared from lysates of the infected cells were done and seeded in Dubos solid medium at 37°C. Colonies were enumerated following a 3 week incubation period.

Purification of mycobacterial DNA from infected cells.

0.5 to 1 ml of infected macrophage lysates were disrupted with 0.5 ml of zirconium beads for different periods of time using 2 ml Sarstedt Micro tubes (Newton, NC). Following disruption, supernatants were transferred to new vials and heated in boiling water for 10 min to inactivate proteases. DNA was subsequently extracted twice with 1

vol of TE-equilibrated phenol, pH 8.0 (14), and once with chloroform:isoamyl alcohol (24:1). DNA was precipitated and the DNA pellet was resuspended in DEPC-treated water. In some experiments, DNA was neither extracted nor purified. After lysis and boiling, 10 μl of crude extract were used for DNA amplification.

Quantitation of amplification products.

To quantitate products of the amplification reactions, blots were hybridized with purified PCR product labelled with [^{32}P]dCTP (sp. act. aprox. 3000 Ci/mmol, Amersham, Arlington Heights, IL). The 123-bp PCR product was purified by electrophoresis in 3% low melting agarose gel (NuSieve, FMC, Rockland, ME). After electrophoresis, the band corresponding to the amplified product was cut out and purified by a modification of the method of Heery et al. (10) using glass beads and DNA was precipitated with ethanol. To quantitate amplification reactions, DNA was separated by electrophoresis in 1% agarose gels and transferred to nylon membranes (Nytran, Schleicher and Schuell, Keene, NH). The membrane was removed from the blotting apparatus and baked at 80°C for 30 min, prehybridized for 4h at 48°C in prehybridization buffer (Hybridisol, Oncor, Gaitherburg, MD), containing 100 μg/ml of denaturated Salmon Sperm DNA, and hybridized for 24h with the same buffer containing 10^7 cpm of the 123 bp probe labelled by nick translation using [^{32}P]dCTP. The membranes were washed twice in 10X SSPE, 1%SDS, 15 min at room temperature, once in 1X SSPE, 1%SDS, 15 min at 37°C and once in 0.1X SSPE, 1% SDS, 15 min at 65°C. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). In some experiments, the intensity of bands present in the autoradiograms were quantitated using laser densitometry (LKB UltroScan XL Laser Densitometer).

RESULTS AND DISCUSSION

Since DNA amplification by the polymerase chain reaction (PCR) is a highly specific and sensitive method, it is applicable even when small amounts of DNA are

present. This fact is particularly important in the case of detection of bacterial or viral DNA from cells when a low level of infectivity is suspected (9,11). Moreover, often only limited numbers of macrophages from key organs such as the spleen are available for *in vitro* studies on infection and replication of intracellular parasites. We present here the conditions which allow a quantitative estimation of the mycobacterial DNA content in infected resident peritoneal macrophages and in infected macrophage lines.

The 20-bp oligonucleotide primers specific for *M. bovis* BCG and *M. tuberculosis* which recognize a 123-bp repeated sequence were recently reported by Eisenach et al (6). As a first step we studied the sensitivity of the method using our specific conditions. Genomic DNA was extracted from 10^7 *M. bovis* BCG, purified, and the DNA concentration was estimated. Decreasing amounts of DNA (1ng to 10fg) were used as templates for PCR reactions. As shown in Fig.1 even 10 femtograms of template mycobacterial DNA could be detected using this method. Since the size of the *M. bovis* BCG genome measures approximately 2,800 kb (2), 10 femtograms correspond to roughly 100 bacteria. Based on these results and those published by Eisenach et al (6) and Williams et al (17), it is reasonable to expect that small numbers of bacteria may be detected.

Since in the experimental conditions routinely used in bactericidal assays, the average number of bacteria ranges from 10^5-10^7, per sample, we tested whether or not PCR amplification of mycobacterial DNA would remain quantitative within this range. Initially, we used logarithmic dilutions of bacteria in the range of 10^9 to 10^5. As shown in Fig.2, 10^5 bacteria were detected by hybridization with the labelled PCR fragment. Moreover, the intensity of the detected signal was directly proportional to the number of bacteria used for DNA extraction.

Having established basic parameters for the quantitative detection of mycobacterial genomic DNA, we next turned our attention to the quantification of BCG-specific DNA

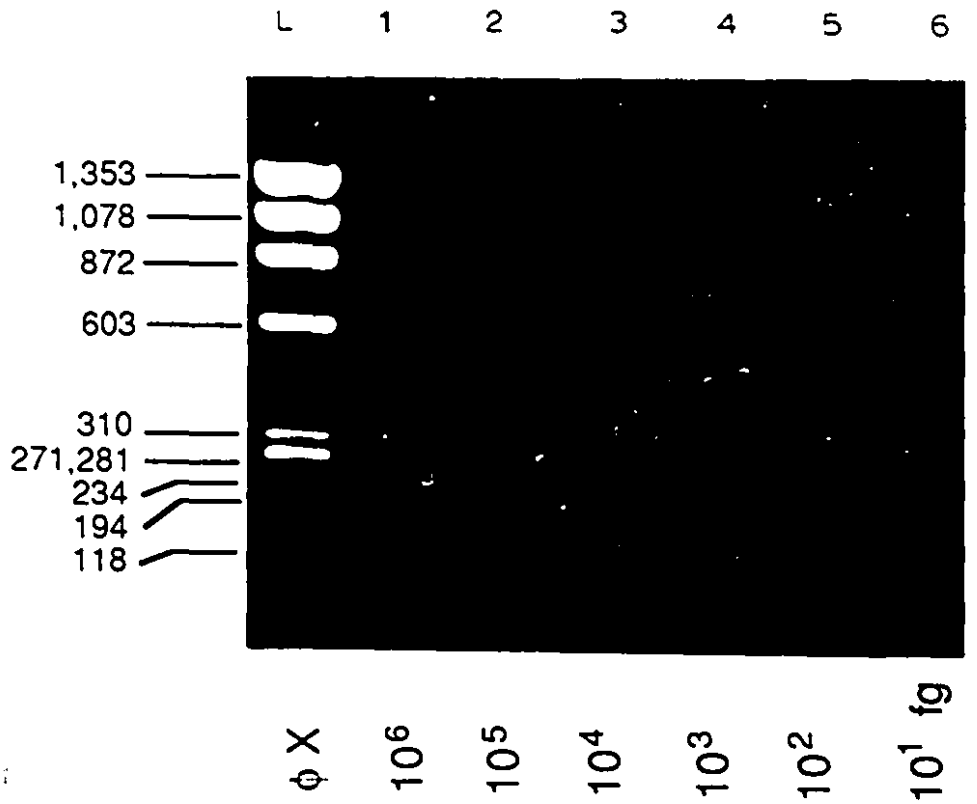

FIGURE 1. Detection of purified genomic DNA from *M. bovis* BCG by PCR. 1/10 vol of each reaction was separated in a 1% agarose gel. L, ϕX174 Hae III digested DNA ladder. Input DNA: 1, 1ng; 2, 100pg; 3, 10pg; 4, 1pg; 5, 100fg; 6, 10fg.

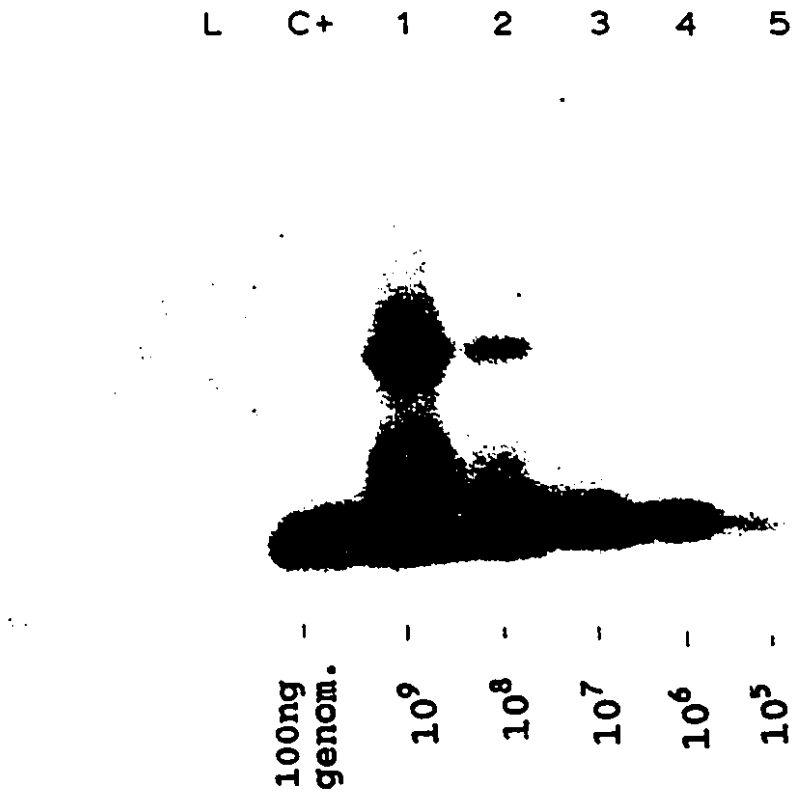

FIGURE 2. Detection of *M. bovis* BCG by PCR. PCR products were separated in 1% agarose gel, labelled, prehybridized and finally hybridized with [^{32}P]-labeled 123-bp purified PCR fragment. L, ϕX174 Hae III digested DNA ladder; C_+, 100 ng of input genomic DNA from BCG. Lanes 1-5, serial dilutions of BCG: 1, 10^9; 2, 10^8; 3, 10^7; 4, 10^6; 5, 10^5.

sequences in a variety of infected macrophage populations, at various times post-infection.

When resident peritoneal macrophages were infected for 4 or 48h using bacteria:macrophage ratios of 1:1 (lanes 1 and 4), 5:1 (lanes 2 and 5) or 10:1 (lanes 3 and 6), the amplification of mycobacterial DNA was proportional to the infectious doses 4 hours after the initial infection and, even more importantly, we could monitor the relative proliferation of BCG inside of the macrophages two days after the initial infection (Fig 3). Since it has been reported that an excess of eukaryotic DNA could interfere with the amplification of bacterial DNA (17), we performed experiments in which a constant concentration of mycobacterial DNA was amplified in the presence of increasing amounts (50ng-1μg) of genomic DNA isolated from macrophages lines.

No modification of the level of amplified mycobacterial DNA content was detected in the presence of eukaryotic genomic DNA within this range of concentrations (results not shown).

Since macrophage genomic DNA content did not seem to affect the PCR amplification of bacterial DNA, our next step was to establish whether or not crude lysates of infected macrophages which also contain proteins, could be used as templates for PCR amplification reactions. To test this possibility, macrophages were infected for 96h and mycobacterial growth was estimated using purified DNA or the crude macrophage lysate. The results indicated that the use of either purified DNA (Fig.4A) or the crude macrophage lysate (Fig.4B) resulted in similar amounts of amplification by the PCR technique. These results clearly indicated that the procedure could be simplified, since DNA purification was not required for adequate amplification.

Next, we infected different macrophages lines with BCG and monitored the replication of the organism by PCR. Following 24h infection with BCG a clear amplification signal was detected in all macrophage lysates tested (Fig.5A). Similarly,

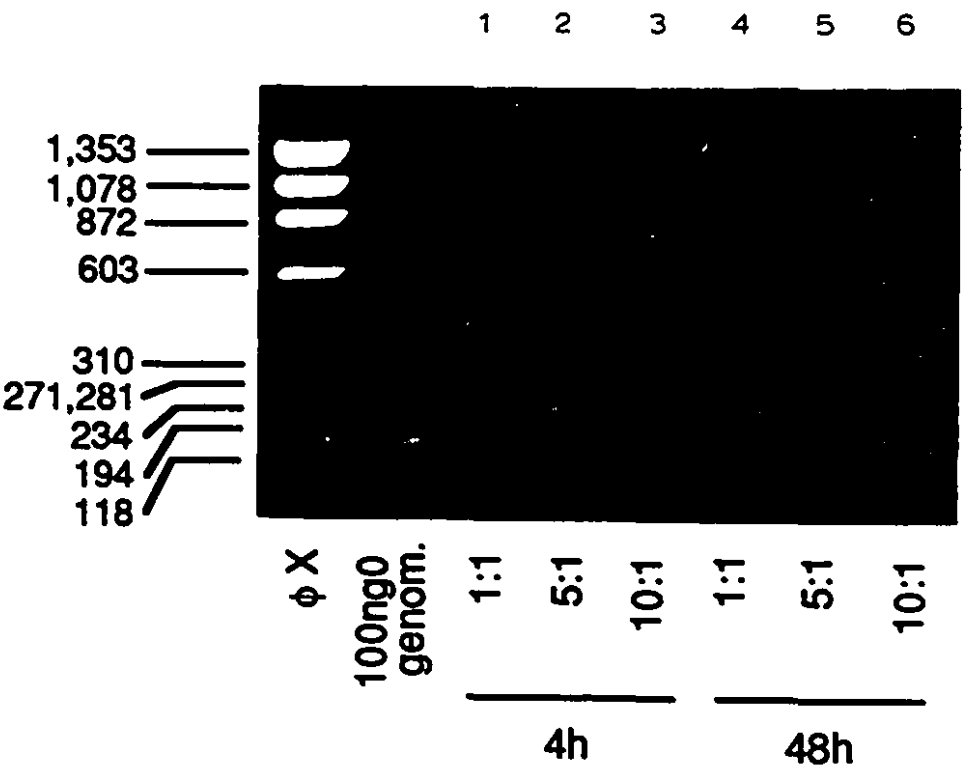

FIGURE 3. Mycobacterial DNA amplification of resident peritoneal macrophages infected with *M. bovis* BCG. Macrophages were infected for 4h (lanes 1-3) or 48h (lanes 4-6) with a mycobacteria to macrophage ratio of 1:1 (lanes 1 and 4), 5:1 (lanes 2 and 5) and 10:1 (lanes 3 and 6). Half of the final volume (10 μl) was used for the amplification reaction (35 cycles).

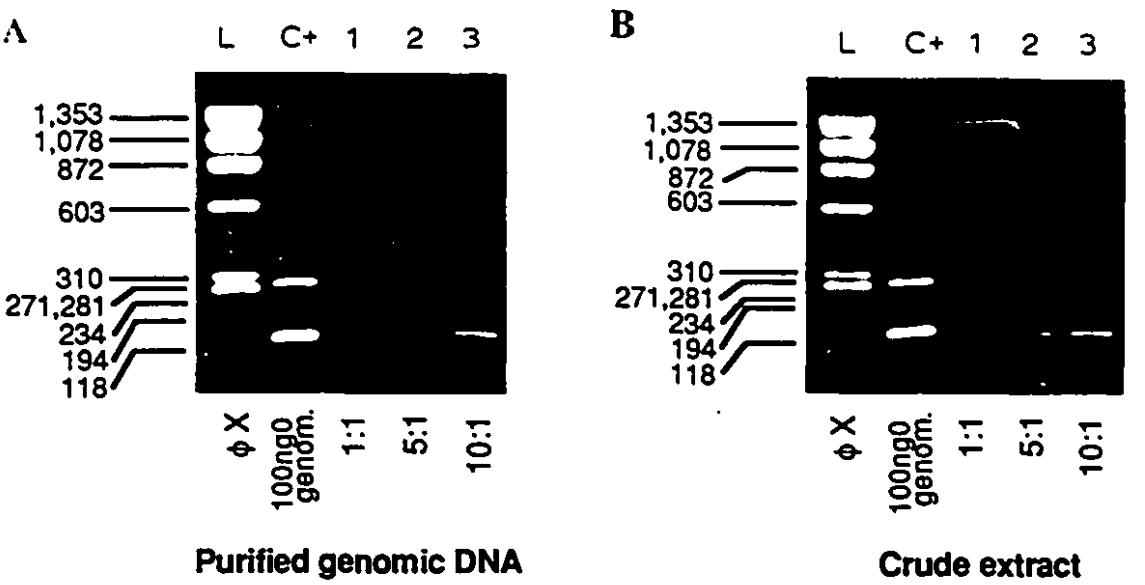

FIGURE 4. Amplification of DNA from *M. bovis* BCG from infected macrophages using the crude extract. Resident peritoneal macrophages ($1x10^6$/well) were infected for 4h, washed and cultured for 4 additional days. At the end of the culture period, cells were lysed and PCR performed as indicated.

Panel A: Amplification using purified DNA. Half of the final volume (20 μl) was used for the amplification reactions (35 cycles). Lane 1, 1:1 infection; lane 2, 5:1 infection; lane 3, 10:1 infection.

Panel B: Shows results generated when crude extracts were used. Ten microliters (1/50 of the final volume) were used for the amplification reactions (35 cycles). L, ϕX174 HaeIII digested DNA ladder; C+, 100ng of input purified genomic DNA from BCG.

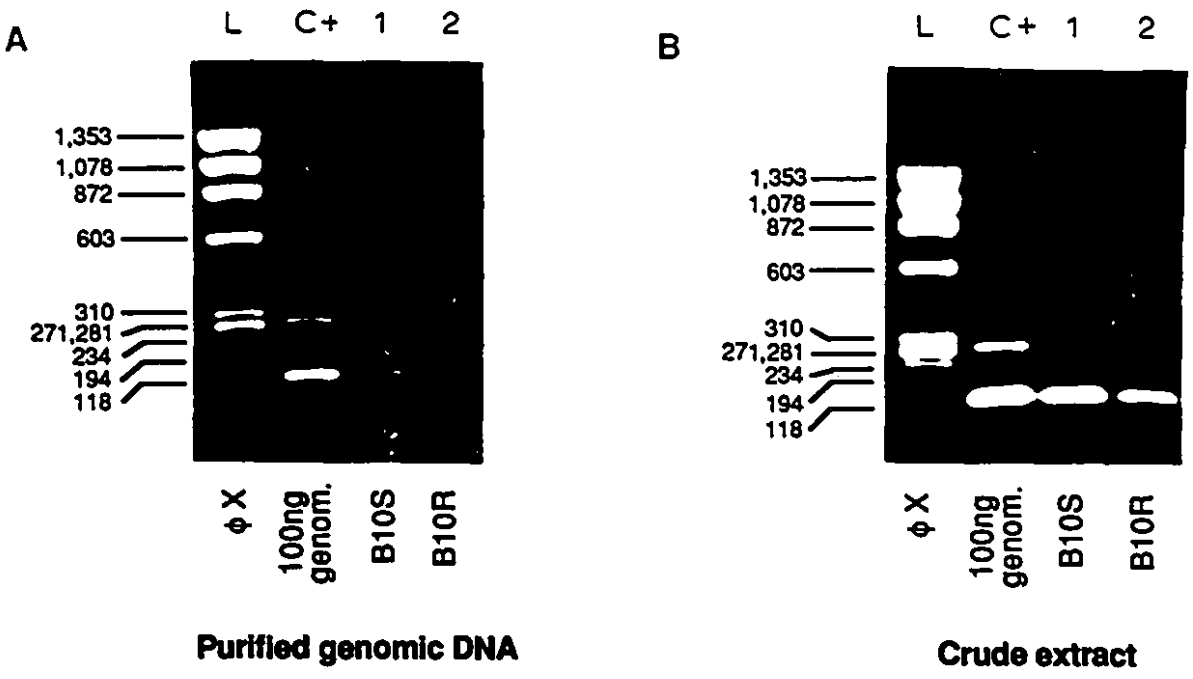

FIGURE 5. Mycobacterial DNA amplification from infected macrophages lines. Macrophages ($1x10^6$/well) were infected with BCG (mycobacteria to macrophage ratio, 10:1) for 24h, lysed and mycobacterial DNA amplification (35 cycles) performed using half of the final volume (20 μl) (Panel A), or 10 μl of the crude extract (1/50 of the final volume) (Panel B). L, ϕX174 HaeIII digested DNA ladder; C_+, 100 ng of input genomic DNA purified from *M. bovis* BCG; 1, B10S; 2, B10R.

when samples comprised of the crude extract were used in the reaction, an almost identical amplification was obtained (Fig.5B), indicating that neither macrophage-derived DNA nor other cellular components, such as proteins, interfered with the amplification of the mycobacterial DNA. Note that the quantity of BCG-DNA amplified in the macrophage line derived from the susceptible mouse strain was greater than that obtained from the line derived from its resistant congenic counterpart.

A final series of experiments was undertaken in order to follow the bacterial growth within macrophages, using three complementary methods in parallel: namely, PCR, [^{3}H]uracil uptake and CFU.

The macrophages were infected for 8h with different numbers of BCG, washed to eliminate extracellular bacilli and the bacterial growth was monitored 4 days later. The results 8h following infection of the macrophages demonstrated that there exists a direct relationship between the size of the inoculum of BCG, and the results obtained from the densitometric scanning, isotopic uracil uptake and the enumeration of the CFU (Fig.6A). The results obtained 4 days after the infection are shown in Fig.6B. An increase in all the parameters measured (total DNA load, metabolically active and live bacteria) led us to conclude that multiplication of the intracellular bacteria took place during this period and thus, a comprehensive view of the fate of the intracellular mycobacteria could be reached by using the three methods.

So far, most reports detailing PCR amplification of DNA sequences derived from infected tissue have emphasized the practical use of the methodology for clinical diagnostics (1, 6, 15, 17). In this report, we present the conditions which allow the use of PCR technology for quantitative monitoring of the mycobacterial infection of macrophages derived from peritoneal cavity, and of macrophage lines derived from genetically-resistant or -susceptible inbred strains of mice.

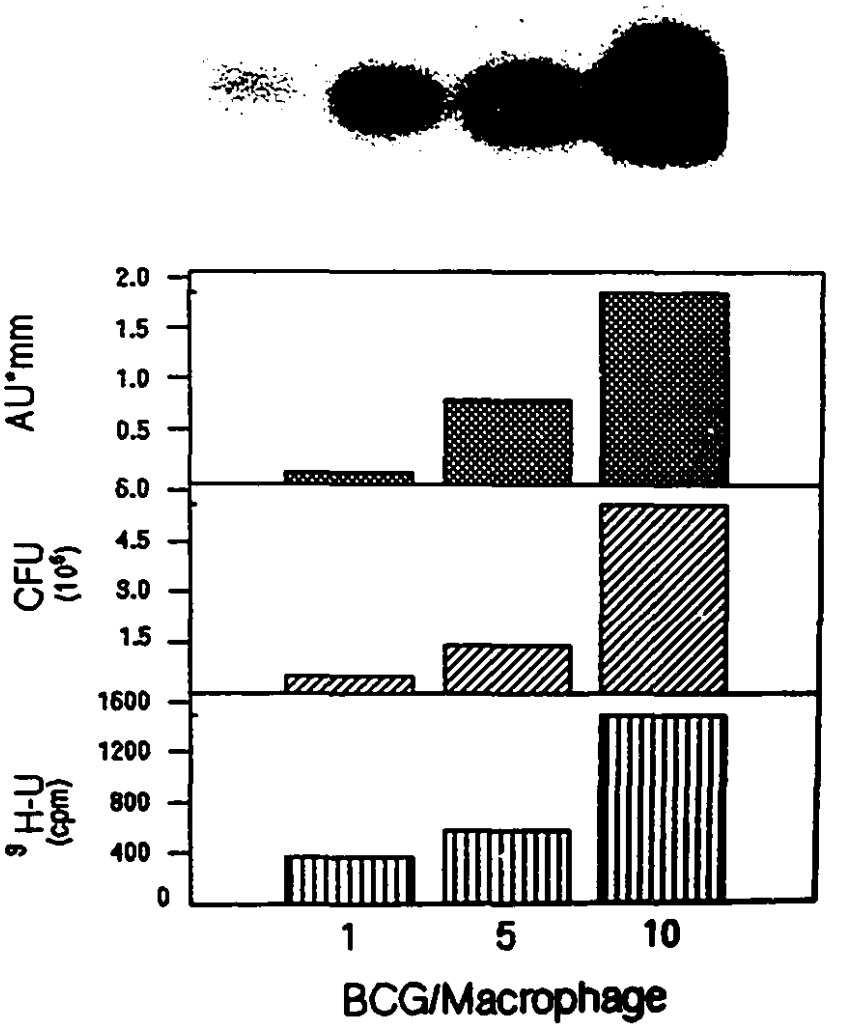

FIGURE 6A. Comparison of three different methods used for assessment of mycobacterial growth within macrophages. Results obtained 8 hours following infection. The B10S macrophage line ($1x10^5$) was infected with different ratios (1:1, 5:1, 10:1) of BCG for 8h. After lysis, intracellular mycobacterial growth was quantified by densitometric analysis of the autoradiograms obtained after 25 cycles of PCR, [^{3}H]uracil uptake and CFU. AU*mm=absorbance units/millimetre; [^{3}H]uracil uptake in cpm; CFU=colony forming unit.

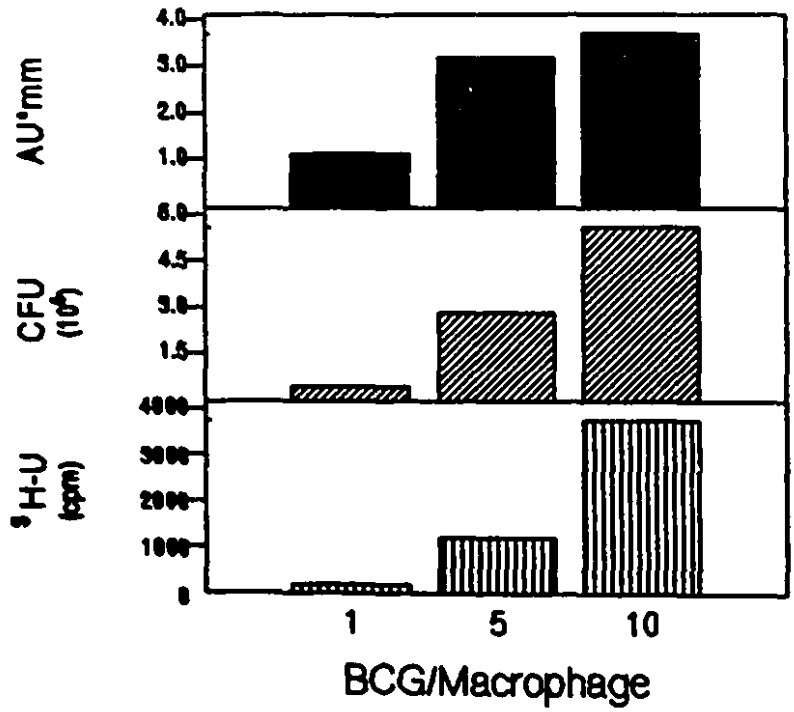

FIGURE 6B. Comparison of three different methods used for assessment of mycobacterial growth within macrophages. Results obtained 4 days following infection. 10^5 cells (B10S macrophage line) were infected with different ratios of BCG (1:1, 5:1, 10:1) for 8h, washed and cultured for 96 hours. After lysis, intracellular mycobacterial growth was quantified by densitometric analysis of the autoradiograms obtained after 30 cycles of PCR reactions, [^{3}H]uracil uptake and CFU. AU*mm=absorbance units/millimetre; [^{3}H]uracil uptake in cpm; CFU=colony forming unit.

The results of our experiments clearly show that the procedure can be applied to the study of mycobacterial infection and proliferation in peritoneal macrophages, as well as in macrophage lines. This method is fast, reliable and correlates with data obtained using CFU and [^{3}H]uracil incorporation methods. Use of this method will facilitate study of the course of mycobacterial infection in different macrophage subpopulations especially splenic macrophages. Several reports describing the course of *in vivo* infection with BCG indicate that spleen macrophages are one of the main effector cell populations during the early growth phase of the organism (5, 7, 8). *In vitro* studies in this system have been hampered by the low yields and poor quality of splenic macrophages due to the extensive manipulation required for their purification. Our adaptation of the PCR method is an excellent tool in the study of infection and multiplication of *M. bovis* BCG in macrophage lines activated for bactericidal function by treatment with cytokines such as interferon-gamma. The results of the present study open the possibility that PCR technology may be used as a quantitative, fast, reliable method of typing the resistance or susceptibility of hosts to infection by a variety of microorganisms, under a variety of conditions designed to test the immunologically-and biochemically-mediated mechanisms of defense.

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CHAPTER III

NITRITE PRODUCTION BY MACROPHAGES DERIVED FROM BCG-RESISTANT AND -SUSCEPTIBLE CONGENIC MOUSE STRAINS IN RESPONSE TO IFN-γ AND INFECTION WITH BCG.

PREFACE

Recent results from a number of laboratories suggest that the secretion of reactive nitrogen intermediates maybe an important means by which macrophage microbicidal activity is generated. We have found that exposure of *M. bovis* BCG to the nitric oxide-generating agent, namely $NaNO_2$ causes a dose-dependent inhibition of bacterial proliferation. This discovery confirms the importance of reactive nitrogen intermediates in host resistant to mycobacterial infection. Furthermore, the B10R macrophages produced significantly more NO_2^- than their B10S counterparts. This result reflected the finding that the expression of nitric oxide synthase mRNA, tested in several B10R and B10S macrophage clones, was higher in B10R as compared to B10S independent macrophages treated with IFN-γ. These data suggest that the differential production of reactive nitrogen intermediates by B10R and B10S macrophages may account for the observed phenotypic differences in their ability to inhibit mycobacterial growth.

SUMMARY

Reactive nitrogen intermediates (RNI) have been implicated in the IFN-γ-induced antimicrobial 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 as compared with B10R macrophages in response to IFN-γ. By 48 hours following treatment with 10 U/ml IFN-γ, B10R macrophages produce approximately three fold 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 *M.bovis* BCG, was higher in B10R macrophages as compared to B10S macrophages. The bacteriostatic activity of B10R and B10S macrophages correlated with the amount of nitric oxide produced by the macrophages. The antimycobacterial activity was inhibited by N^{g}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^{g}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 *M. tuberculosis*, *M. leprae* and *M. bovis*. Macrophages also constitute the first and essential line of defense 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 mice from 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).

Paralell studies of the host response to *Salmonella typhimurium* (3) and *Leishmania 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 MHC antigens and I-A_s mRNA expression are elevated in Bcg^r strain macrophages when stimulated with PMA and/or 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 ($NO^{\cdot}$) (11). Current investigations indicate that $NO^{\cdot}$ 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 microorganisms (17). Recently, a candidate for the putative *Bcg* gene (denominated *Nramp-1*) was cloned by Vidal et al.(18). The *Nramp-1* gene was found to be expressed exclusively in macrophage populations from reticuloendothelial organs and macrophage lines. Interestingly, a structural similarity was found between *Nramp-1*

and the membrane transporter CrnA, responsible for nitrate import in the eukaryote *A.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*^{r}) and B10.A.*Bcg*^{r} congenic mice.

We have 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^gMMLA), 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 Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Richmond, CA), Bovine Serum Albumin, sodium nitrite, d3-phosphoric acid, L-glutami-

ne, saponin, sulfanilamide, and N-(1-naphtyl) ethylenediamine were all purchased from Sigma (St. Louis, MO). [5,6-^{3}H]Uracil (sp. act., 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 form Fisher Scientific (Fair Lawn, NJ). Recombinant IFN-γ was purchased from AMGEN Biologicals (Thousand Oaks, CA); the specific activity was $>4x10^6$ units/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). B10A.*Bcg*^{r} mice were bred in the Montreal General Hospital Research Institute Animal Facility, under specific pathogen free (SPF) condition.

Macrophage cell lines.

Macrophage lines were derived from the bone marrow of B10.A and B10A.*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 was washed two times in PBS without calcium and magnesium.

Quantification of nitrite production by macrophages.

The estimation of NO_2^- in supernatants of stimulated and nonstimulated macrophages was performed by colorimetric spectrophotometry at 543nm using the Griess reagent (19). Background values were subtracted from those obtained for all experimental samples.

Results are expressed as the nitrite production (mmol) per total protein content of macrophages (estimated by the Bradford method at 595nm).

Infection and/or stimulation of macrophage lines and mouse cells.

To study the production of nitrite, $1x10^6$ macrophages were seeded in one 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 bottom plates. After 2h of adherence, macrophage lines were infected with *M.bovis* BCG (Montreal strain) for 48h 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 have also measured the nitrites production in macrophages seeded into 96-well flat bottom microtiter plates (Nunclon, Nunc, Denmark). In this case, macrophages were initially infected with BCG for 4 h using a bacteria to macrophage ratio of 20 to 1 then they were washed 6 times. The macrophages were stimulated with IFN-γ and/or plated in the presence of N^g-monomethyl arginine (N^gMMLA) to a final volume of 200 μl. After 4h, a period of time sufficient to allow phagocytosis of the bacteria, the cells were extensively washed (6 times) with 200 μl volumes of warm CM to eliminate extracellular bacilli, and IFN-γ or N^gMMLA was replaced to the original concentration. To ascertain for the presence of extracellular bacilli into the washed wells, the number of 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 ^{3}H-uracil. After 16h, 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 hours at 37 °C, 5% CO_2, in 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.

$1x10^5$ macrophages were seeded in 200 μl of medium containing 10% FBS but without antibiotic. Macrophage infection, lysis and quantification of the incorporation of ^{3}H-uracil after 48 h were done as previously described in Materials and Methods section. IFN-γ, 10 U/ml, N^{g}MMLA, 200 μM, and increasing concentrations of L-arginine (0.1 mM and up to 10 mM) was added to the wells (final volume, 200 μl). Results were expressed as the percentage of bacteriostatic activity and they were compared to the amount of nitrite present in the wells. The bacteriostatic activity of the macrophages was calculated as follows:

% bacteristasis =

$$[1 - (\text{c.p.m. M}\phi + \text{bacteria} + \text{modulator})/(\text{c.p.m. M}\phi + \text{bacteria})] \times 100$$

where the modulator is N^{g}MMLA, IFN-γ, or L-arginine, depending on the particular experiment.

Four replicas were done for each treatment. Results represent the average of 3 independent experiments. The Neuman and Tytell medium used for cell culture contains 200 μM of L-arginine.

Northern blot analysis.

A 2.3Kb fragment of the putative macrophage nitric oxide synthase gene (20) was subcloned in pCL-BS3 plasmid conferring Amp^r (kindly provided by Dr. S. Snyder, Johns Hopkins University, Baltimore, MD.). To perform Northern analysis of iNOS, $1x10^7$ macrophages were seeded in 100mm 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 (21). Briefly, the RNA was isolated by centrifugation on CsCl cushions and separated on 1.2% agarose gels containing 2.2 M formaldehyde. $1x10^6$ cpm per ml of the NOS probe labelled by random priming was added to the hybridization buffer. The GADPH probe was generated

by PCR using the following oligonucleotide primers: sense primer, 5' CCC TTC ATT GAC CTC AAC TAC ATG G 3', antisense 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, that 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 & Schuell, Keene, NH), and hybridized with probes which were labelled to high specific activity ($>10^8$ cpm/μg) by the random priming technique using the Random Priming System (BRL, Gaithersburg, MD).

Effect of acidified NO_2^- on *M.bovis* BCG.

$1x10^6$ colony forming units (CFU) of *M.bovis* BCG Montreal were seeded into flat-bottom microtiter plates and incubated at 37 °C for 48 h in 200 μl of CM medium (without antibiotics) containing different concentrations of sodium nitrite (range 0.1 to 10mM) titrated to a pH 5.0. Bacteria were pulsed with 1.0 μCi of [^{3}H]Uracil for 16 h. The antimycobacterial effect of NO_2^- was determined by the decrease of [^{3}H]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. 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 *Mycobacterium 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 (*Bcg*^{s}) and B10.A.*Bcg*^{r} 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^- production by the macrophages infected for 48h 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 as compared to nonstimulated controls. Comparison of the nitrite production by B10R and B10S macrophages in response to IFN-γ indicated that B10R produced a significant 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 nonstimulated controls both in B10S and B10R macrophages (3.8-fold increase in B10S vs 4.5-fold increase in B10R). Although B10R macrophages produced approximately 4.6 times more NO_2^- as compared with B10S, this difference was not significant statistically ($p=0.0931$).

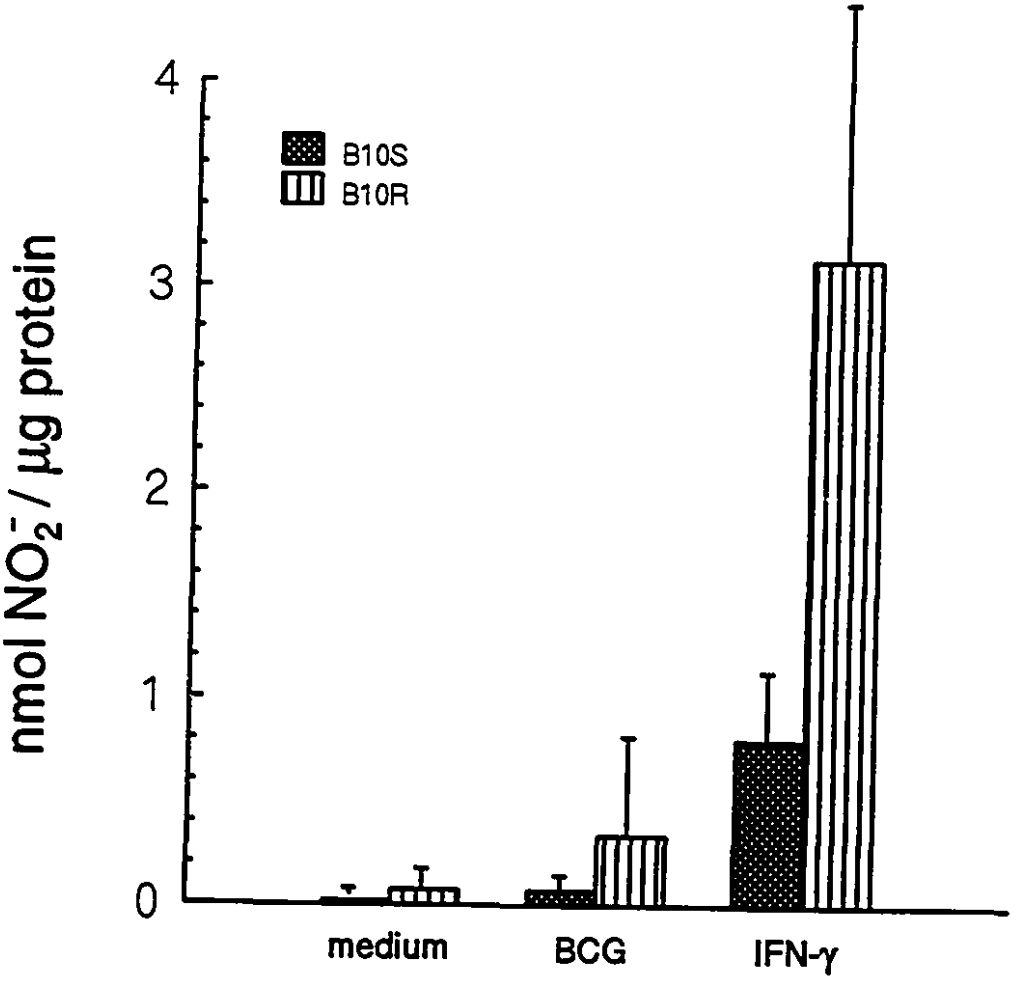

FIGURE 1. NO_2^- production by B10R and B10S macrophage lines in response to *M.bovis* BCG and IFN-γ. The basal production of NO_2^- as well as their response to BCG and IFN-γ after 48h of stimulation is depicted. Values represents a minimum of 3 and a maximum of 6 experiments performed in each macrophage line. The p values were 0.065 for basal production, 0.0931 for BCG stimulation, and 0.0022 for IFN-γ stimulation. Bars represent standard deviation (SD).

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 hours 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 10mM $NaNO_2$. 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 physiologic pH (pH 7.2).

Inhibition of macrophage IFN-γ-induced nitrite production by N^g-monomethyl arginine.

N^gMMLA has been described as an 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 completely, the production of NO_2^- by the macrophage lines. Maximal inhibition was observed at 500 μM N^gMMLA (82.9 vs 89.7% inhibition of NO_2^- production by B10R and B10S lines, respectively).

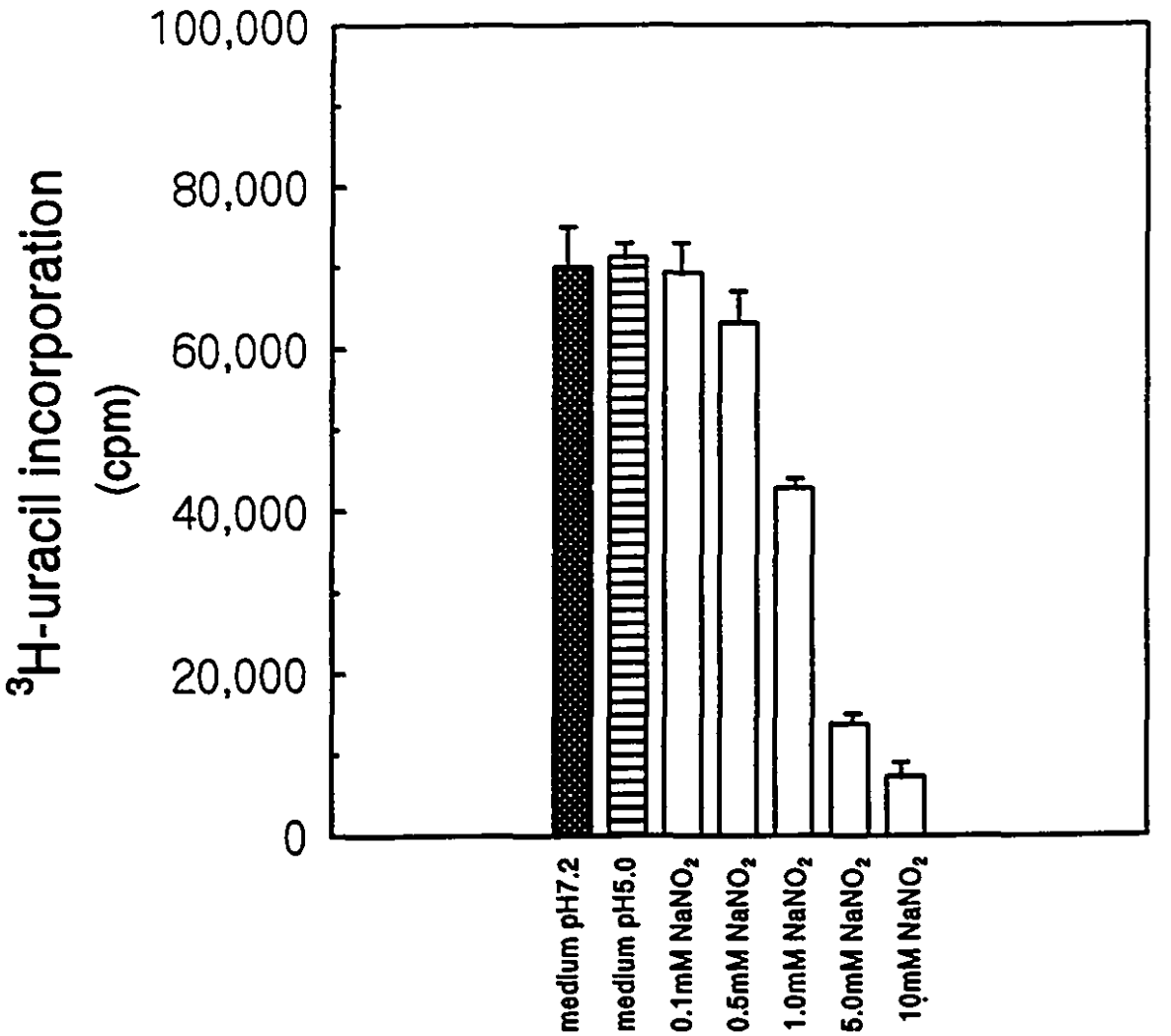

FIGURE 2. Inhibition of the proliferation of *M.bovis* BCG by $NaNO_2$. $1x10^6$ colony forming unit (CFU) of BCG were seeded in 200 μl of CM medium in 96-well plates in the absence or presence of different concentrations of $NaNO_2$. After 48h, 1 μCi/well of ^{3}H-uracil was added to the cultures and the isotope incorporation was measured during the last 16h of the culture period. Hexaplicate samples were used for each treatment. SD was $<15\%$.

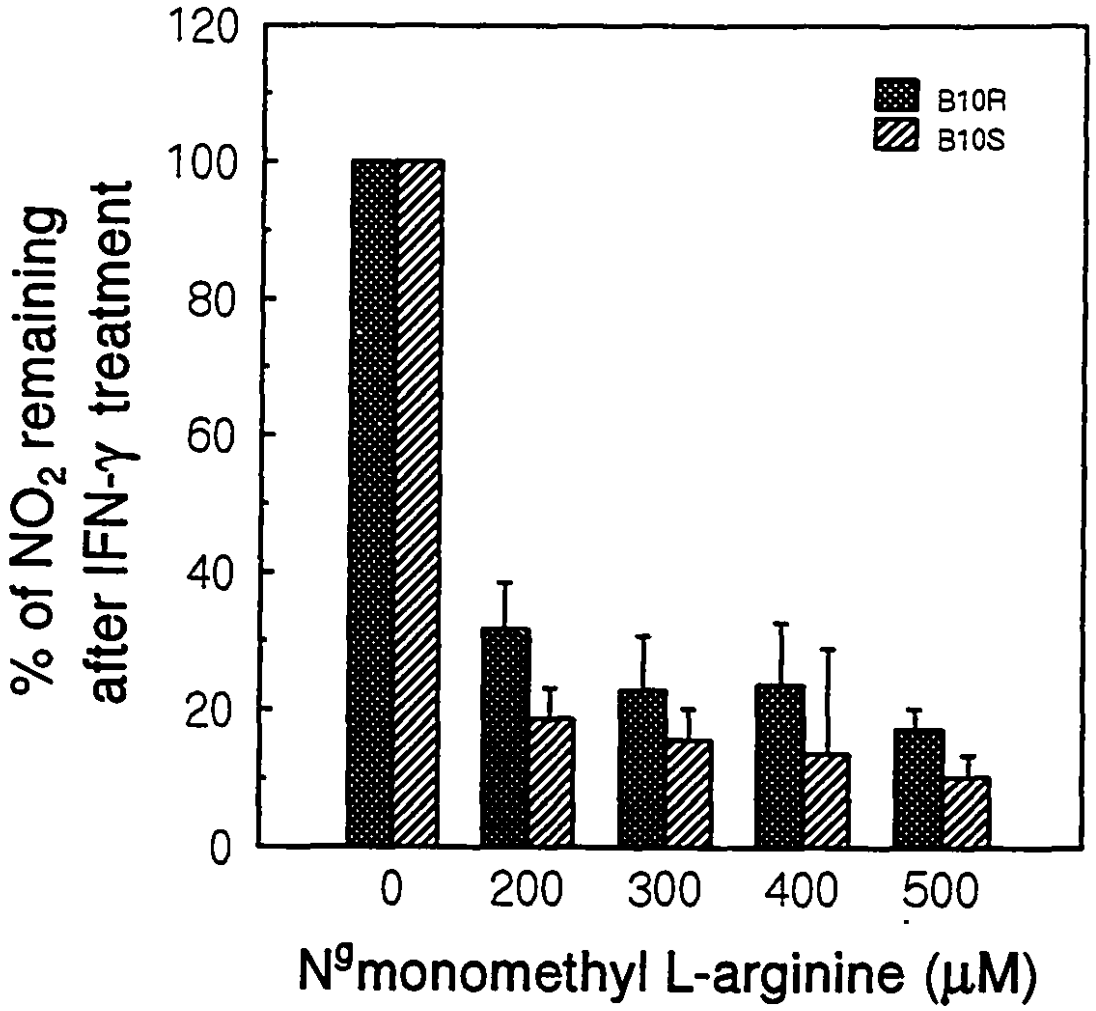

FIGURE 3. Specific inhibition of NO_2^- production in B10R and B10S macrophages by N^g-monomethyl arginine. Macrophages were treated for 48h with 10 U/ml of IFN-γ in the presence or absence of various concentrations of NᵍMMLA. Data represents analysis of triplicate samples. SD was <10%.

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 [a]	NMMA added [b]	NO_2^- production (nmol / 48h) [c]		bacteriostatic activity (%) [d]	
		B10R	B10S	B10R	B10S
-	-	9.5 (7.6) [e]	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)

a, $1x10^5$ macrophages were infected with $2x10^6$ BCG for 4h. Extracellular bacilli were eliminated as described in Materials and Methods. IFN-γ (10 U/ml) and NMMA were added simultaneously.

b, NMMA, 500 μM

c,d, nitrite production and bacteriostatic activity were quantified as described in Materials and Methods. Data represent mean of 3 independent exp. Values are from 4-6 replicas per treatment.

e. (). SD

B10R macrophages are more efficient in controlling BCG proliferation than B10S macrophages. Effect of N^g-monomethyl L-arginine and L-arginine addition.

The next series of experiments was designed to verify the role of nitric oxide on 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 of L-arginine, N^g-monomethyl arginine. 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% vs 27.9%, resp.). The bacteriostatic activity positively correlated with the concentration of nitrite present in wells 48 h 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 have observed that the treatment of B10R macrophages with N^gMMLA resulted in a slight increase in the % 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%, resp.), 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 lapse 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.

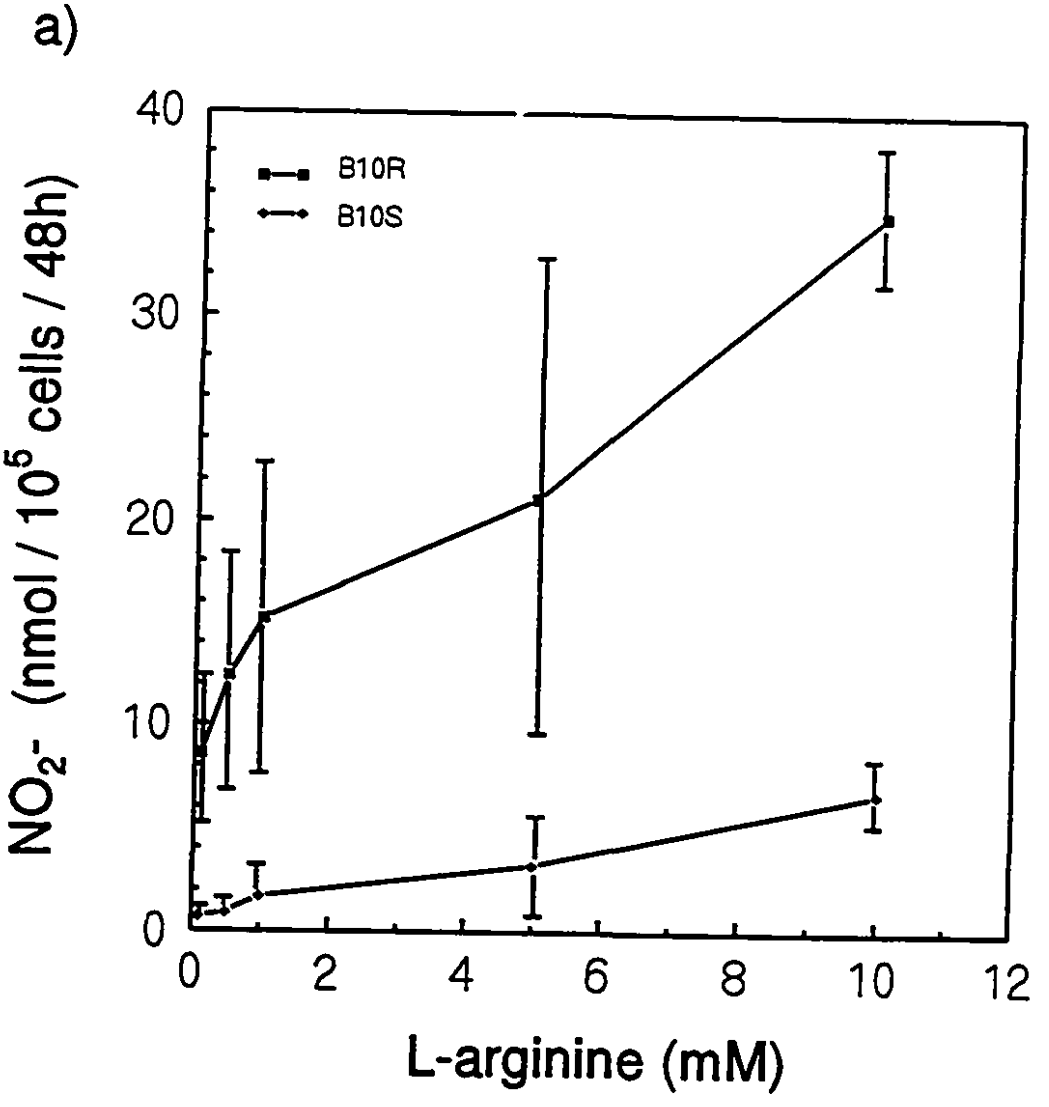

FIGURE 4A. L-arginine addition to culture wells increase nitrite production by macrophages. $1x10^5$ macrophages were infected with $2x10^6$ CFU of *M.bovis* BCG for 4h, washed extensively and then simultaneously treated with IFN-γ (10 U/ml) and N^{ω}MMLA (200 μM). Increasing concentrations of L-arginine were added to selected wells. Nitrite production and bacteriostatic activity by the macrophages were quantified after 48h of treatment. Data represent average of 3 independent experiments. Values represent average of 4 to 6 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 ($0.002 < p < 0.05$)

b)

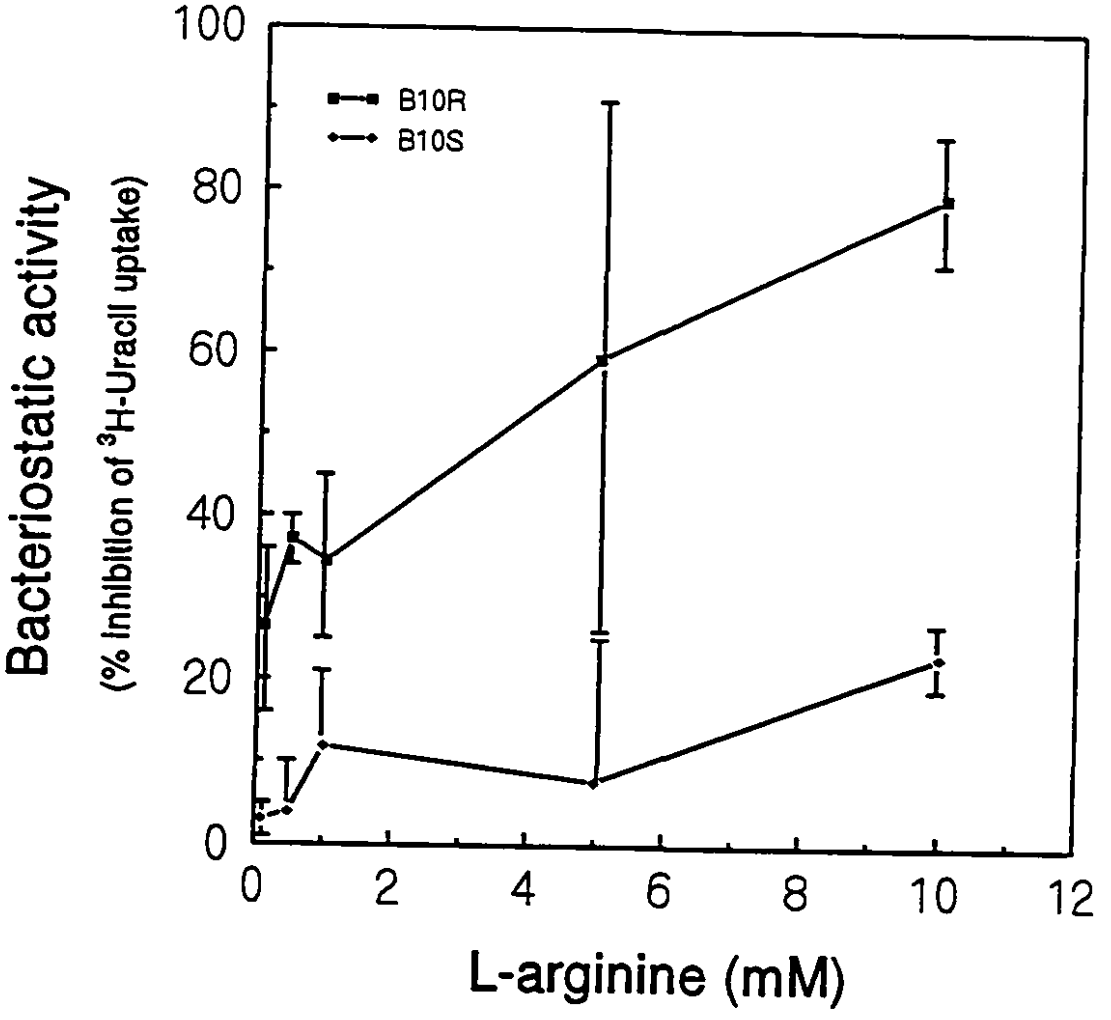

FIGURE 4B. L-arginine addition to culture wells increase bacteriostatic activity by macrophages. $1x10^5$ macrophages were infected with $2x10^6$ CFU of *M:bovis* BCG for 4h, washed extensively and then simultaneously treated with IFN-γ (10 U/ml) and N^gMMLA (200 μM). Increasing concentrations of L-arginine were added to selected wells. Nitrite production and bacteriostatic activity by the macrophages were quantified after 48h of treatment. Data represent average of 3 independent experiments. Values represent average of 4 to 6 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 ($0.002 < p < 0.05$)

Overall these in vitro results indicate that there was 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 are maybe 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 as compared to macrophages obtained from BCG-susceptible mice (6,7,9). It has also been shown that *M.bovis* BCG replicates preferentially in spleen and liver of BCG-susceptible strains (1,2).

Our next objective was to compare the ability of splenic cells, derived from B10A.*Bcg*^{r} and B10.A (*Bcg*^{s}) mice, to produce NO_2^- in response to stimulation by IFN-γ. As shown 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 of IFN-γ than cells derived from BCG-susceptible strain.

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

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 maximum between 8-12 hours (data not shown) and then reached a plateau level by 24h. The levels of nitric oxide synthase expression were higher for all doses used and at all

TABLE 2

Nitrite production by splenic cells stimulated with IFNγ[a]

	NO_2^- (nmol / $5x10^6$ cells / 48h)					
	Expt. #1		Expt. #2		Expt. #3	
	B10R[b]	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

[a] $5x10^6$ unseparated splenic cells per ml were cultured for 48h in absence or presence of IFNγ.

[b] B10R = B10A.*Bcg*^{r} ; B10S = B10.A

[c] nitrite was quantitated as indicated in Materials and Methods.

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 microorganisms. This function is of paramount importance during the onset of the immune response, when macrophages alone or in collaboration with NK cells are able to inhibit the proliferation of and can kill pathogenic intracellular microorganisms 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 reactive oxygen intermediates (ROI) including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) by the macrophages (25,26).

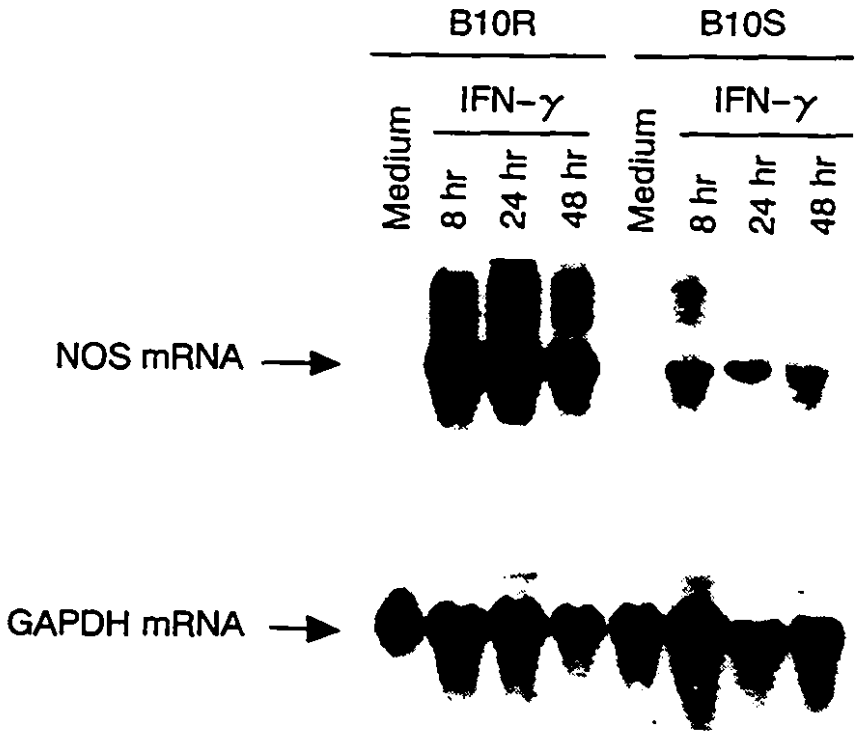

FIGURE 5. Macrophage nitric oxide synthase mRNA expression by B10R and B10S macrophage lines. 10 μg of total RNA purified from IFN-γ-stimulated macrophage lines were hybridized with a ^{32}P-cDNA probe for the macrophage iNOS as described in Materials and Methods.

Recently, reactive nitrogen intermediates (RNI), produced by macrophages and other cell types in response to IFN-γ, or IFN-γ plus TNF-α and IL-1, have been shown to play an important role in the control of the intracellular growth of microorganisms. 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). $NO^{\cdot}$ 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?, and 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. Firstly, 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 with 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 days after infection, in the presence or absence of 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-1*) was recently cloned by Vidal et al. (18). *Nramp-1* 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-1* 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*^{r} macrophages to control intracellular replication of antigenically unrelated ingested microbial targets. It was shown by Vidal and colleagues (18) that susceptibility to infection (*Bcg*^{s}) in 13 *Bcg*^{r} and *Bcg*^{s} strains tested was associated with a nonconservative Gly-10^5 to Asp-10^5 substitution within 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}) strain mice 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 suggest that at least two different, maybe linked, mechanisms of antibacterial activity could be responsible for the resistant and susceptible phenotype of antibacterial 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 had been shown that in SCID mice, 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 nonspecific antimicrobial response within a short period of time after infection. We have observed that IFN-γ mRNA is expressed in cultured spleen cells within 24 hours following *in vitro* infection with BCG (31), and thus, the differential responsiveness of macrophages from susceptible as 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 the resistant animals.

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CHAPTER IV

I-A EXPRESSION REGULATION IN MACROPHAGES DERIVED FROM MICE SUSCEPTIBLE OR RESISTANT TO INFECTION WITH *M. bovis* BCG.

PREFACE

One of the most important pleiotropic effects of the *Bcg* gene is related to the ability of *Bcg*^{r} macrophages to express increased levels of MHC class II antigens compared to *Bcg*^{s} macrophages, either constitutively or in response to stimulation signals including IFN-γ. These differences have been suggested to play an important role in the development of specific immunity against invading *Mycobacteria*, *Leishmania*, or *Salmonella*. However, the molecular mechanisms for the differential I-A surface and mRNA expression are not yet precisely delineated. Using the B10R and B10S macrophage lines, we have found that both transcriptional and post-transcriptional mechanisms may explain the reported differences in I-A antigen and mRNA. Specifically, we observed a modest difference in the transcription of the I-A_β gene between B10R and B10S macrophages. The differential expression of I-A_β mRNA detected in the macrophage lines has been traced to the presence of unique proteins functioning as transcription regulatory factors which bind to specific regions within the I-A_β gene promoters in B10R and B10S macrophages. Our studies have focused on identifying interactions between proteins and motifs of the I-A_β promoter containing the X, Y and S boxes, using a gel retardation assay. We have gathered evidence suggesting that nuclear proteins bind strongly to X and Y box sequences of the I-A_β promoter, and that the differences between B10R and B10S macrophages are quantitative rather than qualitative in nature. Furthermore, significant differences in the I-A_β and I-A_α mRNA stability were found between B10R and B10S macrophages. B10R macrophages

transfected with an antisense *Nramp-1/Bcg* DNA containing a ribozyme construct expressed decreased surface I-A levels compared to controls. This result may suggest that MHC class II expression in macrophages is dependent on *Nramp-1/Bcg* gene.

SUMMARY

The innate capacity of mice to control mycobacterial multiplication early after infection is controlled by the resistant allele of the *Nramp-1/Bcg* gene. Thus, inbred mouse strains are either resistant (*Bcg*^{r}) or susceptible (*Bcg*^{s}) to mycobacterial multiplication. The *Bcg* gene seems to be involved in a pathway leading to macrophage activation for microbiostatic/microbicidal function. It differentially affects the ability of BCG-resistant and -susceptible strains of mice to express important macrophage genes including Major Histocompatibility Complex (MHC) class II genes. We have investigated the molecular mechanisms involved in IFN-γ-induced MHC class II expression using macrophages derived from mice resistant or susceptible to mycobacterial infections (B10R and B10S, respectively). We have found that differences at the level of I-A_s gene transcription, and I-A mRNA stability may be responsible for observed differences between steady-state levels of I-A_s mRNA in B10R and B10S macrophages and consequently in the Ia surface protein expression. Furthermore, we have studied the binding of proteins prepared from nuclear extracts of non-stimulated and IFN-γ-stimulated B10R and B10S macrophages to the S, X and Y cis-acting elements of the I-A_s promoter. Differences observed in protein binding to the X box may explain the difference in transcription activation of the I-A_s gene. In addition, we found that B10R macrophages transfected with an *Nramp-1* antisense cDNA containing a ribozyme construct expressed lower amounts of Ia antigen compared to mock-transfected macrophages in response to IFN-γ. Overall, these findings strongly suggest involvement of the *Nramp-1/Bcg* gene in the control of Ia antigen expression in macrophages.

INTRODUCTION

The class II genes of the Major Histocompatibility Complex (MHC) encode highly polymorphic, cell surface glycoproteins (called Ia antigens). These molecules play a central role in the immune response by forming the fundamental part of the ligand for the antigen-specific T cell receptor. The Ia antigen T-cell receptor interaction is required both for the development of the T-cell repertoire in the thymus and the presentation of antigenic peptides to helper T cells in the periphery. Proper function of the Ia antigens depends on the polymorphic nature of their structures and their ability to bind the antigenic peptide. Overall, the regulation of expression of these proteins on the surface of antigen-presenting cells, specifically macrophages, is not fully understood.

In the mouse, there are two isotypic forms of Ia, I-A and I-E. Each is a heterodimer composed of an α chain (33 to 34 kd) and a ß chain (28 kd) associated non-covalently on the cell surface. The four murine class II genes are coordinately regulated and display a complex pattern of expression (reviewed in 1).

Class II gene expression is developmentally regulated in the cells of B cell lineage. Immature pre-B cells are class II^- (2), while mature B cells express class II antigens constitutively (3,4). Terminally differentiated plasma cells lose their capacity to express class II antigens (5). While class II molecules are expressed constitutively in B cells, they can be induced in macrophages by several stimuli. Both macrophage-like cell lines and tissue macrophages can be induced to express class II antigens by IFN-γ (6-8). Other cytokines including tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) (9) have been reported to modulate Ia antigen as well.

The regulation of surface class II expression is achieved through the combination of transcriptional and post-transcriptional effects. Transcriptional effects are mediated by

factors that interact in a sequence-specific manner with elements found in the gene promoter, or within introns. Additional enhancer elements, located both in the introns and the positions some distance upstream of the transcription start site, have been described (9-11). Most work on the class II cis-acting elements has involved the proximal promoter region, encompassing approximately 200 bp upstream from the transcription start site.

Initial comparison of the sequences of the human and murine class II genes revealed two highly conserved sequence elements in the region spanning 40 to 150 nucleotides 5' of the start site of transcription. These elements have been called the X and Y boxes, and several studies have shown them to be important for both the constitutive and induced expression of class II genes in the human and murine cells (12-14). Some other sequence motifs have also been considered important for class II gene expression. Experiments involving deletions at the 5' end of the X box have revealed that a stretch of 30-40 bp, the Z/W area, including the 7 bp S box, play an important role in class II regulation (13,15,16).

Studies performed during the last decade have demonstrated that the ability of recombinant inbred and congenic strains of mice to control intracellular growth of *M.bovis* BCG during the early phase of infection depended on the activity of a single, autosomal, dominant gene called the *Bcg* gene (17-19). Interestingly, the same locus controls growth of *Leishmania donovani* (*Lsh*) and *Salmonella typhimurium* (*Ity*) (19-23). The cellular studies searching for the cell type expressing the resistant or susceptible phenotype pointed to the mature macrophage as the cell type directly involved in the phenotypic expression of the *Bcg/Lsh/Ity* gene (24-27). These studies also suggested that the activity of the *Bcg* gene influenced several important functional characteristics of macrophages, including the ability of macrophages to express MHC class II antigens (28). Three lines of evidence have been reported regarding the difference in Ia expression between macrophages derived from resistant mice and their susceptible counterparts: 1)

the percentage of Ia^+ macrophages in the spleens of *Bcg*^{r} congenic mouse strains was higher than the percentage in the spleens of their *Bcg*^{s} counterparts (28), 2) the stability of IFN-γ-induced class II antigens expressed by macrophages derived from BCG-resistant mouse strains has been shown to differ from that of the BCG-susceptible mouse strains (29-32). In this particular case, the membrane class II antigens are expressed for a longer period of time on macrophages resistant compared to susceptible strains following IFN-γ stimulation, and 3) we have reported that the steady-state levels of the IFN-γ-induced IA_s-mRNA were higher in macrophages derived from the B10.A.*Bcg*^{r} mouse strain (B10R macrophage line) compared to levels in macrophages derived from the B10.A (*Bcg*^{s}) mouse strain (B10S macrophage line) (33).

Overall, the fact that the differential expression of Ia antigens was found in the susceptible mice compared to its congenic resistant counterpart which differs only in a short segment of DNA containing the *Bcg* gene, suggested that the *Bcg* gene might play an important role in the regulation of Ia. Recently, a candidate for the putative *Bcg* gene (designated *Nramp-1*) was cloned (34,35). The *Nramp-1* gene was found to be expressed exclusively in macrophage populations from reticuloendothelial organs and macrophage lines. Based on the sequence of the cDNA encoding *Nramp-1*, five consensus sites for protein kinase C (PKC) phosphorylation as well as one Src homology 3 (SH3) binding domain were found, suggesting that both PKC and tyrosine kinases (TKs) may play a role in regulation of the *Nramp-1* gene product (34,35).

In this report, we present evidence indicating that B10R macrophages expressed significantly more surface Ia antigen than B10S macrophages when stimulated with IFN-γ. The difference in Ia surface expression correlated with the steady-state levels for the $I\text{-}A_s$ mRNA in B10R and B10S macrophages. A superior capability of B10R macrophages to express $I\text{-}A_s$ mRNA following IFN-γ stimulation was found in both kinetic as well as IFN-γ dose-response studies. Our data indicate that the transcription and mRNA stability may be responsible for the difference in the $I\text{-}A_s$ mRNA steady-state

levels between B10R and B10S macrophages. In addition, we performed kinetic analysis of the binding of proteins present in nuclear extract preparations of B10R and B10S macrophages to the S, X and Y consensus element motifs of the I-A_s gene promoter. Some quantitative differences in the DNA-protein binding activity to the X box were detected between B10R and B10S macrophages. The role of the *Bcg* gene in the differential ability of B10R and B10S macrophages to express surface MHC class II antigens was further supported by the fact that B10R macrophages transfected with an *Nramp-1* antisense cDNA containing a ribozyme construct revealed significantly reduced amounts of Ia antigen following IFN-γ stimulation compared to their mock-transfected controls.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagles Medium (DMEM), penicillin/streptomycin, L-glutamine, phosphate buffer saline (PBS), Hepes buffer, were purchased from ICN (Costa Mesa, CA); Neuman and Tytell serumless medium was purchased from GIBCO (Grand Island, NY). Bovine serum albumin (BSA), actinomycin D, aminoguanidine (hemisulfate salt), Nonidet P-40 (NP-40), goat IgG were obtained from Sigma (St. Louis, MO); guanidine isothiocyanate, N,N',methilenebisacrylamide, glycerol, agarose, nick translation kit, end labelling kit, were purchased from BRL (Gaithesburg, MD). Recombinant rat interferon-(IFN-γ), was obtained from Amgen (Thousand Oaks, CA); cesium chloride (CsCl), sodium dodecyl sulfate (SDS), were purchased from USB (Cleveland, OH); formaldehyde, magnesium chloride ($MgCl_2$), sodium azide were obtained from Fisher Scientific (Fair Lawn, NJ). Tris base, (Ethylenedinitrilo)tetraacetic acid disodium salt (EDTA), dithiotreitol (DTT), poly[d(I-C)], acrylamide, were obtained from Boehringer Mannheim (Mannheim, Germany). Sodium chloride (NaCl) and boric acid were obtained from Anachemia (Rouses Point, NY). Bradford Reagent was purchased from Bio-Rad (Hercules, CA). [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]dATP (5000 Ci/mmol), [α-^{32}P]UTP

(3000 Ci/mmol) were purchased from Amersham (Amersham, UK). Nytran nylon membranes were obtained from Schleicher & Schuell (Keene, NH). Fluoresceine isothiocyanate (FITC) conjugated mouse anti-mouse I-$A^{k,r,f,s}$ was purchased from Pharmingen (San Diego, CA). Kodak XAR films were obtained from Kodak (Rochester, NY).

Cells

Macrophage lines were derived from the bone marrow of B10.A (B10S macrophage lines) and B10A.*Bcg*^{r} strain mice (B10R macrophage lines), congenic at the *Bcg* (*Nramp-1*) locus, as previously described in detail (33). The cultured cells were used in the experiments when they approached confluence. Viability, determined by trypan blue exclusion, was greater than 90%.

B10R macrophages, stably transfected with a fragment of *Nramp-1* antisense cDNA containing a hammerhead ribozyme construct targeting a GUC site at position 434 of the *Nramp-1* mRNA (B10R-NrampRb), were established in our laboratory. The *Nramp-1*-specific ribozyme comprises the antisense to *Nramp-1* sequence 12 bp upstream and 14 bp downstream of the cleavage site, and the 22 bp long sequence of hammerhead ribozyme catalytic domain substituting for a nucleotide complementary to C of the ribozyme cleavage motif (GUC) on *Nramp-1* mRNA (Kramnik, I., et al., manuscript in preparation).

Measurement of cell surface Ia expression

Macrophages were seeded at $1x10^6$ per 60-mm tissue culture dishes (Falcon, Lincoln Park, NJ) and treated for 24 h with IFN-γ. Cells were removed from dishes by scraping with a rubber policeman (Sarstedt, Newton, NC), washed in PBS and fixed in PBS containing 5% BSA and 0.1% sodium azide. After blocking Fc receptors with goat IgG for 20 min, cells were washed, pelleted, resuspended in the buffer containing optimal amounts of FITC-conjugated mAbs, and were then incubated for 30 min on ice. Stained

cells were analyzed using a dual laser FACstar Plus flow cytometer (Becton Dickinson). Fluorescence (log-amplified; four decade range) and light scatter signals were acquired and stored in list mode fashion using the Consort 40 Vax Data Management System (Becton Dickinson).

DNA Probes

The pGEM-A_β containing a 500 bp Pst I fragment and pGEM-A_α containing the 800 Bst EII-Pvu II fragment of the I-A_β^d gene were kindly provided by Dr. R. Germain. The 18s rRNA probe (EcoRI-SalI) was kindly provided by Dr. Arnheim (State University of New York). pBluescript (KS^+) was purchased from Stratagene. The GAPDH probe was generated by PCR using the following oligonucleotide primers: sense primer, 5' CCC TTC ATT GAC CTC AAC TAC ATG G 3', antisense primer, 5' AGT CTT CTG GGT GGC AGT GAT GG 3'. After PCR amplification, the 456 bp PCR product was subcloned by PCR in pBluescript KS^+ and sequenced.

Northern Blot Analysis

To perform Northern analysis of I-A_β, 1-2x10^7 macrophages were seeded in 150mm plastic Petri dishes (Falcon, Thousand Oaks,, CA) 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 previously described (36). Briefly, total RNA was isolated by centrifugation on CsCl cushions and separated on 1.2% agarose gels containing 2.2 M formaldehyde. 1x10^6 cpm per ml of the I-A_β or I-A_α probes labelled by nick translation was added to the hybridization buffer.

Measurement of I-A_α and I-A_β mRNA stability

Macrophages (2x10^7) were cultured in Neuman and Tytell medium supplemented with 10% FBS and antibiotics using 150-mm plastic culture dish in the absence or presence of 100 U/ml of IFN-γ for 24 hours. At this point, 5 μg/ml of actinomycin D was added to the cells for a defined period of time. Then, cells were lysed, total RNA was purified and Northern blot analysis was performed as described in Materials and Methods. To

control the amount of RNA loaded, the blots were rehybridized with a probe specific for the 18S rRNA. The viability of the cells decreased dramatically when the macrophages were treated with actinomycin D for more than 10 hrs. Since it has been reported that the loss of viability of macrophages in culture after stimulation with IFN-γ is partly due to nitric oxide production (37), aminoguanidine (500 μM), an inhibitor of nitric oxide production (38,39) was used in some experiments. Under these conditions, enough RNA was recovered even after 24 hours in the presence of actinomycin D. No significant differences in I-A surface expression nor in the decay of I-A_β mRNA were detected during the first 10 hours after the actinomycin D treatment when macrophages were cultured in Dulbecco's modified Eagles Medium or in the conditions reported here (results not shown).

***In vitro* nuclear transcription essay**

Macrophages ($12x10^6$) were cultured in a 150-mm plastic culture dish, and stimulated with 100 U/ml of IFN-γ for the stated times. All subsequent steps and solutions were at 4°C. The nuclei were isolated essentially as described (40) and resuspended in 100 μl of storage buffer containing 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA and 40% glycerol, immediately frozen in liquid nitrogen and stored at -80 °C.

For the labelling reaction equal numbers of nuclei per sample were thawed on ice and reactions were carried out as previously described (40). Using a solution of 2x SSC with 0.1% SDS the filters were washed 3 times at 45°C for 20 minutes followed by 3 washes each time for 20 minutes at 65°C in 0.1x SSC with 0.1% SDS. The filters were then exposed to X ray film using an intensifying screen at -70°C.

Nuclear extract preparation

Macrophage nuclear extracts were prepared using the procedure of Dignam et al. (41). Protein concentration was measured by the Bradford method utilizing a commercial kit (Bio-Rad Laboratories, Richmond, CA), using BSA as a standard.

Electrophoresis Mobility Shift Assay

The binding reaction was performed in a total volume of 25 μl in 125mM HEPES pH 7.5, 250 mM NaCl, 5 mM DTT, 0.25% Nonidet P-40, 50% glycerol, the indicated amounts of poly[d(I-C)] or poly[d(A-T)], 20,000-40,000 cpm of ^{32}P-labelled DNA, and the defined amounts of nuclear extract. Oligonucleotides were labelled with [γ-^{32}P]dATP (5,000 Ci/mmol; Amersham) by the end labelling using T4 polynucleotide kinase. Binding was allowed to proceed for 30 min at 20°C. Samples were then applied onto a 4% polyacrylamide gel (acrylamide/bisacrylamide, 29:1), 0.5x TBE (0.045 M Tris-borate, 0.001 M EDTA) containing 0.01% NP-40, and electrophoresis was carried out at 200 V (10 V/cm)for 2 h at room temperature. The gels were then fixed in 10% ethanol, 7% acetic acid for 15 min, dried and exposed to X-ray film (XAR5; Kodak) at -70°C. The amount of nuclear extract, poly [d(I-C)], and ^{32}P-labelled oligonucleotide were titrated for each one of the MHC class II I-A_β promoter sequence motifs studied. Optimal amounts were used for the binding reactions under study.

The sequences of the double stranded (ds) oligonucleotides used to study DNA binding activity of proteins present in nuclear extracts as well as those used for the competition assays were as follows: X box (27-mer), 5'-CAATGTCTACCCAGAGACAGATGAC-AG-3', spanning from -110 to -84 of the I-A_β promoter; Y box (18-mer), 5'-ATGCTGATTGGTTC-CTCA-3', spanning from -71 to -54; S box (36-mer), 5-'AGACCATGCCG-CGCATAGAGAGCCTTTGTAAACAAT-3', spanning from -142 to -107. All the sequences were synthesized based on the sequence published by Celada et al. (42).

RESULTS

B10R macrophages express higher levels of sIa and I-A_g mRNA than B10S macrophages.

The level of surface Ia (sIa) expression in non-stimulated or IFN-γ-stimulated B10R and B10S macrophages was studied by cytofluorometry. As shown in Fig.1, the levels of sIa induced by 24-hour treatment with IFN-γ were significantly higher in B10R macrophages compared to B10S macrophages.

To test whether the differential expression of sIa in B10R and B10S macrophages resulted from a differential expression of mRNA, we have assessed the steady-state levels of I-A_g mRNA by Northern blot analysis using a probe specific for I-A_g (Fig.2). A kinetic analysis of the I-A_g mRNA expression in B10R and B10S macrophages is shown in Fig.2A. No I-A_g mRNA expression was detected in non-stimulated macrophages. We observed a time-dependent increase in the levels of I-A_g mRNA in B10R and B10S macrophages starting 8 hours following IFN-γ stimulation. Twenty four hours after IFN-γ stimulation, the accumulation of I-A_g mRNA was maximal in both types of macrophages. Levels of I-A_g mRNA were maintained in B10R macrophages but started to decline in B10S macrophages after 48 hours in the presence of IFN-γ. The steady-state levels of I-A_g mRNA were higher in B10R macrophages at each of the time points tested following IFN-γ stimulation.

To study responsiveness to IFN-γ, B10R and B10S macrophages were stimulated with 1 to 100 U/ml of IFN-γ for 48h. As shown in Fig.2B, increased levels of I-A_g mRNA were detected in response to 1 U/ml of IFN-γ and the maximal expression was achieved at the range of 10-100 U/ml. The levels of I-A_g mRNA were higher in B10R macrophages at all concentrations of IFN-γ tested.

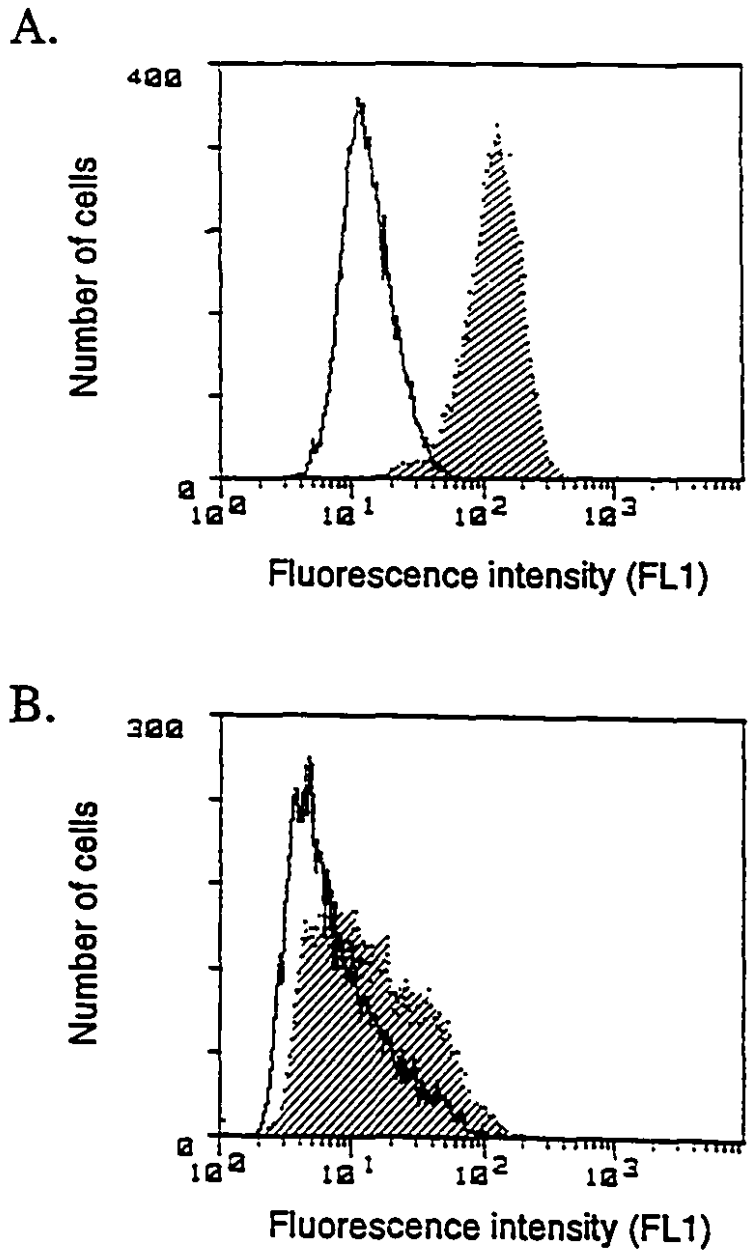

FIGURE 1. Induction of MHC class II I-A antigen expression in B10R and B10S macrophages. Cells were incubated for 24 hr in the absence or presence of IFN-γ (10 U/ml), and the expression of I-A was analyzed by flow cytometry after staining for surface I-A by direct immunofluorescence, using a FITC-$IA^{k,r,s,f}$ monoclonal antibody as described in Materials and Methods. Shaded histograms indicate the expression of I-A antigens on B10R (A) or B10S (B) macrophages treated with IFN-γ. Histograms show cell number (Y-axis) vs log fluorescence (X-axis).

A.

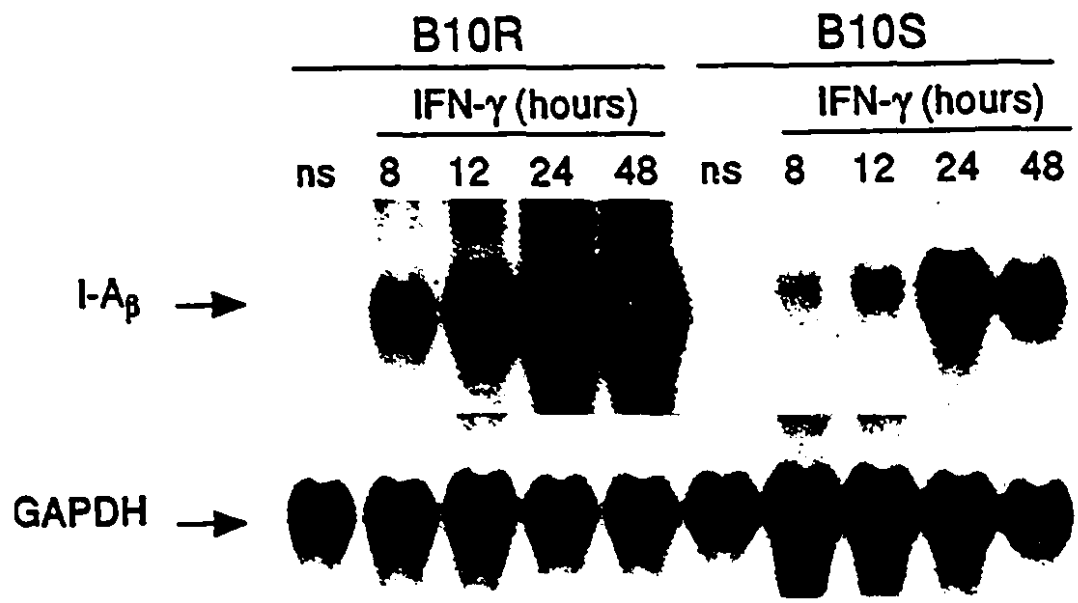

FIGURE 2A. Northern blot analysis of the MHC class II I-A_s mRNA steady-state levels in B10R and B10S macrophages. Kinetics of induction of I-A_s mRNA by the macrophages. Cells were cultured for the indicated periods of time in the absence (non-stimulated, ns) or presence of IFN-γ (10 U/ml). Ten micrograms of total RNA was blotted on nylon membranes and hybridized with a α^{32}P-cDNA I-A_s probe as described in Materials and Methods.

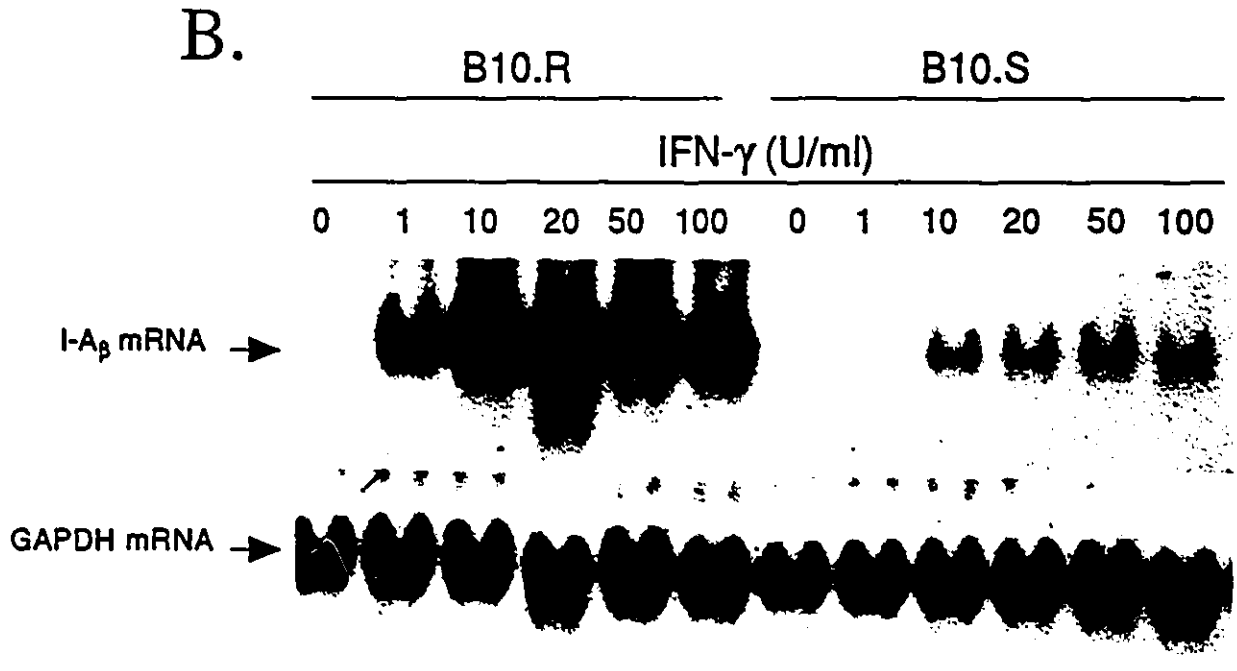

FIGURE 2B. Northern blot analysis of the MHC class II I-A_g mRNA steady-state levels in B10R and B10S macrophages. Levels of I-A_g mRNA in response to various doses of IFN-γ. Cells were cultured for 48 hr in the absence (ns) or presence of IFN-γ. Ten micrograms of total RNA was blotted on nylon membranes and hybridized with a α^{32}P-cDNA I-A_g probe as described in Materials and Methods.

The transcription of the I-A_g gene is more efficient in nuclei from B10R macrophages.

One of the mechanisms that could explain the difference in the I-A_g mRNA steady-state levels between B10R and B10S macrophages might be differential transcription of the I-A_g gene. Therefore, we conducted experiments to measure the transcriptional elongation of the I-A_g mRNA using nuclei prepared from non-stimulated and IFN-γ-stimulated B10R and B10S macrophages. RNA was prepared from the nuclei and hybridized to probes specific for I-A_g, GAPDH, and pBluescript control plasmid DNA.

As shown in Fig.3, the transcription of the I-A_g gene reached a maximum 12 hours after IFN-γ stimulation in B10R macrophages. The transcription of the I-A_g gene in B10S macrophages followed a different kinetic, reaching maximum transcription 24 hours after IFN-γ stimulation. An analysis of the densitometric values obtained after normalization of the I-A_g values against the values obtained with GAPDH, indicated an approximately 3.1-fold increase in the transcription in B10R macrophages as compared to 1.9-fold increase in B10S macrophages following 12 hrs of IFN-γ stimulation. No significant differences were found between B10R and B10S macrophages 4 hours after the stimulation with IFN-γ.

I-A_g mRNA stability in B10R and B10S macrophages treated with IFN-γ.

The difference in transcription of the I-A_g gene between B10R and B10S macrophages can partially explain the differences found in the steady-state level of the I-A_g mRNA. However, lower expression of I-A_g mRNA in B10S than in B10R macrophages could also be due to posttranscriptional events.

To measure the half-life of the I-A_g mRNA, B10R and B10S macrophages were cultured for 48 hours with or without IFN-γ. Total cellular RNA was prepared and

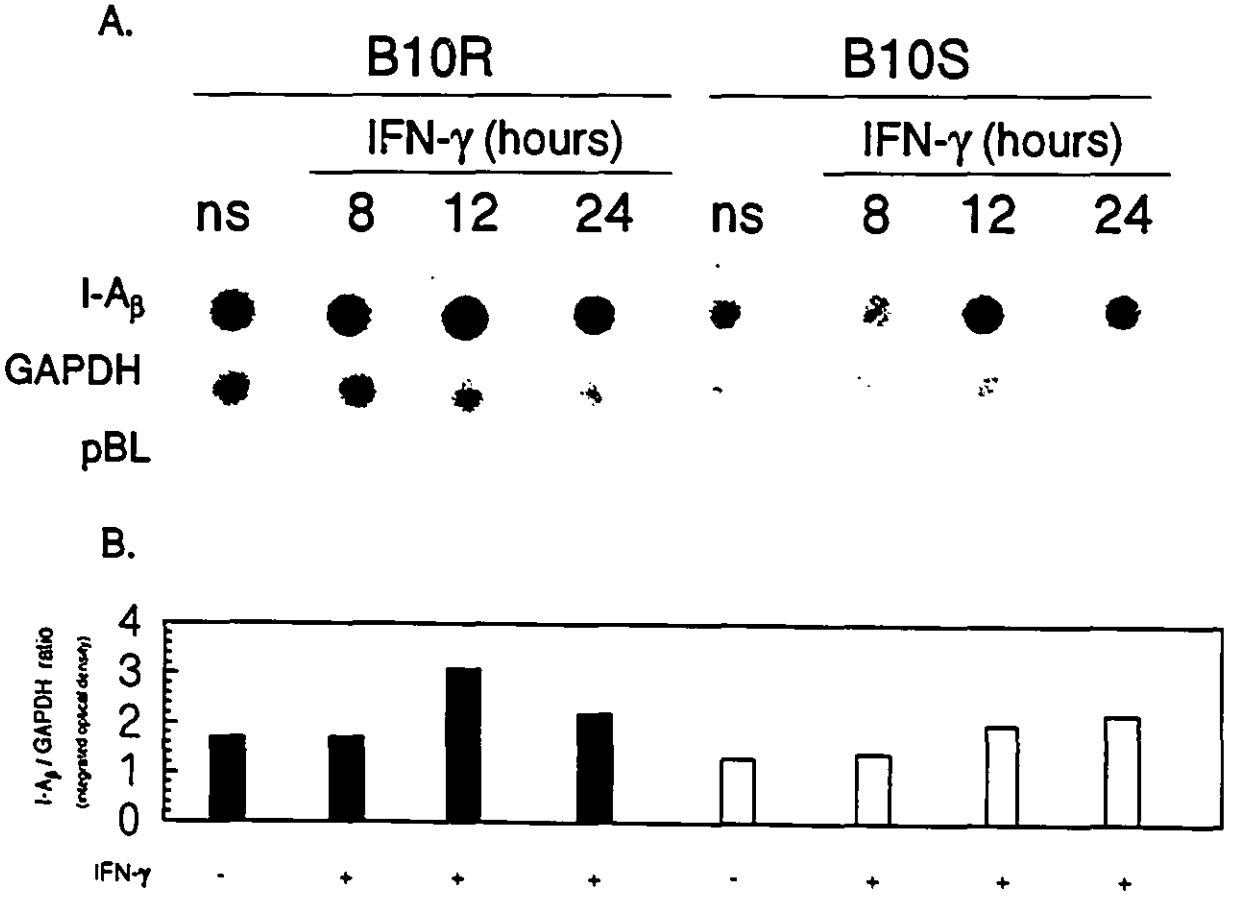

FIGURE 3. Effect of IFN-γ on the rate of I-A_s gene transcription in B10R and B10S macrophages. **Panel A** shows the autoradiograph from a representative nuclear run-on experiment carried out on macrophages treated with medium only (ns) or with IFN-γ (100 U/ml) for the indicated periods of time before extraction of nuclei. Labelled transcripts were hybridized to nylon filters containing excess probe. **Panel B** shows the densitometric quantification data. The X-axis represents the time (hours) after treatment with IFN-γ. The Y-axis represents the ratio of I-A_s to GAPDH transcripts for each time point studied. pBL denotes DNA from pBluescript.

subjected to Northern blot analysis. As shown in Fig.4A, we observed a linear decay of the I-A_β mRNA in both B10R and B10S macrophages. We found a faster decay of IFN-γ-induced I-A_β mRNA in B10S macrophages (13.7 hours) than in B10R macrophages (18.9 hours). In addition, we also estimated the half-life of the I-A_α mRNA in B10R and B10S macrophages stimulated with IFN-γ. We found a significant difference in the predicted half-lives for the I-A mRNA between B10R and B10S macrophages (24.7 h vs 17.5 h for B10R and B10S macrophages, respectively) (Fig.4B).

Overall, these data suggest that the different half-lives of the I-A_β and I-A_α mRNAs in B10R and B10S macrophages might significantly contribute to the difference in the steady-state level of I-A_β and I-A_α mRNAs of B10R and B10S macrophages.

Cold specific I-A_β promoter elements compete with the radiolabelled I-A_β promoter elements for the binding to specific transcription factors.

The S, X and Y sequence motifs of the MHC class II genes have been shown to play an important role in their transcriptional activation. Since we found that the transcription of the I-A_β gene was different between B10R and B10S macrophages, we performed studies to establish the differences and similarities in DNA-protein interactions for the S, X and Y cis-acting elements of the I-A_β promoter.

To characterize DNA-binding factors that might bind to the X, Y and S sequence elements, oligonucleotides representing the respective sequence motifs were radiolabelled and used in a mobility shift assay. The nuclear extracts were prepared using untreated or IFN-γ-stimulated B10R and B10S macrophages as described above. In preliminary experiments we observed a series of potential protein-DNA complexes when each one of the radiolabelled X, Y, or S sequence motifs were used.

The specificity of the complex formation can be assessed by monitoring the binding of the nuclear proteins to the radiolabelled elements of a promoter in presence

A.

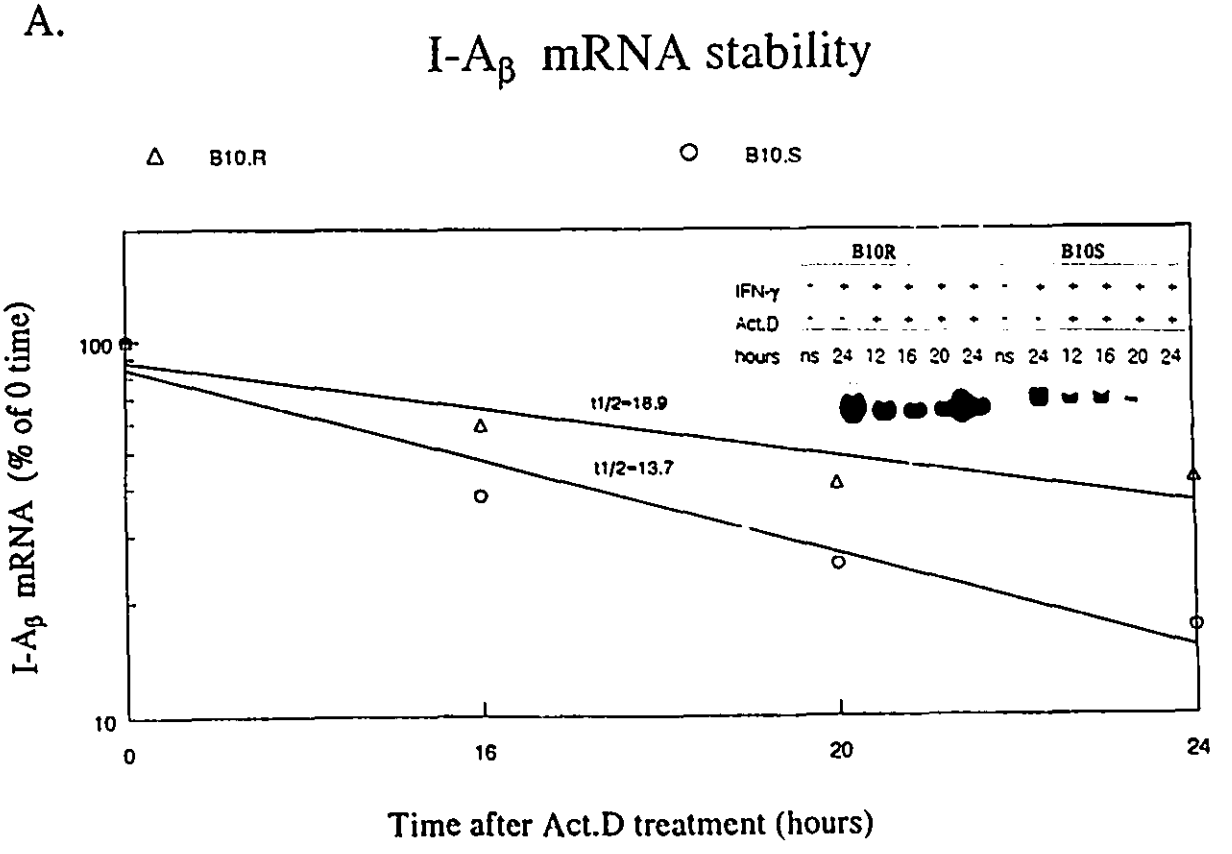

FIGURE 4A. MHC class II mRNA stability in the B10R and B10S macrophages. Decay and predicted half-life of I-A_β. Cells were cultured in the presence of IFN-γ (100 U/ml) for 24 hr before addition of Actinomycin D (5 μg/ml) for the indicated periods of time. Ten micrograms (B10R) or twenty micrograms (B10S) of total RNA were hybridized to ^{32}P-cDNAs specific for I-A_β mRNA. Densitometric scans were used to calculate the percentage of specific mRNA remaining after Actinomycin D treatment. The hybridization signals for I-A_β mRNA were normalized to the amounts of GAPDH mRNA and expressed as a percentage of the t_0 value (100%). The half-life ($t_{1/2}$) calculated for these experiments was extrapolated from the logarithmically transformed best fit line by linear regression analysis. ns: no stimulation.

B.

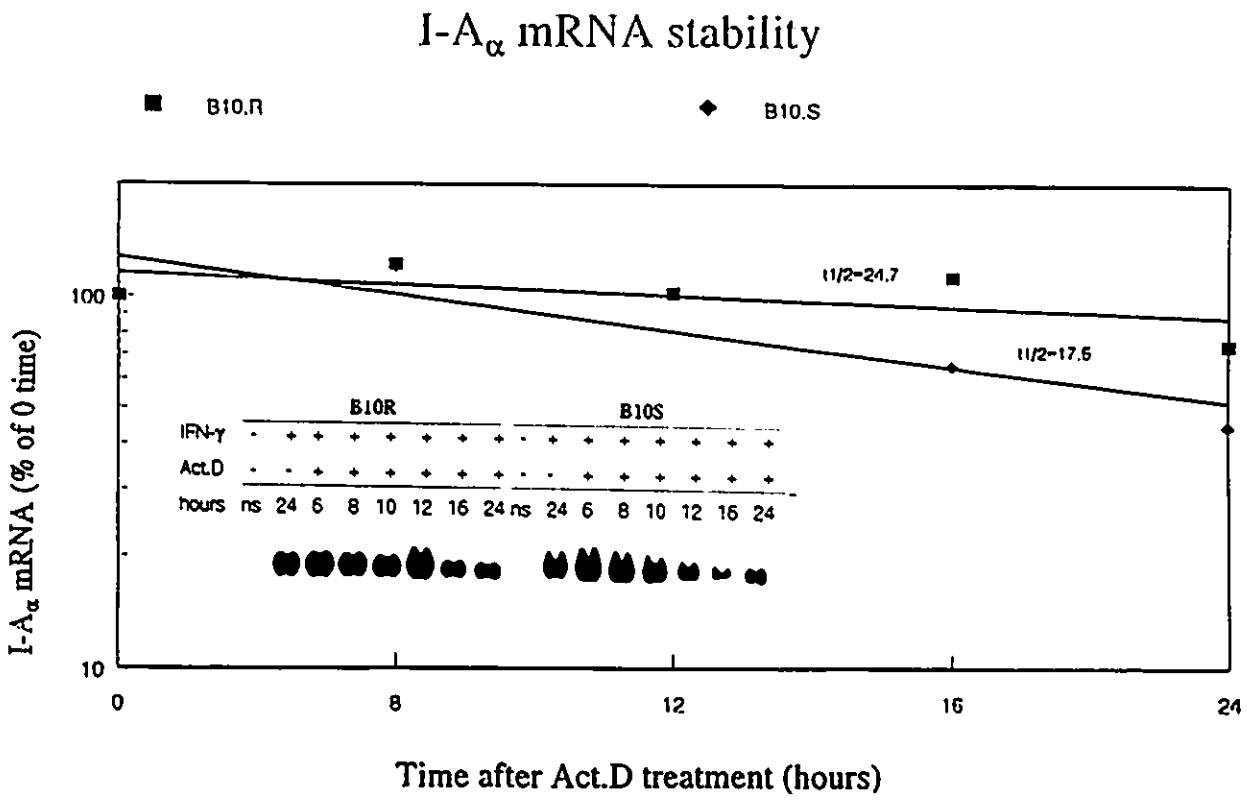

FIGURE 4B. MHC class II mRNA stability in the B10R and B10S macrophages. Decay and predicted half-life of I-A_α. Cells were cultured in the presence of IFN-γ (100 U/ml) for 24 hr before addition of Actinomycin D (5 μg/ml) for the indicated periods of time. Ten micrograms (B10R) or twenty micrograms (B10S) of total RNA were hybridized to ^{32}P-cDNAs specific for I-A_α mRNA. Densitometric scans were used to calculate the percentage of specific mRNA remaining after Actinomycin D treatment. The hybridization signals for I-A_α mRNA were normalized to the amounts of GAPDH mRNA and expressed as a percentage of the t_0 value (100%). The half-life ($t_{1/2}$) calculated for these experiments was extrapolated from the logarithmically transformed best fit line by linear regression analysis. ns: no stimulation.

of the increasing concentrations of the cold elements of the same promoter region. Nuclear extracts obtained from non-stimulated or IFN-γ-stimulated B10R and B10S macrophages were preincubated with an excess of unlabelled competitor DNA. The labelled probe was then added, and complex formation was assessed by the gel mobility assay. In these experiments, an unlabelled specific DNA fragment should compete for factor binding to the labelled probe.

Four complexes (X-1 to X-4) were efficiently competed when protein binding to the X box was analyzed. We have found that an addition of a 10-fold or 50-fold molar excess of the unlabelled X box oligonucleotide effectively inhibited formation of complexes X-1 to X-4 in both B10R and B10S macrophages. Competition for complexes formed was more efficient when extracts from B10S macrophages were used, suggesting a lower amount of protein and/or lower affinities of binding in the case of B10S macrophages. Complex X-1, was the most efficiently competed by the cold X box oligonucleotide, while the efficiency of competition was the lowest for complex X-3. The efficiency of competition was intermediate for complex X-2. A representative example of results which were similar for B10R and B10S macrophages is shown in Fig.5A.

Three prominent complexes (Y-1 to Y-3) were efficiently competed when protein binding to Y box was analyzed. The efficient displacement of binding for the Y box required at least a 100-fold molar excess of the cold Y oligonucleotide. A 500-fold molar excess of the cold Y oligonucleotide was even more efficient. Since results using B10R and B10S macrophages were similar, only results for the competition studies using extracts from B10R macrophages are presented (Fig.5B). We found that the complex Y-3 was the most efficiently competed when a 500-fold molar excess of the cold Y oligonucleotide was used. Thus, densitometric analysis indicated a 7.4-fold reduction in the binding for non-stimulated, and 12.3 and 16-fold reductions 1 hour and 24 hours after IFN-γ stimulation, respectively (data not shown). The competition for complex Y-2 showed the lowest efficiency (1.7-fold reduction for non-stimulated, and 3.3, and 4.8-fold reductions for 1 hour and 24 hours after IFN-γ stimulation, respectively). The

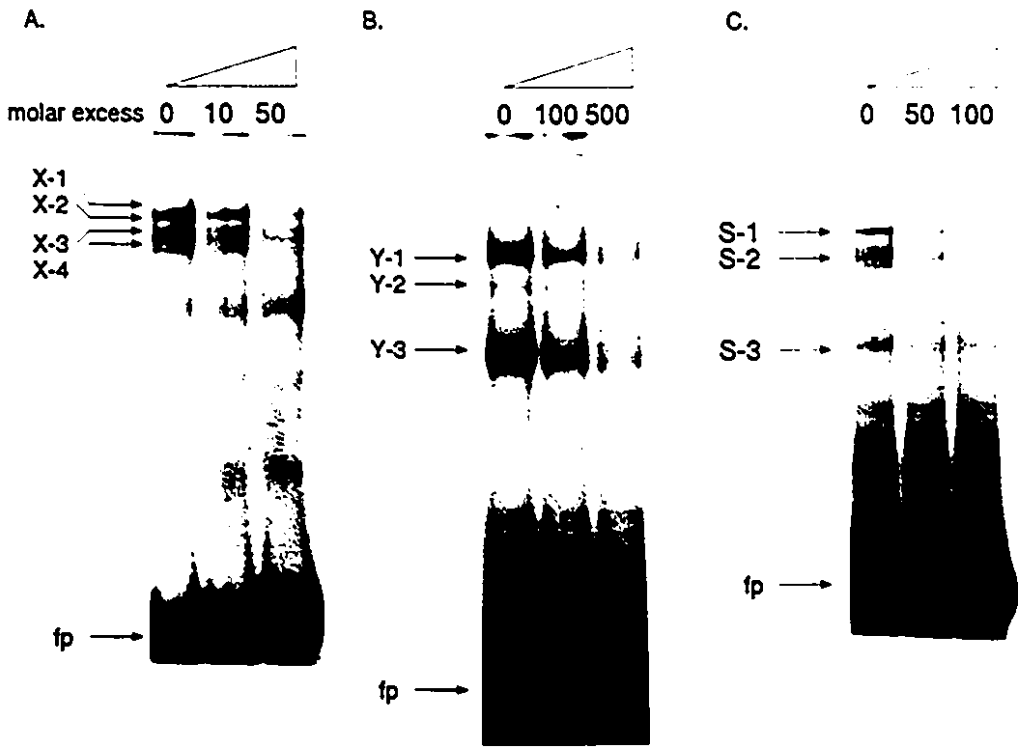

FIGURE 5. Specific complexes are formed between proteins present in nuclear extracts from B10R and B10S macrophages, and the X, Y, and S cis-acting elements of the I-A_β promoter. EMSAs and competition assays were performed with radiolabelled X, Y, or S oligonucleotides as described in Materials and Methods. A representative example the specific complex formation is shown in the panels A-C. **Panel A** shows competition for the X box. Five micrograms of nuclear extract from B10R macrophages were incubated with 20,000 cpm (0.1-0.2 ng) of radiolabelled oligonucleotide probe in the presence of 32 ng of poly[d(I-C)]. Competitor DNA was present in 10 or 50x molar excess of cold X box oligonucleotide. **Panel B** illustrates the competition studies for the Y box. Five micrograms of nuclear extract from B10R macrophages were incubated with 20,000 cpm (0.1-0.2 ng) of radiolabelled Y box oligonucleotide in the presence of 2 μg of poly[d(I-C)]. Competitor DNA was 100 or 500x excess of cold Y box. **Panel C** shows competition for the S box. Five micrograms of nuclear extract from B10R macrophages were incubated with 40,000 cpm (0.1-0.2 ng) of radiolabelled S box oligonucleotide in the presence of 125 ng of poly[d(I-C)]. Competitor DNA was 50 or 100x molar excess of cold S box. Fp, free probe. Arrows indicate the position of specific complexes as determined by the competition experiments. Complexes are identified by a capital letter (X, Y or S), followed by a number (1-4) identifying the position of the complex in the autoradiogram.

competition for complex Y-1 was intermediate between complexes Y-3 and Y-2 (5.4-fold reduction for non-stimulated, and 7.2 and 8.9-fold reductions for 1 hour and 24 hours after IFN-γ stimulation, respectively).

Three prominent complexes were observed when protein binding to S box was analyzed. Results of the competition studies using the S box are shown in Fig.5C. A decrease in a formation of complexes by a cold competitor was observed when 50-fold to 100-fold molar excess of cold S oligonucleotide was used. Only results involving 100-fold molar excess of S oligonucleotide are described. No significant differences between B10R and B10S macrophages were observed. A representative example from results obtained using B10R macrophages is shown in Fig.5C. Complex S-1, the slowest migrating complex, was the most efficiently competed. Densitometric analysis indicated a 7.3, 11.4, and 12.2-fold decrease in binding for unstimulated, 1 hour and 24 hours after IFN-γ stimulation, respectively (data not shown). Complex S-2 presented the lowest competition with a 2-fold reduction in binding present in nuclear extracts from macrophages stimulated with IFN-γ for 1 hour (data not shown). This complex was barely observed in nuclear extracts from cells stimulated for 24 hours with IFN-γ, thus making it more difficult to quantify the inhibition precisely. The efficiency of competition was intermediate for complex S-3, with a 2.6 and 6.5-fold reduction in binding for unstimulated and 1 hour stimulation with IFN-γ, respectively (data not shown).

DNA-protein interactions and transcriptional activation of the I-A_s activation gene in B10R and B10S macrophages.

Once the numbers of specific DNA-protein complexes were established for each of the X, Y, and S boxes, a kinetic analyses of DNA-protein binding was performed for B10R and B10S macrophages.

Two time points for study following stimulation of macrophages with IFN-γ were selected. One hour and 24 hours post-stimulation with IFN-γ were chosen to follow both the very early and late interactions between the nuclear proteins and X, Y and S

sequences. It was also important to analyze very early modulation in the binding of nuclear proteins that could result from the phosphorylation of proteins present in the cell at the moment of stimulation, or from the rapid translation of immediate early genes following IFN-γ stimulation.

When nuclear extracts from non-stimulated B10S macrophages were used for the study of DNA-protein interactions with the X box, two prominent complexes were identified (X-2 and X-3) (Fig.6). Two additional complexes were clearly observed in some nuclear extract preparations (denominated here X-1, and X-4). The number of complexes was the same when nuclear extracts from B10S macrophages stimulated with IFN-γ for 1 hour were analyzed. An increase in the binding was observed for all complexes, suggesting that treatment of macrophages with IFN-γ enhanced the binding of these complexes to the promoter motifs. Interestingly, the number of complexes observed, as well as the intensity of the binding, were modified when extracts prepared from B10S macrophages stimulated with IFN-γ for 24 hours were analyzed. Thus, complexes X-1 and X-4 were no longer observed, and the intensity of the binding to X-2 and X-3 complexes decreased compared to their respective intensities when extracts from macrophages stimulated for 1 hour with IFN-γ were analyzed. Three prominent complexes (X-2 to X-4) were present when nuclear extracts prepared from unstimulated B10R macrophages were analyzed in the gel shift assay (Fig.6). One hour following the IFN-γ stimulation, there was a dramatic increase in the binding for complex X-2 and X-3 and no increase in the binding of the fastest migrating complex X-4. When extracts from B10R macrophages stimulated with IFN-γ for 24 hours were analyzed in the gel shift assay, a sharp decrease in the intensity of the binding was observed for all complexes. Complexes X-2 and X-3 were barely detectable, while the decrease in binding for complex X-3 was not as marked as the reduction in complexes X-2 and X-3.

The binding of nuclear proteins to the Y box is presented in Fig.7. Three prominent complexes (Y-1 to Y-3) were observed when extracts from unstimulated B10S macrophages were analyzed, with the Y-2 complex being the less prominent. No

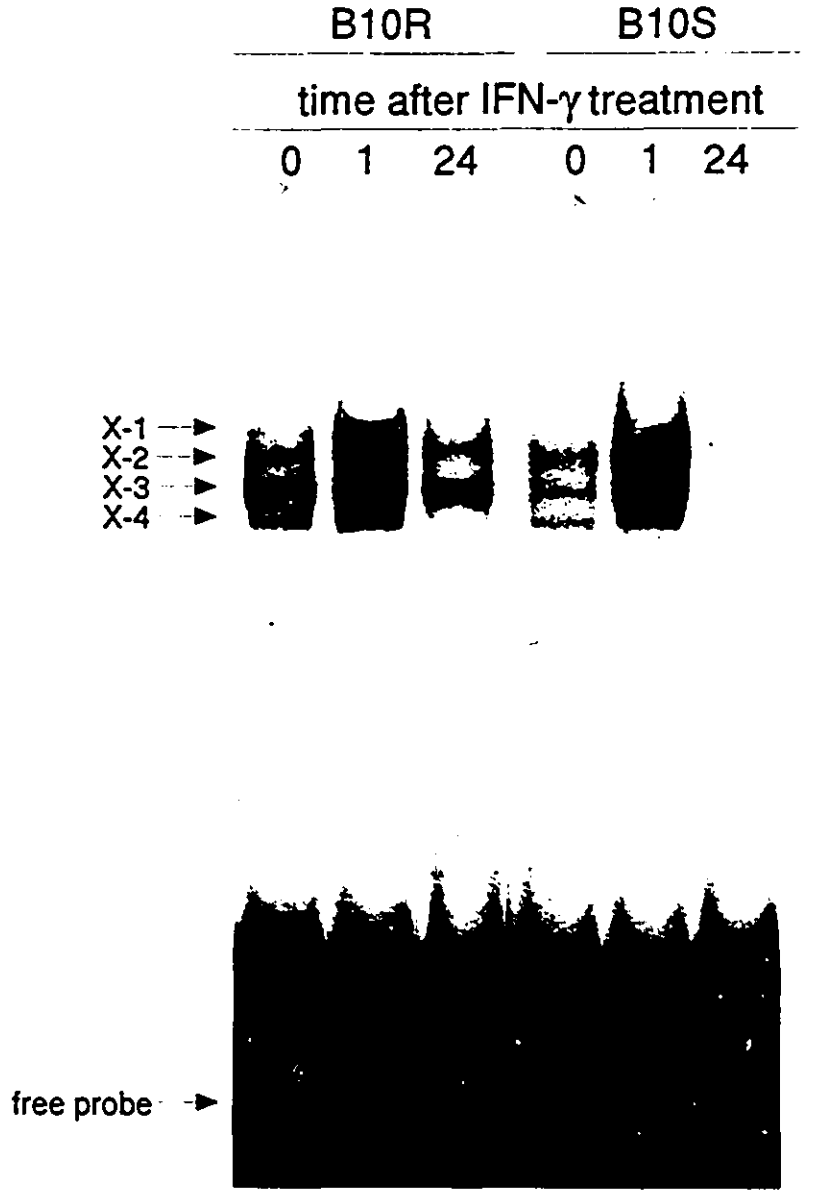

FIGURE 6. Nuclear protein binding to the X box in the I-A_s promoter. EMSA analysis of binding to X box (-116 to -84) in the I-A_s promoter. Nuclear extracts (2 μg) from non-stimulated (0 hr) or IFN-γ-stimulated (1 or 24 hr) B10R and B10S macrophages were used in the binding studies. Binding reaction containing about 0.2 ng of labelled probe and 125 ng of poly[d(I-C)] was performed as described in Materials and Methods. Fp, free probe; X-1, complex X-1; X-2, complex X-2; X-3, complex X-3; X-4, complex X-4.

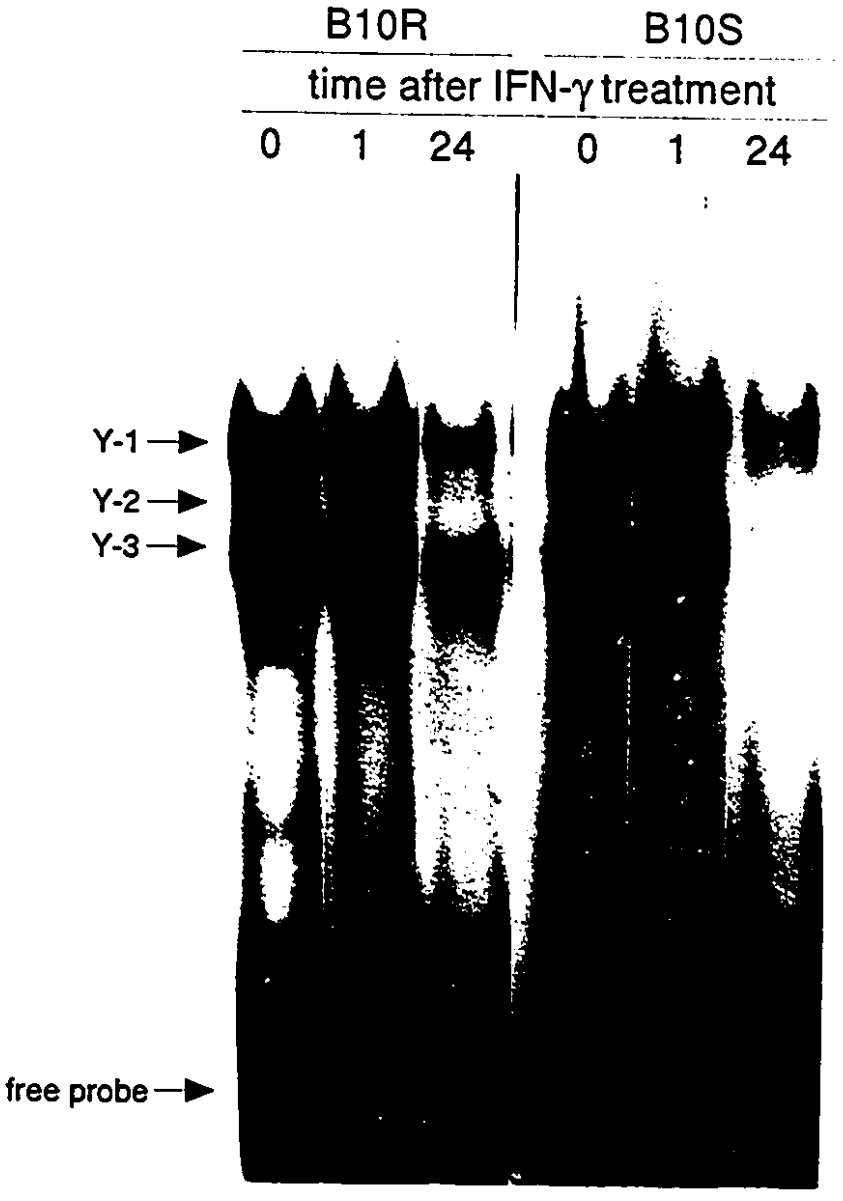

FIGURE 7. Factor binding to the I-A_g promoter Y box. EMSA analysis of binding to Y box in the I-A_g promoter (-71 to -54). Nuclear extracts (10 μg) from non-stimulated (0) or IFN-γ-stimulated (1 or 24 hr) B10R and B10S macrophages were used in the binding studies. Binding reaction containing about 0.2 ng of labelled probe and 2 μg of poly[d(I-C)] was performed as described in Materials and Methods. Fp, free probe; Y-1, complex Y-1; Y-2, complex Y-2; Y-3, complex Y-3.

modification in the number of complexes nor a significant increase in the intensity of the binding was observed in extracts from macrophages stimulated with IFN-γ for 1 hour. However, when nuclear extracts from macrophages stimulated with IFN-γ for 24 hours were analyzed, complex Y-2 was very faint and the intensity of the binding to Y-1 and Y-3 complexes was dramatically reduced. A similar picture was observed when nuclear extracts from B10R macrophages, unstimulated or IFN-γ-stimulated (1 hr) were analyzed. Three prominent complexes were observed with almost no change in the intensity of the binding for complexes Y-2 and Y-3, while a significant increase in the binding was observed for complex Y-1. As in nuclear extracts prepared from B10S macrophages, there was a dramatic decrease in the binding for complexes Y-1 and Y-2 in extracts from B10R macrophages stimulated with IFN-γ were analyzed. Interestingly, complex Y-3 was no longer detected, suggesting that proteins forming this DNA-protein complex could be associated with the differences in transcription observed between B10R and B10S macrophages.

As shown in Fig.8, three prominent DNA-protein complexes (S-1 to S-3) were observed when extracts from unstimulated B10S macrophages were analyzed for binding to the S box. The same number of complexes were observed when nuclear extract preparations from macrophages stimulated with IFN-γ for 1 hour or 24 hours were analyzed. However, the intensity of DNA-protein binding decreased when extracts from macrophages stimulated with IFN-γ for 24 hours were analyzed. The same number of complexes (S-1 to S-3) were also observed when nuclear extracts from unstimulated B10R macrophages were analyzed. One hour following IFN-γ stimulation of B10R macrophages, augmentation in the amount of S-1 and S-2 complexes could be observed.

B10R macrophages transfected with an Nramp-1 antisense DNA containing a ribozyme construct express lower amounts of surface Ia.

Finally, the ability of the *Nramp-1/Bcg* gene to directly modulate the expression of MHC class II antigens was tested by transfection studies. B10R macrophages stably

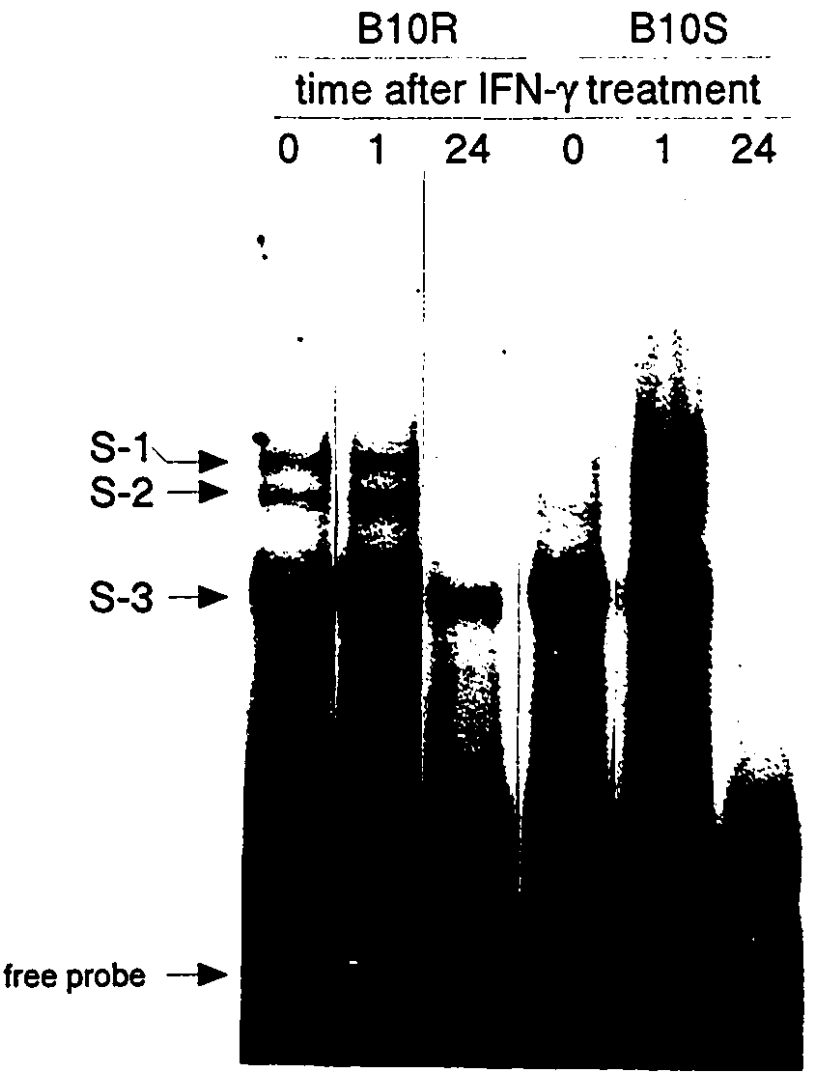

FIGURE 8. Factor binding to the I-A_s promoter S box. EMSA analysis of binding to the S box in the I-A_s promoter (-110 to -90). Nuclear extracts (3 μg) from non-stimulated (0 hr) or IFN-γ-stimulated (1 or 24 hr) B10R and B10S macrophages were used in the binding studies. Binding reaction containing about 0.2 ng of labelled probe and 1 μg of poly[d(I-C)] was performed as described in Materials and Methods. Fp, free probe; S-1, complex S-1; S-2, complex S-2; S-3, complex S-3.

transfected with a fragment of the *Nramp-1* cDNA containing the ribozyme target GUC site at the position 434 (B10R-NrampRb), and mock-vector control B10R macrophages were stimulated for 24 hours with IFN-γ. The surface expression of the Ia antigen was then assessed by cytofluorometry. A significant reduction in the amount of the Ia antigen occurred in the B10R-NrampRb macrophages as compared to mock- transfected macrophages (Kramnik, I., et al., manuscript in preparation). These results strongly suggest that the *Nramp-1/Bcg* gene may be involved in the regulation of Ia antigen expression.

DISCUSSION

Previous studies in our laboratory and others (reviewed in 43,44) have suggested involvement of the gene *Bcg/Lsh/Ity* in the mechanisms of priming for macrophage activation. This conclusion has been made based on several observations, including the superior ability of tissue macrophages and macrophage cell lines derived from BCG-resistant animals to more efficiently control *in vivo* and *in vitro* the intracellular proliferation of microorganisms such as *Mycobacteria*, *Leishmania* and *Salmonella*. Furthermore, a number of reports showed many pleiotropic differences between tissue macrophages from congenic resistant mice and their susceptible counterparts. They included production of toxic reactive nitrogen (45,46) and oxygen (47) intermediates, surface expression of activation markers such as the AcM-1 antigen (43) and 5'nucleotid-ase (48), expression of the early response gene *KC*, and production of IL-1ß and TNF-α (49). Differences in Ia surface antigen (28,29) and I-A_g mRNA expression (33) as well as differences in antigen presentation ability (50) have been observed in both tissue macrophages and macrophage lines derived from BCG-resistant and susceptible inbred and congenic mouse strains.

Using macrophage lines generated from B10.A and its congenic resistant B10A.*Bcg*^{r} mouse strains, we have investigated in detail the molecular mechanisms underlying differences in the steady-state levels of I-A mRNA.

It was therefore important to determine whether differences in the steady-state level of the I-A_β mRNA between B10R and B10S macrophages could be explained by a differential transcription of the I-A_β gene. Results obtained from run-on experiments indicated that the transcription of the I-A_β gene was higher in B10R compared to B10S macrophages. The transcriptional induction of the MHC class II genes by IFN-γ is well documented. It has been proposed as the main mode of regulation of class II genes both in mouse and humans (51-56). The difference in the steady-state mRNA levels may be due to post-transcriptional events resulting in the alteration of the half-life of a mRNA. Our experiments showed us that the half-life of I-A_β mRNA in B10R macrophages was significantly longer than in B10S macrophages. In order to determine whether the differences in the stability were restricted to I-A_β mRNA, we performed similar studies on the I-A_α mRNA. We found that the half-life of the I-A_α mRNA in B10R macrophages was longer than in B10S macrophages. Calman and Peterlin (57) reported a half-life of 5 hours for the HLA DR gene mRNA of human mutant B cell lines. In the study reported by Kern et al.(51) half-lives of class II mRNAs in the myelomonocytic cell line WEHI-3 and B cell lymphoma line A20/2J were studied. Half-lives of 16-20 hours for A_α and E_α mRNAs in WEHI-3 were reported and an estimate of more than 10 hours for the half-lives of A_α, A_β, E_α, and E_β in A20/2J was found (51). It is important to stress that the values obtained for WEHI-3 were obtained from the rate of decay of class II RNA without the use of actinomycin D.

We have found the difference between B10R and B10S macrophages both at the level of transcription and I-A_β mRNA stability. Differences in the protein binding to important regulatory sequence motifs of the I-A_β promoter, namely the X, Y and S boxes, may explain the difference in I-A_β gene transcription between B10R and B10S macrophages.

Several cis-acting elements in the proximal promoter region of the MHC class II genes seem to play a critical regulatory role in their expression (reviewed in 9,10,58). The conserved S (H), X, and Y sequence motifs, located between approximately 40 and 160 base pairs upstream of the start site of transcription of all class II gene promoters, have been implicated in IFN-γ responsiveness and the transcriptional activation of class II genes (reviewed in 9). These transcription regulatory elements are recognized by tissue-specific and nonspecific trans-acting factors (59-64). Our studies using nuclear extracts from non-stimulated and IFN-γ-stimulated macrophages allowed us to identify two to four specific DNA-protein complexes for the S, X, and Y sequence motifs. Some of the proteins binding to MHC class II regulatory elements have been cloned and characterized (65-70). At least three different proteins interacting with the X box have been described (12,14,16,66). We were able to detect several quantitative as well as qualitative differences in the DNA-protein binding profiles between B10R and B10S macrophages. Interestingly, the increase in the protein binding was usually detected early after IFN-γ treatment of macrophages (0.5 to 2 hours), and was reduced at later time points (4 to 48h, data not shown). Second, we consistently observed a reduction in binding activity that peaked 24 hours after IFN-γ stimulation. This binding activity increased in later time points (36 to 48 hours, data not shown). These differences in the amount of complexes and/or binding activity may explain the differences in transcription displayed by B10R and B10S macrophages. Other investigators have been able to detect increases in binding activities from cells treated with IFN-γ both *in vitro* and *in vivo* (71,72). This periodicity in DNA-protein interactions observed by us *in vitro*, is reminiscent of the observations made by Kara and Glimcher (72) who were studying the *in vivo* occupancy of the DR promoter by *in vivo* footprinting using murine B cell lines. Similarly, Ombra et al (73) studying transcriptional activation of a defective MHC class II-negative human B cell mutant, observed an inverse correlation between the level of DNA-protein complex formed and the level of MHC class II gene mRNA. Therefore, our observations suggest common mechanisms of transcriptional activation of MHC class II genes between macrophages and B cells.

Evidence generated in our laboratory (28,33), and by others (29,74,75) has shown a differential ability of macrophages obtained from resistant and susceptible animals to express surface MHC class II antigens. The studies on Ia antigen expression in B10R-NrampRb transfected macrophages further support the possibility that the *Bcg* gene is involved in the control of MHC class II antigens in macrophages. The mechanism(s) responsible for the difference has not been elucidated. We have presented results indicating that the basis for differential class II expression between B10R and B10S macrophages may lie in differences found both at the level of I-A_s transcription and its stability. Alternative explanations, such as Ia antigen stability have been proposed (29,30,32). Nath and colleagues reported differences in Ia antigen expression between peritoneal macrophages of BCG-resistant and BCG-susceptible strains but neither the transcription nor mRNA stability were analyzed (76).

The molecular mechanism(s) through which the *Nramp-1/Bcg* gene influences expression of MHC class II antigens is not known. Previously published results suggest that the tissue macrophages and macrophage lines obtained from BCG-resistant animals respond more efficiently to activation stimuli, including IFN-γ, compared to tissue macrophages or macrophage lines obtained from BCG-susceptible macrophages (43). These findings suggest that the *Nramp-1/Bcg* gene is involved in the priming for macrophage activation. Based on the deduced amino acid sequence for the *Bcg* gene candidate, the *Nramp-1* gene (34,35), two hypothetical functions have been proposed for the *Nramp-1/Bcg* gene. Vidal et al. (34), based on the structural similarity between *Nramp-1* and the membrane transporter responsible for nitrate import in the eukaryote *A. nidulans*, CrnA, proposed that the two proteins may be functionally related and implicated in the transport of simple nitrogen compounds. Therefore, Nramp could function as a nitrite/nitrate concentrator for phagolysosomes and alterations in this putative transport system would affect the capacity of *Bcg*^{r} macrophages to control intracellular replication of antigenically unrelated ingested microbial targets. Barton et al.(35), based on the finding of an Src homology 3 (SH3) binding domain located in the

5' region of the *Nramp-1* cDNA, propose that the SH3 binding domain of Nramp could mediate specific protein-protein interactions with molecules involved in macrophage signal transduction such as Hck and Fgr, or that phosphorylation of the Nramp-1 SH3-binding domain on tyrosines might itself regulate transport of important substrates such as L-arginine. It is known that in the mouse system bacteriostatic/bactericidal activities, MHC class II expression, production of reactive nitrogen intermediates and TNF-α, are mostly dependent on binding of IFN-γ to its receptor (reviewed in 77-80). It is tempting to speculate that the *Nramp-1/Bcg* gene product could collaborate with the IFN-γ receptor to achieve an efficient signal transduction. Successful generation of antibodies against *Nramp-1/Bcg* as well as the development of *Nramp-1/Bcg* knock-out mice (both in progress) will help to establish the precise function of *Bcg* gene.

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CHAPTER V

GENERAL DISCUSSION

POLYMERASE CHAIN REACTION AS A TOOL TO STUDY MYCOBACTERIAL INFECTION AND MULTIPLICATION IN MACROPHAGES

PCR-based methodologies have previously been used to detect mycobacterial DNA present in tissue samples only for the purpose of clinical diagnosis (Brisson-Noel et al., 1989; Eisenach et al., 1990; Williams et al., 1990; Shankar et al., 1991; Narita et al., 1992). Using primers specific for mycobacterial DNA sequences (Eisenach et al., 1990), we developed a simple, reproducible and rapid tool for the assessment of mycobacterial DNA content in infected macrophages. We have found a linear correlation between the number of CFU, ^{3}H-uracil incorporation by the bacteria, and densitometric analysis of mycobacterial DNA present in infected macrophages. Therefore, the use of PCR represents a fast and extremely sensitive approach to study mycobacterial infection and multiplication in macrophages. Because of the sensitivity of the method, the number of macrophages required for the analysis can be dramatically reduced. Furthermore, this method has obvious advantages compared to CFU estimations which usually take 2-5 weeks. The development of a quantitative PCR methodology allowed us to monitor precisely the amount of mycobacterial DNA (mycobacterial genomic DNA equivalents) present in infected BCG-resistant and -susceptible macrophages under different experimental conditions. Since the amount of bacterial DNA present correlates with the number of CFU (the most reliable estimation of viability), quantitative PCR can provide us with information concerning the critical issue of bacteriostatic and/or bactericidal activity of macrophages.

MACROPHAGE LINES AS A MODEL TO STUDY FUNCTIONAL EXPRESSION OF THE *Bcg* GENE

Macrophage lineage consists of several different populations of cells, at different stages of differentiation. It is not fully understood how this heterogeneity arises. It has been postulated that it may arise either as a response to differentiation signals encountered by the macrophages in the microenvironment, or as a genetically-determined pathway of differentiation, or thirdly, nature and nurture together collaborate for the final macrophage phenotype (reviewed in Rutherford et al., 1993).

Crocker et al. (1987) compared the *in vitro* expression of the *Lsh* gene for its ability to control the proliferation of *L. donovani* by different macrophage populations. They observed that Kupffer cells, splenic and lung macrophages, as well as short (7 days) and long term (6 weeks) cultured bone marrow-derived macrophages supported growth of *L. donovani*. However, resident peritoneal macrophages grown in adherent or in suspension cultures neither supported growth of *L. donovani* nor showed any evidence of *Lsh* gene expression *in vitro*. Denis et al. (1988a) reported that splenic but not peritoneal macrophages derived from the BALB/c.*Bcg*^{r} mouse strain more efficiently presented soluble and particulate antigens to T cells compared to macrophages derived from the BALB/c mouse strain. Roach et al. (1991) observed that bone marrow-derived macrophages from B10A.*Lsh*^{r} strain mice were more potent leishmanicidal effector cells than their B10.A (*Lsh*^{s}) counterparts. Furthermore, a direct correlation between leishmanicidal activity and nitrite production was also found. In contrast, the analysis of 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.

To overcome the difficulties associated with the heterogeneity of freshly isolated macrophages as well as the low yield of macrophages isolated from certain tissues, we

have generated macrophage lines from the bone marrow of the B10.A (*Bcg*^{s}, B10S macrophages) mouse strain and its congenic resistant counterpart B10A.*Bcg*^{r} (B10R macrophages).

Both B10R and B10S cell lines morphologically resemble typical macrophages. They display surface markers associated with the macrophage phenotype such as F4/80, complement and Fc receptors, MHC class II antigens, as well as biochemical markers such as lysozyme production. In addition, they perform typical macrophage functions such as phagocytosis, tumoricidal and bacteriostatic/bactericidal activities. Overall, these characteristics allow us to conclude the B10R and B10S macrophage lines represent tissue macrophages (appendix). Importantly, differences observed between B10R and B10S macrophages concerning phenotypic functions such as MHC class II expression and NO production have been maintained.

Bcg GENE, ANTIMYCOBACTERIAL ACTIVITY AND NITRIC OXIDE

Until the discovery of NO as one of the major antimycobacterial molecules produced by macrophages, ROI were believed to play an important role in the control of intracellular proliferation of mycobacteria as well as other intracellular pathogens. However, most of the evidence collected during recent years tends to support the conclusion that ROI do not play an essential role in the antimycobacterial activity of macrophages (Rutherford et al., 1993; Appelberg and Orme, 1993; Chan et al., 1992; O'Brien et al., 1991; Flesch and Kaufmann, 1988; Stokes et al., 1986).

There is now ample evidence, mostly from *in vitro* experiments, showing a close correlation between production of RNI by macrophages and antimicrobial activity (Green et al., 1993). Our results, using the B10R and B10S macrophages support the notion the NO is a key antimycobacterial molecule produced by activated macrophages. We were

able to positively correlate the production of nitrite with the capacity of B10R and B10S macrophages to arrest the intracellular proliferation of *M. bovis* BCG. This antimycobacterial function of the activated macrophages was inhibited by monomethyl L-arginine (N^gMMLA), a specific competitive inhibitor of NO, and promoted by L-arginine, in conditions in which the nitric oxide production by the macrophages was inhibited. Similar conclusions were made by Roach and colleagues (1991) using a model of *Leishmania* infection.

Evidence collected from in vitro experiments suggests that the capacity of mouse strains carrying the *Bcg/Lsh/Ity* resistant allele to control intracellular proliferation of BCG, *L. donovani*, and *S. typhimurium* results from their superior ability to produce NO *in vivo*. So far, no direct evidence has been provided to unequivocally implicate NO as an antimycobacterial agent *in vivo*. Evidence provided by Liew et al. (1991) and Stenger et al. (1994) using mice infected with *L. major*, suggests that NO plays a critical role in the capacity of resistant and susceptible animals to control proliferation of the protozoan parasite. Similar results were published by Green et al. (1993) using *F. tularensis*, and Beckerman et al. (1993) in the *Listeria* model. Our results indicate that the production of NOS mRNA and NO in response to IFN-γ was higher in B10R compared to B10S macrophages. Splenocytes obtained from B10A.*Bcg*^{r} mice were also superior producers of NO in response to IFN-γ compared to splenocytes obtained from the congenic B10.A (*Bcg*^{s}). This result suggests that NO production by tissue macrophages from BCG-resistant and -susceptible mouse strains may also differ.

Recently, Vidal et al. (1993) found in the *Nramp-1* gene a sequence encoding a 20 amino acid consensus motif termed the binding-protein-dependent transport system inner membrane component signature. This motif was found in prokaryotes as well in eukaryotes (Vidal et al., 1993). Interestingly, the same transporter motif is present in the *CrnA* gene of the fungi *Aspergillus nidulans* (Vidal et al., 1993). Since *CrnA* is involved in nitrate import, the authors speculated that Nramp-1 could function as a nitrite/nitrate

concentrator in phagolysosomes. The defect in this putative transport system of *Bcg*^{r} macrophages would affect their capacity to control intracellular multiplication of antigenically unrelated microbial targets (Vidal et al., 1993). Furthermore, we have found that B10R macrophages transfected with antisense *Nramp-1* cDNA containing a ribozyme construct (B10R-NrampRb) produce significantly lower levels of NO compared to mock-transfected B10R or non-transfected B10R macrophages after stimulation with IFN-γ (Kramnik et al., manuscript in preparation). These results suggest that production of NO is under the control of the *Nramp-1/Bcg* gene.

Brown et al. (1993) studied the production of NO_2^- in splenic macrophages in unrestrained (low plasma levels of glucocorticoids) and restrained (high plasma levels of glucocorticoids), BALB/c.*Bcg*^{r} and BALB/c.*Bcg*^{s} animals in the presence of IFN-γ and/or LPS. No difference in NO production by the splenic macrophages was found in unrestrained control animals. Since the basal levels of NO_2^- production are not reported by the authors, it is not possible to ascertain whether the extent of stimulation was the same in the BCG-resistant and -susceptible macrophages. Furthermore, we have found that the culture conditions, such as the duration of *in vitro* culture, will critically influence the ability of splenic adherent cells to respond to stimulation (L.F. Barrera, personal observations).

De Chastellier and colleagues (1993) found a difference in the level of phagosome-lysosome fusion between *Bcg*^{r} and *Bcg*^{s} macrophages that could explain, at least in part, a superior control of mycobacterial infection by *Bcg*^{r} macrophages. Mycobacterial proliferation inside macrophages was also shown to depend on pH (Crowle et al., 1991; Appelberg and Orme, 1993) and iron levels (Lepper et al., 1988). Interestingly, both the regulation of iron levels and pH may depend on the presence of NO or RNI. For example, it has been shown that NO activates IRP/IRF, and therefore, endogenously produced NO may modulate the post-transcriptional regulation of genes involved in iron homeostasis (Drapier et al., 1993; Weiss et al., 1993). In addition, the

intracellular level of iron regulates transcription of iNOS, and therefore, the quantity of iNOS in macrophages (Weiss et al., 1994). The dependency of *Mycobacteria* on external sources of iron has been very well established (Ratledge, 1982). *Mycobacteria* rely on the production of iron chelating molecules such as the siderophores, mycobactins and exochelins (Neilands, 1981). It has been reported that an increase in the concentration of iron caused an appreciable increase in the growth of several strains of mycobacteria including *M. tuberculosis* (Raghu et al., 1993). The same study also shows that the production of siderophores was significantly higher in virulent compared to avirulent strains, suggesting that the capacity of pathogenic *Mycobacteria* to chelate iron may be related to its capacity to proliferate intracellularly (Raghu et al., 1993).

Regulation of cytoplasmic pH in macrophages has been associated with monocyte differentiation, proliferation of monocytes and macrophages, and macrophage activation, as well as microbicidal activity (reviewed in Swallow et al., 1990). Prpic et al. (1989) reported that concentrations of IFN-γ which activated murine peritoneal macrophages for tumoricidal activity also caused a 0.1 pH unit cytoplasmic alkalinization. Furthermore, these investigators found that co-incubation with amiloride, an inhibitor of the Na^+/H^+ antiport, resulted in a 60-70% inhibition of the IFN-γ-induced accumulation of mRNA for the early response gene *JE*, and the mRNA for the MHC class II gene I-A_g. Although the mechanisms regulating intracellular pH in macrophages are not fully understood, different transport systems including the Na^+/H^+ antiport, the Na^+-dependent anion exchange, or the vacuolar-type H^+ ATPases, were shown to play an active role in the maintenance of cytoplasmic pH (Swallow et al., 1990, 1991). Moreover, it has been reported that phagosomal acidification is mediated by a vacuolar-type the H^+-ATPase in murine macrophages (Lukacs et al., 1990; Swallow et al., 1990). Interestingly, Swallow et al. (1991) reported that NO derived from L-arginine decreased H^+-ATPase-mediated recovery rate to normal physiologic pH in acid-loaded peritoneal macrophages.

The role of phagosomal and/or phagolysosomal acidification in the control of intracellular *Mycobacteria*, has been well illustrated by the observations of Crowle et al. (1991), and Appelberg and Orme (1993). In the study of Crowle et al. (1991), immunocytochemical analysis showed that phagosomal acidity was associated with killed but not living mycobacteria in macrophages. Appelberg and Orme (1993) found that the addition of a weak acid enhanced the mycobacteriostatic effect of mouse macrophages stimulated with IFN-γ. The authors suggested that the bacteriostatic effect of IFN-γ may be mediated through acidification of the infected phagosome, perhaps through activation of proton pumps in the phagosomal membrane (Appelberg and Orme, 1993).

Overall, the precise mechanism(s) involved in the mycobacteriostatic/microbicidal activity of macrophages remains to be elucidated. The data collected from recent years point to NO or RNI as the effector molecule(s) for the antimicrobial activity of macrophages. The microbicidal activity by NO or RNI is mediated indirectly. NO produced by macrophages may participate in phagosomal or phagolysosomal acidification. The acidification may, in turn, interfere with the iron availability for the invading microorganism. It is known that under acidic conditions, the soluble ferric iron (Fe^{3+}) is reduced to the most insoluble ferrous iron (Fe^{2+}), and therefore, the availability of soluble iron for the bacteria might become a limiting factor for growth. Another possibility is that NO or RNI directly interferes with the siderophores produced by some *Mycobacteria*. Although mycobactins, siderophores present in mycobacteria, do not have [Fe-S] clusters in their structure, it has not been tested whether NO could affect their iron-chelating ability. In addition, little is known about the chemistry and mechanism(s) of iron chelation by exochelins, siderophores secreted by mycobacteria. Our results indicate that *Bcg*^{r} macrophages produce more NO than *Bcg*^{s} macrophages in response to IFN-γ stimulation. Since Nramp-1 may be a protein involved in the transport of RNI, it is possible that the *Nramp-1/Bcg* gene influences bacterial multiplication in the MPS through production and/or transport of RNI to phagosomes and the subsequent iron deprivation of the bacteria.

MHC CLASS II EXPRESSION AND *Bcg* GENE

One of the most prominent pleiotropic effects of the *Bcg* gene is associated with the upregulation of MHC class II genes in *Bcg*^{r} macrophages. Johnson and Zwilling (1985) reported that peritoneal macrophages obtained 7 days after the i.p. injection of BCG transiently expressed I-A, while macrophages obtained 28 days after injection, continuousiy expressed I-A for up to 10 days in culture. Furthermore, the continuous expression of I-A by macrophages following the injection of BCG correlated with the genetic resistance of certain strains of mice to this microorganism (Johnson and Zwilling, 1985). Not only BCG but some other stimuli, including *Corynebacterium parvum* or IFN-γ, induced continuous I-A expression by macrophages from BCG-resistant but not from BCG-susceptible mice (Zwilling et al., 1987). Using mice congenic for the *Lsh* gene, Kaye et al. (1988) found that resistant mice showed a rapid increase in their accessory cell activity, subsequently allowing T-cell expansion. This change in the accessory cell function activity correlated with increased class II antigen expression relative to susceptible mice, both *in vivo* during early infection and *in vitro* in response to induction by IFN-γ. In addition, Denis et al. (1988) observed that purified splenic macrophages from uninfected *Bcg*^{r} mice contained a significantly greater percentage of Ia$^+$-bearing macrophages compared to *Bcg*^{s} mice.

The results from our studies using B10R and B10S macrophages stimulated with IFN-γ indicated that cytokine-induced surface levels of I-A antigen were higher in B10R compared to B10S macrophages. This difference in surface I-A expression correlated with differences in the I-A_β and I-A_α mRNA steady-state levels. B10R macrophages not only expressed more I-A_β mRNA in response to all doses of IFN-γ tested (1-100 U/ml), but were superior in I-A_β mRNA expression in all time points tested (8-48 hours).

Most of the evidence published indicate that the MHC class II antigens are mainly regulated at the transcriptional level (Kara and Glimcher, 1993). Therefore, we

performed studies to determine the molecular basis for the difference in I-A_α mRNA steady-state levels between B10R and B10S macrophages. Run-on analysis of the I-A_β gene mRNA in nuclei from B10R and B10S macrophages stimulated with IFN-γ, showed a modest difference in transcription of the I-A_β gene between B10R and B10S macrophages. This difference may partially explain the difference detected at the I-A_β mRNA steady-state levels. The estimation of the transcriptional induction of MHC class II antigens either in murine macrophages (Fertsch-Ruggio et al., 1988; Figuereido et al., 1989; Celada et al., 1989; Woodward et al., 1989) or macrophage lines (Bottger et al., 1988; Woodward et al., 1989), varies depending on the macrophage population or the particular gene studied. Induction of transcription ranges from 2 to 8-fold, with the peak of transcription ranging from 8 to 48 hours (Fertsch-Ruggio et al., 1988; Bottger et al., 1988; Figuereido et al., 1989; Celada et al., 1989; Woodward et al., 1989).

It is widely accepted that the regulation of surface class II expression is achieved mainly through transcription, although post-transcriptional effects have also been observed (reviewed in Glimcher and Kara, 1992). The transcriptional effects are mainly mediated by highly conserved S, X and Y sequence elements located in the proximal promoter region of the MHC class II gene promoters (reviewed in Glimcher and Kara, 1992; Ting and Baldwin, 1993). The difference between B10R and B10S macrophages in protein binding to important regulatory sequences of the I-A_β gene promoter may explain the difference in the transcription rate observed between B10R and B10S macrophages. We observed several quantitative as well as qualitative differences in the DNA-protein binding profiles of B10R and B10S macrophages. The transfection of cells with DNA constructs containing reporter genes, expressed under the control of the sequences of interest (i.e., X, Y, S sequence motifs), would allow the relative contributions of these particular sequence motifs in the transcriptional activation of the I-A_β gene in B10R and B10S macrophages to be properly addressed. Our attempt to perform transient transfection assays in these macrophages have not yet been successfull even though we could perform stable transfection quite easily (data not shown).

The difference in the I-A_s steady-state mRNA levels between B10R and B10S macrophages may also be due to post-transcriptional events resulting in the alteration of the I-A_s mRNA half-life. The results of our experiments clearly indicate that the I-A_s mRNA of B10R macrophages was significantly more stable than the I-A_s mRNA of B10S macrophages. We also have found a significant difference in the half-life of the I-A_α gene mRNA between B10R and B10S macrophages. Kern et al. (1989) reported a half-life of more than 10 hours (16-20 hours) for the I-A and I-E mRNAs in the WEHI-3 myelomonocytic cells stimulated with IFN-γ. The half-life value was obtained from the decay rate of steady-state levels of I-A and I-E mRNA following IFN-γ treatment but in the absence of actinomycin D due to its toxicity (Kern et al., 1989). It has also been reported that the half-life for DR mRNA of the human B-cell line, Raji, and subclones is about 5 hours (Calman and Peterlin, 1988).

The precise mechanism(s) responsible for the differences in the MHC class II antigen expression among macrophages from BCG-susceptible and -resistant mice is not fully understood, but post-translational mechanisms have been proposed (Vespa and Zwilling, 1989). Several reports have indicated that peritoneal macrophages explanted from BCG-resistant inbred and congenic mouse strains (Johnson and Zwilling, 1985; Vespa et al., 1987; Zwilling et al., 1987; Vespa and Zwilling, 1989; Brown et al., 1993), or macrophage-macrophage hybrids derived from WEHI-3 (cell line derived from BALB/c mice, *Bcg*^{s}) fused to peritoneal macrophages from the C3H/HeN mouse strain (*Bcg*^{r}) (Faris and Zwilling, 1990, 1991) are able to continuously express Ia antigen in response to i.p. infection with *Mycobacterium bovis BCG* (Johnson and Zwilling, 1985), *H. capsulatum* (Johnson and Zwilling, 1985), or *C. parvum* (Johnson and Zwilling, 1985; Zwilling et al., 1987), or *in vitro* treatment with high doses of IFN-γ (Zwilling et al., 1987; Vespa and Zwilling, 1989; Faris and Zwilling, 1990; Brown et al., 1993) compared to BCG-susceptible strains. Nath and colleagues compared the steady-state mRNA levels for I-A between BALB/c and C.D2 mice in response to stimulation with IFN-γ (Nath et al., 1988). These authors found that peritoneal macrophages from C.D2

animals expressed surface Ia antigen even though I-A mRNA was undetectable, while in macrophages from BALB/c animals, there was a parallel reduction of I-A surface antigen and I-A mRNA. Based on these results, they concluded that the differences in I-A expression by macrophages from *Bcg*^{r} and *Bcg*^{s} mice were due to post-transcriptional events (Nath et al., 1988). These conclusions were proposed without an assessment of the transcription of any of the MHC class II genes and secondly, the stability of MHC class II genes was not measured. Our data implicate both transcriptional and post-transcriptional mechanisms in the control of surface I-A antigen expression in *Bcg*^{r} and *Bcg*^{s} macrophages. Given the significant differences in half-lives for the $I\text{-}A_\beta$ and $I\text{-}A_\alpha$ gene mRNAs between *Bcg*^{r} and *Bcg*^{s} macrophages, mRNA stability may be the main component involved in differential I-A surface expression. However, we did not perform any studies directed at testing I-A protein stability, and therefore, we can not formally exclude protein antigen stability as a factor involved in MHC class II antigen expression in *Bcg*^{r} and *Bcg*^{s} macrophages. Furthermore, several potential problems should be solved concerning the hypothesis proposed by Zwilling et al. (1987) for class II antigen stability. Firstly, differences in I-A expression are only reached in the presence of high concentrations of IFN-γ (more than 100 U/ml), unlikely to occur in *in vivo* situations. Secondly, most of the evidence indicates that the acquisition of peptide by recycling of class II antigens is an unlikely event (reviewed in Neefjes and Momburg, 1993), and therefore consequences of antigen presentation are compromised. Thirdly, it has been reported that the half-life of class II/antigen complexes *in vivo* in the spleen is short (3-8 hours) (Muller et al., 1993); therefore new synthesis is necessary to maintain levels of surface class II molecules.

The potential immunological importance of the difference in expression of MHC class II antigens has been highlighted by the results obtained from *in vitro* and *in vivo* experiments. Thus, Denis and colleagues (1988) reported that splenic cells obtained from BCG-resistant mice were more efficient antigen-presenting cells (APC) for soluble and particulate antigens than those obtained from BCG-susceptible mice. Buschman and

Skamene (1988) established that during BCG infection, specific T helper cell responses were elicited earlier in BALB/c.*Bcg*^{r} compared to BALB/c.*Bcg*^{s} animals. In addition, these authors showed by FACS analysis that the number of splenic T lymphocytes increased after infection in the resistant mice (Buschman and Skamene, 1988). Similar results were obtained by Kaye and colleagues (1988) who showed that splenic adherent cells obtained from *Lsh*^{r} mice infected with *L. donovani*, displayed an increase in accessory cell activity that generated a greater T-cell expansion compared to *Lsh*^{s} mice. This accessory cell activity correlated with Ia antigen expression by splenic adherent cells *in vivo* and by enhanced responsiveness of peritoneal macrophages to IFN-γ *in vitro* (Kaye et al., 1988).

The development of acquired immunity in response to BCG or *L. donovani* infections appears to be more efficient in BCG-resistant compared to -susceptible mice. However, these results contradict somewhat the results published by Pelletier et al. (1982) who observed that markers of acquired immunity such as the number of granulomas in the liver and the spleen, the delayed-type hypersensitivity to PPD, and the resistance to a challenge with BCG or *L. monocytogenes* were superior in BCG-susceptible inbred mice compared to BCG-resistant mice. The evidence presented by Unanue et al. (1985) showing a direct correlation between the density of class II molecules and antigen-specific T cell proliferation, support the possibility of a more efficient development of acquired immunity in *Bcg*^{r} mice. On the other hand, it has been estimated that T cells were activated by antigen presenting cells in which as low as 0.1% of the I-A molecules presented specific antigen (Harding and Unanue, 1990; Srinivasan et al., 1991).

The MHC class II difference at the level of gene expression in these macrophages may be seen as markers of differential responsiveness of *Bcg/Lsh/Ity* gene expressing cells to stimulatory cytokines such as IFN-γ (Buschman and Skamene, 1989) and/or may be causally implicated in the acquisition of specific immunity.

NRAMP-1 AND IFN-γ, A POTENTIAL LINK?

Most of the data generated in the murine system indicate that IFN-γ is the major physiological activator of macrophages (reviewed in Farrar and Schreiber, 1993). It has been established that NO production, MHC class II expression, and bacteriostatic and/or bactericidal activities (reviewed in Farrar and Schreiber, 1993) depend on the presence of IFN-γ. Particularly relevant for this discussion has been the *in vivo* data collected in recent years using knock-out mice for IFN-γ (Dalton et al., 1993) or IFN-γR (Kamijo et al., 1993; Huang et al., 1993). Results obtained by Dalton and colleagues, who generated mice by a targeted disruption of the IFN-γ gene, showed that mice deficient in IFN-γ had impaired production of NO and reduced expression of MHC class II antigens (Dalton et al., 1993). Furthermore, the same investigators observed that IFN-γ-deficient mice were killed by a sublethal dose of *M.bovis* BCG (Dalton et al., 1993). Similarly, Kamijo and colleagues demonstrated increased mortality of IFN-γR knock-out mice in response to an infection with BCG that was correlated with a drastically reduced production of TNF-α compared to control mice (Kamijo et al., 1993).

One of the main conclusions from the research of the *Bcg* gene function(s), is that the *Bcg*^{r} allele confers an increased ability to respond to stimulatory signals such as IFN-γ. The differences found between B10R and B10S macrophages in NO production, iNOS mRNA expression (chapter 4), Ia surface antigen and I-A mRNA expression (chapter 5), as well as enhanced mycobacteriostatic function of B10R macrophages in the presence of IFN-γ (chapter 4), suggest that the presence of a fully functional *Nramp-1/Bcg* gene allows B10R macrophages to respond faster, to lower doses, or to mount a higher response to this physiological stimulator.

Immunoregulatory functions induced by IFN-γ, such as the induction of MHC class I and class II antigens, activation of macrophages, regulation of immunoglobulin class switching, and upregulation of Fc receptor expression are involved in modulating

a variety of other host defense mechanisms (reviewed in Farrar and Schreiber, 1993). The first event in inducing these responses is the specific binding of IFN-γ to its cell-surface receptor encoded on mouse chromosome 10 (Mariano et al., 1987). Recently, it was shown that signals elicited through the IFN-γR need the presence of an accessory factor (Kalina et al., 1993; Soh et al., 1993; Hemmi et al., 1994). Transfection of cells with specific accessory factor induced a variety of biological effects including 2,5'-oligoadenylate-synthetase, resistance to virus cytopathic effect, and MHC class I antigens (Kalina et al., 1993; Soh et al., 1993)

While the IFN-γR accessory factor(s) participating in MHC class II expression, nitric oxide production, TNF-α production and bacteriostatic/bactericidal activity of macrophages has not been described, it is tempting to speculate that *Nramp-1/Bcg* could collaborate with the IFN-γR for the efficient signal transduction.

Better understanding of the *Nramp-1/Bcg* gene function will allow the elucidation of the mechanisms of recognition, phagocytosis and killing of intracellular parasites by macrophages.

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APPENDIX

GENETIC RESISTANCE/SUSCEPTIBILITY TO MYCOBACTERIA: PHENOTYPIC EXPRESSION IN BONE MARROW DERIVED MACROPHAGE LINES.

PREFACE

Alternative alleles of the *Bcg* gene, *Bcg*^{r}, conferring resistance, and *Bcg*^{s}, conferring susceptibility to infection with *Mycobacteria*, *Leishmania*, and *Salmonella*, are expressed in host macrophages. Circumstantial evidence has suggested that the gene may regulate the innate level of activation of the phagocytes for bactericidal activity. My interest has been to explore this hypothesis by undertaking a systematic comparison of the molecular parameters of activation in macrophages expressing alternative alleles of the *Bcg* gene.

As a first step in the pursuit of this goal, we have established immortalized macrophage lines from bone marrow of mice of the BCG-resistant and -susceptible congenic strains, B10A.*Bcg*^{r} and B10A.*Bcg*^{s}, respectively. The genome of the two strains differs only in the segment of chromosome 1 which carries the allele determining resistance or susceptibility to intracellular pathogens. We have completed a full surface marker analysis of the cell lines which establishes their identity as macrophages. In addition, we have confirmed that the phenotype of BCG resistance or susceptibility, apparent following *in vivo* infection of the mouse strains described above, is faithfully reflected by the *in vitro* function of B10R (resistant) and B10S (susceptible) macrophage lines. Specifically, we have demonstrated that the B10R macrophages are more efficient

at killing *Mycobacteria in vitro*, and that the expression of I-A_s mRNA is upregulated in these cells as compared to their B10S counterparts.

SUMMARY

Congenic strains of mice susceptible (B10A.*Bcg*^{s}) or resistant (B10A.*Bcg*^{r}) to BCG were established. Here we describe the model system which has been established to analyze the functional activities of macrophages in the two strains. We have immortalized bone marrow macrophages from B10A.*Bcg*^{s} and B10A.*Bcg*^{r} congenic strains of mice and derived cloned macrophage lines designated B10S and B10R, respectively. B10R and B10S cell lines exhibited surface markers and morphology typical of macrophages. B10S and B10R were similar in their phagocytic activity, in the level of c-fms, in TGF-β mRNAs expression and in their expression of tumoricidal activity in response to IFN-γ plus LPS. However B10R macrophages expressed higher level of Ia mRNA when activated with IFN-γ compared to B10S macrophages. Analysis of the bacteriostatic activity of the two all lines revealed that B10R macrophages were much more active in inhibiting *M. smegmatis* replication than B10S. To measure the intracellular destruction of bacilli, a bactericidal assay based on hybridization with a oligonucleotide probe specific for mycobacterial ribosomal RNA was designed. The results demonstrated that B10R macrophages were endowed with enhanced constitutive bactericidal activity as compared to B10S.

In conclusion we have obtained macrophage lines from bone marrow of B10A.*Bcg*^{s} and B10A.*Bcg*^{r} mice that express to a similar extent functional and phenotypic characteristic of macrophages. However we demonstrate that relative to B10S macrophages, the B10R macrophages have higher expression of Ia mRNA and that they are constitutively more active in expressing mycobactericidal activity.

INTRODUCTION

The *Bcg* gene is located on the centromeric part of murine chromosome 1, which has homology with a linkage group located on the telomeric end of the long arm (q) of human chromosome 2 (27). The development of murine strains congenic for *Bcg*, genetically identical except for the segment of chromosome 1, containing the *Bcg* gene (24), opened new opportunities for studies on the cellular and molecular mechanism controlled by the *Bcg* gene. The analysis of recombinant inbred mice showed that *Bcg* gene is identical with or very closely linked to two other host resistance genes, one controlling resistance and susceptibility to infection with *S. typhimurium* designated as *Ity* and the other controlling resistance and susceptibility to *Leishmania donovani*, designated as *Lsh* (5,18,23,33). The results from numerous laboratories clearly demonstrated that macrophages are the cells responsible for the expression of the resistant or susceptible phenotype (1,8,15,21,30). It has been shown that the replication of the bacilli was significantly reduced following exposure to Bcg^r macrophages when compared with Bcg^s macrophages (9,11,30,32). The mechanisms of macrophage activation for mycobactericidal function are not clearly defined as yet. Studies to determine the intracellular mechanisms responsible for mycobacterial killing are complicated by the heterogeneity of freshly isolated macrophages, by the low yield of macrophages isolated from certain tissues and by the genetically-diverse origin of macrophages isolated from various mouse strains.

We have previously reported that infection of bone marrow cells with J2 virus leads to the immortalization of macrophage cell lines (2,3,4,7). We have used this protocol to generate macrophage lines from congenic strains of mice differing in their susceptibility to infection with *M bovis BCG* and *Mycobacterium smegmatis*.

The purpose of this study was to compare the properties of homogenous populations of macrophages immortalized from bone marrow of B10A.Bcg^r and

B10A.*Bcg*^{r} congenic mice and check whether the phenotypic expression of genetic resistance/susceptibility to mycobacteria was maintained by bone-marrow derived macrophage cell lines. Using this experimental model we were able to show that the *Bcg*^{r} macrophages (B10R) but not *Bcg*^{s} macrophages (B10S) caused a reduction in the proliferative abilities of the bacilli (CFU) and to demonstrate enhanced intracellular killing of mycobacteria by B10R macrophages.

MATERIALS AND METHODS

Mice.

C57BL/10.A mice (B10A.*Bcg*^{s}) were obtained from Jackson Laboratories, Bar Harbor, Maine. Congenic B10A.*Bcg*^{r} mice were constructed by transferring the *Bcg*^{r} allele of the A/J strain into the C57BL/10.A background using the Nx backcross system as described previously (20).

Cells.

Cultures of fresh bone marrow cells were prepared in medium consisting of Dulbecco's Modified Minimal Essential Medium, 15% heat-inactivated horse serum, 1% heat-inactivated fetal bovine serum, 2mM glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and 20% L cell-conditioned medium (a source of CSF-1). After 3 days, cultures were washed free of nonadherent cells. Adherent cells were cultured for additional 12 days and then screened for surface phenotype by flow cytometry analysis.

Immortalization of macrophage lines.

B10S and B10R macrophage cell lines were derived from the bone marrow of B10A.*Bcg*^{s} and B10A.*Bcg*^{r} mice, respectively. Bone marrow cells were infected with J2 retrovirus as described previously (4). After 36 hours of J2 virus infection at 37°C, cells were washed and cultured in fresh complete medium at a concentration of 10^6 cells per ml. Proliferating cells were then expanded and subcloned in soft agar (4). Isolated clones

were examined by microscopy after cytospin, and stained (Diff-Quik stain set, American Scientific Products, McGaw Park, IL).

Flow cytometry analysis.

After two subsequent cloning steps in soft agar, five clones of B10S and five clones of B10R macrophage cells were selected and studied. The cells were washed in phosphate buffered saline (PBS) calcium/magnesium free, containing 0.5% bovine serum albumin and 0.1% sodium azide (Sigma, St.Louis, MO). Freshly isolated bone marrow derived macrophages cultured in 20% L-cell conditioned media 15 days were used in the analysis as a comparison. One million cells were seeded into each well of 96-well round-bottomed microtiter plate. The following antibodies were used for flow cytometry analysis: anti-Lyt1.1, anti-Thy1.2 (Cedarline, Hornby, Ontario), anti-CD4 (L3T4), anti-CD8 (Ly2), anti-Fc receptor (2.4G2), anti-Mac1, anti-Mac2, anti-Mac3 (Hybritech, San Diego, CA), anti-asialoGM1, anti-F4/80 (kindly provided by Dr. B. Mathieson, NCI-FCRF, Frederick, MD). 10-20 μl of the appropriate antibody was added to the cell pellet, mixed and incubated for 10 min at 4°C. After the incubation cells were washed twice in PBS and 10 μl of secondary antibody labelled with FITC was applied. After 10 min incubation at 4°C, the cells were washed well and resuspended in PBS buffer and analyzed on a cytofluorograph System 30-H (Orto Diagnostic System, Westwood, MA). A green fluorescence histogram of 1,000 channel resolution was collected from 10,000 cells for each sample analyzed.

Lysozyme production.

Micrococcus luteus (Sigma, St.Louis, MO) was resuspended at a final concentration of 0.5 mg/ml in 1% agarose solution as described previously (22). 5 μl samples of standards containing 5, 25, 50, 100 or 500 μg per ml of lysozyme (Sigma, St. Louis) or tested cell line supernatants were seeded into individual wells. The concentration of lysozyme in the triplicate test samples was determined by interpolation from the semilogarithmic plot of diameter versus concentration of reference standard.

RNA purification and Northern blot analysis.

B10R and B10S macrophages were solubilized with guanidine isothiocyanate (Gibco-BRL) and total RNA was purified by centrifugation through a cushion of CsCl according to the method of Chirgwin et al. (6). Twenty micrograms of total RNA was separated on 1.2% agarose gel containing 2.2M formaldehyde as previously described (35). The following probes were used: c-fms (1.4 kb PstI fragment from the pSM3 plasmid), TGF-β (kindly provided by Dr. R. Derynck, Genentech Inc., Ca), Ia (I-A_β)(kindly provided by Dr. R. Germain, NIH, Bethesda) and "25-46" (EcoRI-SalI) probe of 1.9 kb recognizing 18S rRNA (kindly provided by Dr. Arnheim, State University of New York). Purified DNA inserts were labeled with [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) by nick translation. Specific activity of the probes was 5 x 10^8 cpm/μg of DNA. After hybridization, the blots were washed three times in 2 x SSC and 0.1% SDS at room temperature for 10 min and then three times in 0.1 x SSC and 0.1% SDS at 55°C for 15 min. The blots were then exposed to X-ray films with an intensifying screen at -70°C.

Cytotoxic activity against P815 tumor cells.

The tumoricidal activity of the immortalized macrophage lines was determined by ^{111}In release assay as described previously (34). Macrophages were incubated with medium, Lipopolysaccharide (LPS) (Sigma) at the doses ranging from 5ng per ml to 5 μg or with IFN-γ at the doses ranging from 1-50 U per ml plus 5ng per ml of LPS. The cells were incubated with the activators 18 hours at 37°C. After the incubation, the plates were centrifuged at 300 x g for 10 min, supernatants were removed and the plates were washed with warm Hank's solution three times. Finally ^{111}In-labelled P815 target cells were added at the concentration 5 x 10^3 per ml. After 18 hours the plates were centrifuged (300 x g) and 100 μl of supernatant from each well was harvested to assess cytolytic activity. Results were expressed as per cent cytotoxicity calculated by the following formula:

[(cpm released by target cells cultured treated with activated effector cells - spontaneous cpm released by target cells cultured with untreated effector cells) / total cpm incorporated in target cells - spontaneous cpm released by the target cells cultured with untreated effector cells] x 100

Phagocytosis of Mycobacterium smegmatis.

Phagocytosis of *M. smegmatis* was evaluated according to previously described method (17). Briefly, $5x10^4$ of B10R or B10S macrophages were spotted in quadruplicate on plastic petri dishes and after 30 min, the petri dishes were inverted and macrophages were kept in the inverted position for 12-14 hours in media containing no antibiotics. Macrophages were subsequently infected at a mycobacteria:macrophage ratio of 10:1 for 0.5, 1, 2, 3, and 6 hours. After the infection, macrophages were washed with warm Hanks solution followed by 5 min treatment with 0.25% glutaraldehyde at 37°C and again they were washed with warm Hanks solution. The plates were air-dried and mycobacteria were stained with carbolfucsin (Sigma) for 3 min at 50°C, washed with water and destained with ethanol-hyperchlorique acid mixture. After washing with water the macrophages were stained with methylene blue for 10 seconds and infected macrophages were counted per total of 400 cells. All counts were done in quadruplicates. The percentages of infected macrophages were then estimated for B10R and B10S macrophages.

Proliferative activities of *Mycobacterium smegmatis* after exposure to B10R and B10S macrophages.

B10R and B10S macrophages were infected with a ratio 2:1, 3:1, or 5:1 of viable mycobacteria per macrophage cell suspended in Neuman-Tytell medium supplemented with 15% FCS and 4mM glutamine; no antibiotics were added. After 6 hours of phagocytosis at 37°C, macrophages were washed five times with warm PBS. Uptake of bacilli was evaluated at time 0 in half of the dishes after lysis with 2.5% saponin solution. Remaining dishes were incubated for 48 hours and uptake of bacilli was

evaluated at time 48 hours after lysis with 2.5% saponin solution. Both lysates were used for determination of CFU of intracellular mycobacteria Dubos solid medium four days after inoculation and incubation at 37°C.

Bactericidal assay based on hybridization with a probe specific for mycobacterial rRNA.

B10R and B10S macrophage cell line were infected with a ratio 10:1 of viable mycobacteria per macrophage cell suspended in Neuman-Tytell medium supplemented with 15% FCS and 4mM glutamine; no antibiotics were added. After 6 hours of phagocytosis at 37°C, macrophages were washed five times with warm PBS and mycobacteria uptake was evaluated at time O in half of the dishes for each macrophage line. The remaining dishes were cultured for 48 hours in Neuman-Tytell medium supplemented with 15% FCS and 30 g/ml of chloramphenicol. Macrophages were then lysed with 2.5% saponin and the lysates were centrifuged at 900 rpm for 5 min. Supernatants containing mycobacteria were collected and bacteria were pelleted at 9,000 rpm for 20 min (Beckman, JA-20). In order to extract *M. smegmatis* rRNA the pellets were resuspended in sodium acetate-EDTA buffer pH 5.1 and 700 μl of zirconium beads. The mixtures were placed in bead-beater and beaten for 5 min, stopping after 3 min to cool the tube on ice. After phenol extraction, phenol:chloroform extraction and chlorcform extraction aqueous phases were precipitated overnight with 2.5 volume of 100% ethanol and 1/10 of the volume of 3M NaAcetate at -20°C. The pellets were washed twice with 80% ethanol, dried and dissolved in DEPC treated water. RNAs were then spotted onto nitrocellulose paper. Nitrocellulose was baked for 1 hour at 80°C under vacuum, prehybridized for 1.5 hours at 43°C and $2x10^5$ cpm per dot of ^{32}P-labelled 20-nucleotide-long probe specific for mycobacterial rRNA was added to the prehybridization buffer. The probe specific for mycobacteria was kindly provided by Dr. D. Stahl (College of Veterinary Medicine, Urbana, Il). After 12-18 hours of hybridization the membrane was washed once with 1xSSC and 1% SDS solution at room temperature and 3 times with the same buffer at 50°C followed by exposure to XAR film for 4-12 hours. X-ray pictures were then scanned with the laser scanner to estimate the difference in the

hybridization, then the dots were cut out and placed into scintillation vials and counted in β-counter.

RESULTS

Morphology and Surface Phenotype of the Bone Marrow-Derived Macrophage Cell Lines B10S and B10R.

We have studied two murine macrophage cell lines established from bone marrow cells of congenic strains B10A.*Bcg*^{r} and B10A.*Bcg*^{r}. J2 virus-infected bone marrow cells were grown for 7 weeks before they were cloned in 0.3% soft agar. B10R and B10S cell lines were similar morphologically, with typical eccentric nuclei of macrophages, some cytosolic vacuoles and they were strongly adherent to plastic. Diff-Quik differential staining of cytospin preparations revealed that both immortalized cell lines resembled typical macrophages.

The surface phenotype of the B10R and B10S cell lines was determined by flow cytometry analysis. As shown in Table 1 both cell lines exhibited a macrophage-like phenotype since they were strongly positive for Mac-1, Mac-2, Mac-3, F4/80, Fc receptors, Ly5 and they were negative for T cell markers (Lyt1.1, L3T4) and for surface Ig. The expression of cell surface markers by both cell lines was similar to that of 15-day-cultured bone marrow cells. As shown in Table 2 there was no significant difference in the surface phenotype expression among different subclones of B10R and B10S macrophages. Overall, both B10R and B10S lines had complete morphological and phenotypic characteristics of macrophages.

Functional Characteristics of B10S and B10R Cell Lines.

Having obtained macrophage lines from the bone marrow of BCG-resistant and

TABLE 1. Surface Phenotype of the B10R and B10S Cell Lines[a]

Surface marker	CD equivalent	Antibody designation	% Positive cells of B10R cell line	% Positive cells of % Positive cells of B10S cell line	15-day bone marrow cells culture
FcγR II	CDw32	2.4G2	83-97	70-89	75-89
Mac1	CD11b	M1/70	82-95	99	78-91
Mac2	ND	M3/38	52-98	99	29-39
Mac3	ND	M3/84	12-68	98	ND
F4/80	ND	F4/80	25-60	93	28-65
Ly5	CD45	Ly5	87	89	71-79
Lyt1.1	CD5	39/3	0.6-2.5	0.4-2.3	1.5
L3T4	CD4	H129.19	1.1-3.1	1.0-2.9	1.0
sIg		sIg	1.5-2.1	1.1-1.9	1.5-2.2
Ly2.2	CD8	Ly2.2	0.9-1.0	0.5-1.0	1.4-2.6
MT4	ND	MT4	1.0	1.0	1.0-2.5
4D11	ND	4D11	1.0	0.9-1.5	0.9-1.6

[a] As analyzed by flow cytometry described in detail in Materials and Methods.
Abbreviation: ND, not determined

TABLE 2. Comparative Analysis of B10R and B10S Subclones

	B10S subclones					B10R subclones				
	$B10S_1$	$B10S_2$	$B10S_3$	$B10S_4$	$B10S_5$	$B10R_1$	$B10R_2$	$B10R_3$	$B10R_4$	$B10R_5$
Surface phenotype[a]										
Macrophage markers[b]	+	+	+	+	+	+	+	+	+	+
T cell markers[b]	-	-	-	-	-	-	-	-	-	-
B cell markers[b]	-	-	-	-	-	-	-	-	-	-
Lysozyme secretion[c]	7.1	4.2	5.2	5.1	ND	<1	2.0	<1	1.7	1.4
Phagocytosis of mycobacteria[d]	ND	35%	43%	55%	ND	ND	60%	45%	50%	60%
Bactericidal activity[e]	ND	8%	0%	5%	7%	ND	62%	49%	58%	29%
Tumoricidal activity[f]	25%	21%	32%	25%	ND	33%	ND	15%	19%	ND

[a] As measured by flow cytometry, as described in Materials and Methods.

[b] Markers as described in Table 1.

[c] Assay as described in Materials and Methods.

[d] Macrophages were infected for 6 h with mycobacteria, and phagocytosis was estimated as described in Materials and Methods.

[e] Macrophages were infected with mycobacteria, and bactericidal activity was evaluated by hybridization method using an oligonucleotide probe specific for rRNA of mycobacteria as described in details in Materials and Methods.

[f] Macrophages activated with 500 ng of LPS/ml for a period of 18 h. Assay as described in Materials and Methods.

Abbreviation: ND, not determined.

susceptible murine strains our next step was to compare several of the cloned lines for a number of phenotypic and functional properties.

As shown in Table 2 all cell lines tested expressed macrophage but not B cell or T cell markers. When tested for tumoricidal activity there was no significant difference in the ability of lines derived from either mouse strain to kill P815 tumor taget cells.

When the macrophage lines were tested for lysozyme secretion, B10S-derived cells evolved higher levels of lysozyme than their B10R counterparts. Despite a lack of strong differences in phagocytic ability, hovewer an analysis of the bactericidal capacity of the macrophages revealed that B10R-derived lines were significantly more efficient at killing *M. smegmatis* than the B10S subclones.

Two subclones $B10R_4$ and $B10S_4$ were chosen for in-depth study of the functional capability of macrophages harvested from BCG-resistant and -susceptible mouse strains. Our aim was to test whether or not genetic difference between congenic B10A.*Bcg*^{r} and B10A.*Bcg*^{r} mice had been maintained by macrophage cell lines established from bone marrow.

C-fms, TGF-β and Ia mRNAs Expression in B10S and B10R Macrophage Cell Lines.

In order to establish whether there was any difference between B10S and B10R cell lines at the level of gene expression, total RNA was extracted and analyzed by Northern blot analysis. Since the c-fms protooncogene encoding a high affinity receptor for CSF-1 (colony stimulating factor-1) is highly expressed in mononuclear phagocytes and their precursors but not in other leukocyte populations (25,29,31,40), we tested the expression of c-fms in $B10R_4$ and $B10S_4$ cell lines. As shown in Fig.1A c-fms mRNA was expressed at similar levels in both cell lines and it was not modulated by IFN-γ. The same cell lines were tested for the expression of TGF-β mRNA and as shown in Fig.

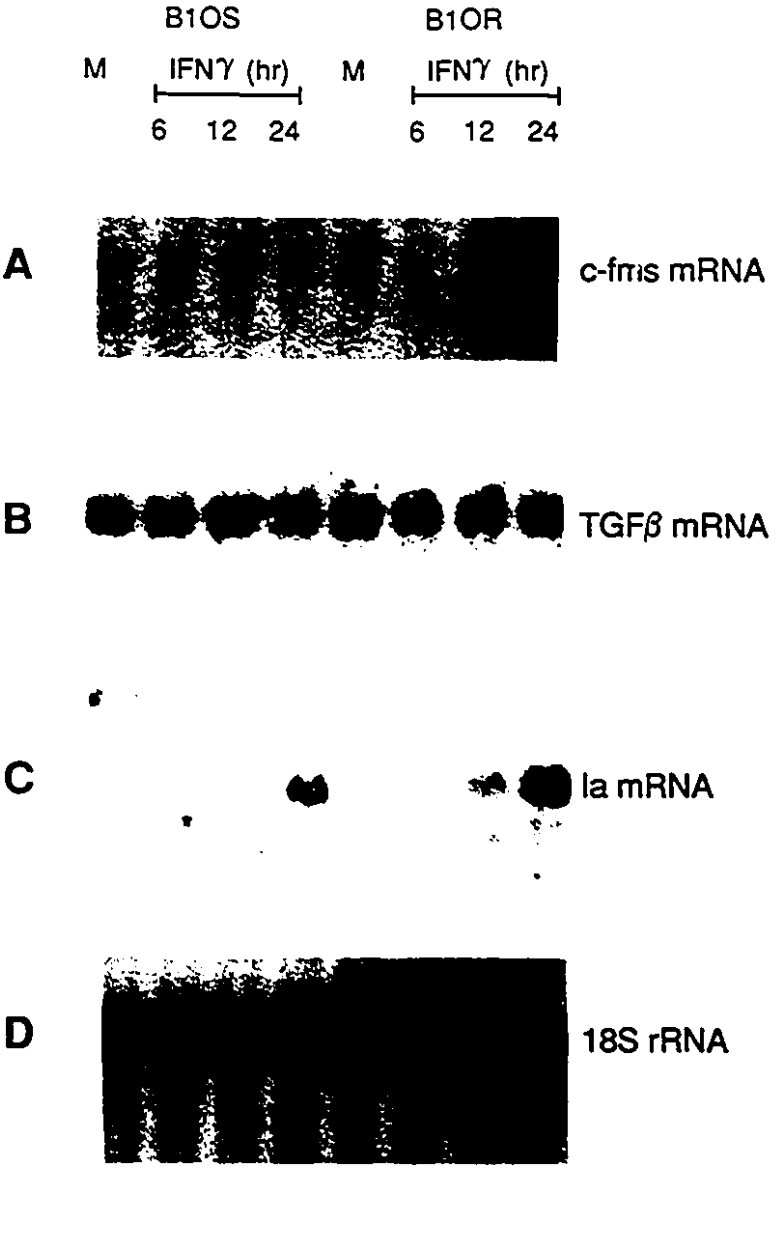

FIGURE 1. Effects of IFN-γ on the expression of c-fms mRNA, TGFß mRNA, Ia_s mRNA and 18S rRNA. 10^7 cells of B10R (subclone No.4) and B10S macrophages (subclone No.4) were treated with 10U per ml of IFN-γ for 6, 12 or 24 hours at 37°C. RNAs were extracted and analyzed by Northern blot for c-fms mRNA, TGFß mRNA, $I\text{-}A_s$ mRNA and 18S rRNA as described in Materials and Methods.

1B, the levels of TGF-β mRNA were similar in B10R and B10S macrophages and they were not modulated by IFN-γ treatment. On the contrary the induction of I-A_s mRNA in the two macrophage lines differed. We have consistently observed 3-4 fold higher expression of I-A_s mRNA in B10R macrophages than in B10S macrophages (Fig.1C). As shown in Fig.1D the same blot was rehybridized with a probe recognizing 18S rRNA, demonstrating an equivalent amount of RNA per sample.

Tumoricidal Activity of B10R and B10S Macrophage lines Activated with LPS or with IFN-γ

In a further analysis of the functional competence of $B10R_4$ and $B10S_4$ macrophages, the cells were tested for their ability to lyse P815 tumor targets in the presence of either LPS or IFN-γ. As shown in Fig.2 macrophage of both lines were capable of significant tumor lysis. There were no consistant differences in the level of P815 lysis by $B10R_4$ and $B10S_4$ macrophages. These data suggest that macrophages of both lines were fully able to respond to activating signals provided by LPS (Fig.2A) or IFN-γ (Fig.2B) over the same dose range.

Phagocytosis of *M. smegmatis* by B10S and B10R macrophages.

Subsequently, we have studied B10R and B10S cell lines for their ability to phagocytose *M. smegmatis*. As shown in Fig.3, we have found that B10S macrophages phagocytosed mycobacteria better at 0.5, 1 and 2 hr, but there was no significant difference at 3 and 6 hours, (see also Table 2).

Effect of B10R and B10S Macrophages on Proliferation of *M. smegmatis*.

As illustrated in Fig.4, the $B10R_4$ cell line showed enhanced bacteriostatic activity against *M. smegmatis* compared to the $B10S_4$ cell line. Macrophage cell lines were

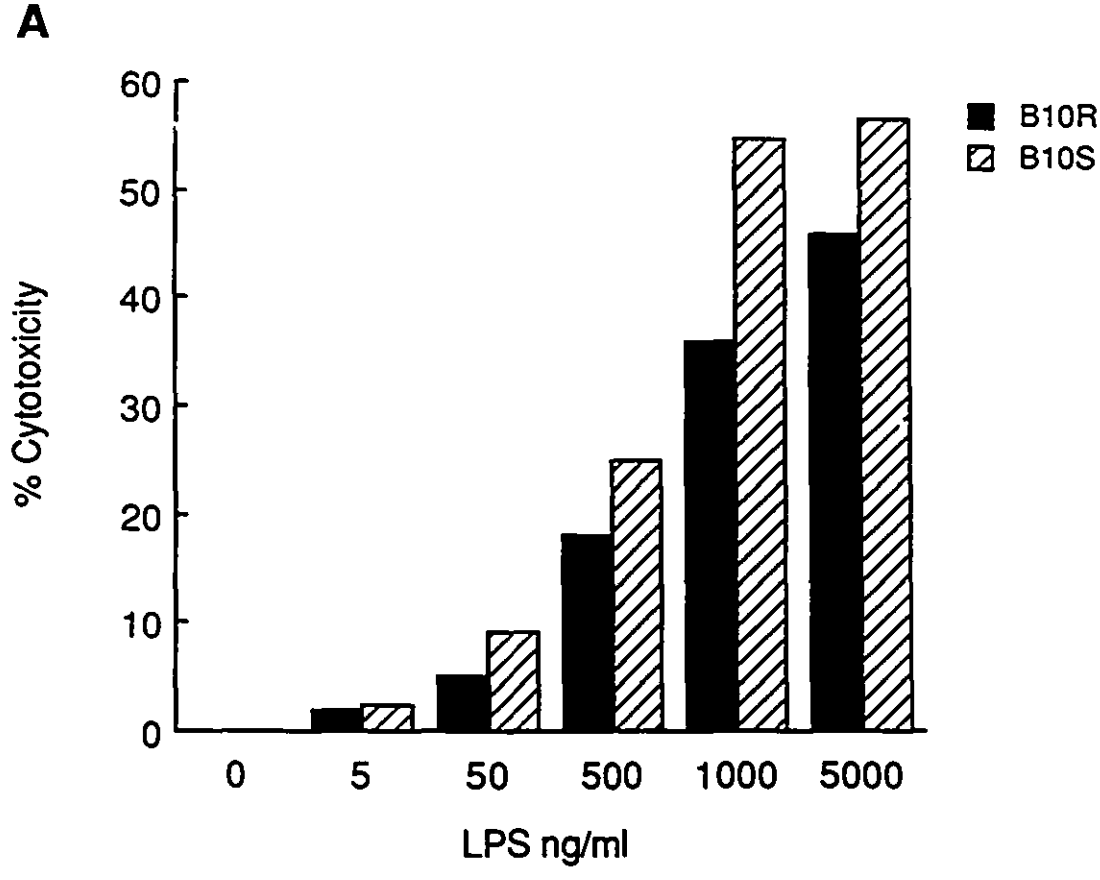

FIGURE 2A. Tumoricidal activity of B10S and B10R macrophages. Comparison of tumoricidal activities of B10S and B10R macrophages activated with LPS. $B10R_4$ and $B10S_4$ macrophages were activated for 18 hours with LPS at the doses ranging from 5ng to 5μg per ml, washed and P815 tumor cells labelled with ^{111}In were added for 18 hours. Supernatant from each well was then harvested and cytolytic activity was calculated as described in Materials and Methods. The results represent mean values calculated from 5 experiments.

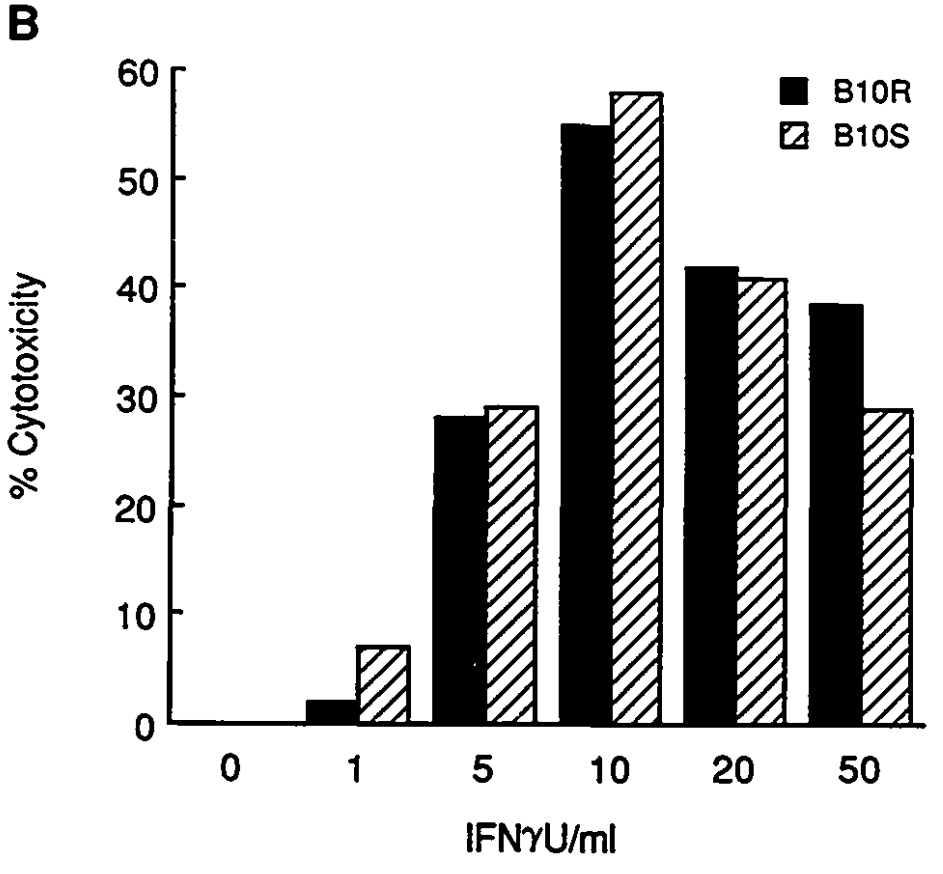

FIGURE 2B. Tumoricidal activity of B10S and B10R macrophages. Comparison of tumoricidal activities of B10S and B10R macrophages activated with IFN-γ. $B10R_4$ and $B10S_4$ macrophages were activated for 18 hours with 1-50 U of IFN-γ and 5 ng of LPS per ml, washed and P815 tumor cells labelled with ^{111}In were added for 18 hours. Supernatant from each well was then harvested and cytolytic activity was calculated as described in Materials and Methods. The results represent mean values calculated from 5 experiments.

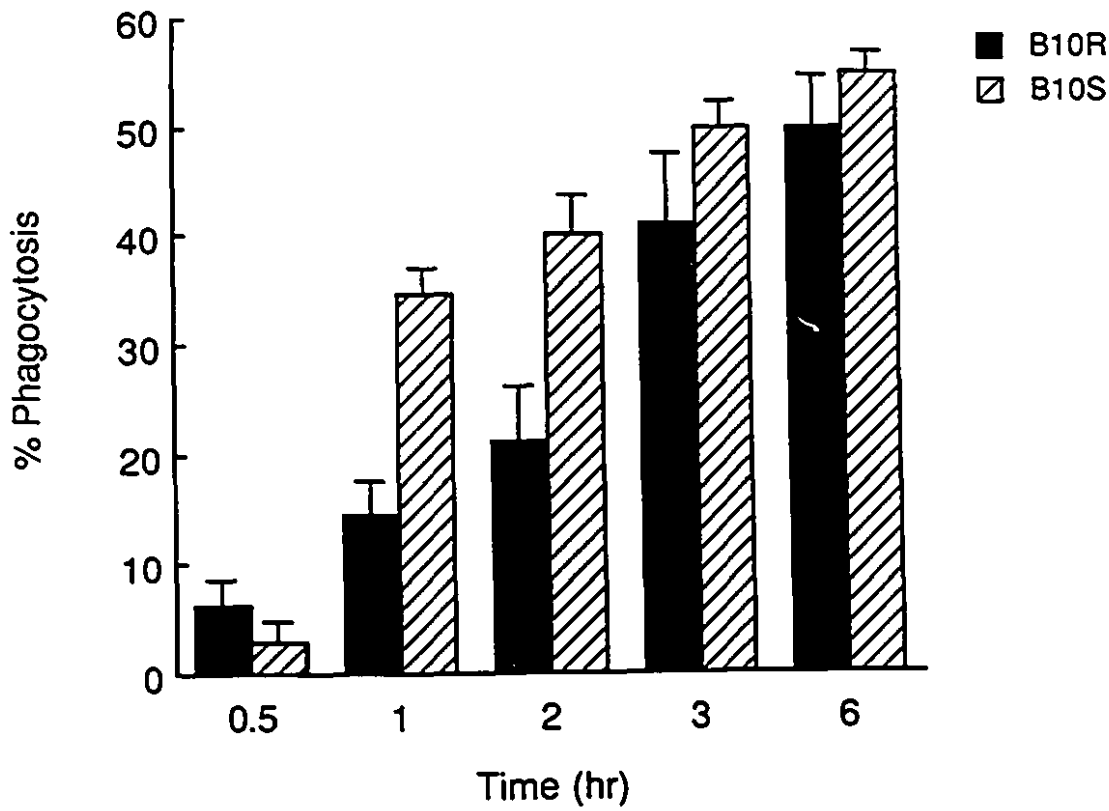

FIGURE 3. Phagocytosis of *M. smegmatis* by B10A and B10S macrophages. Phagocytosis of *Mycobacteria smegmatis* was evaluated after 0.5, 1, 2, 3 and 6 hours infection of $B10S_4$ and $B10R_4$ macrophages with mycobacteria as described in detail in Materials and Methods. Vertical bars represent standard errors for five experiments.

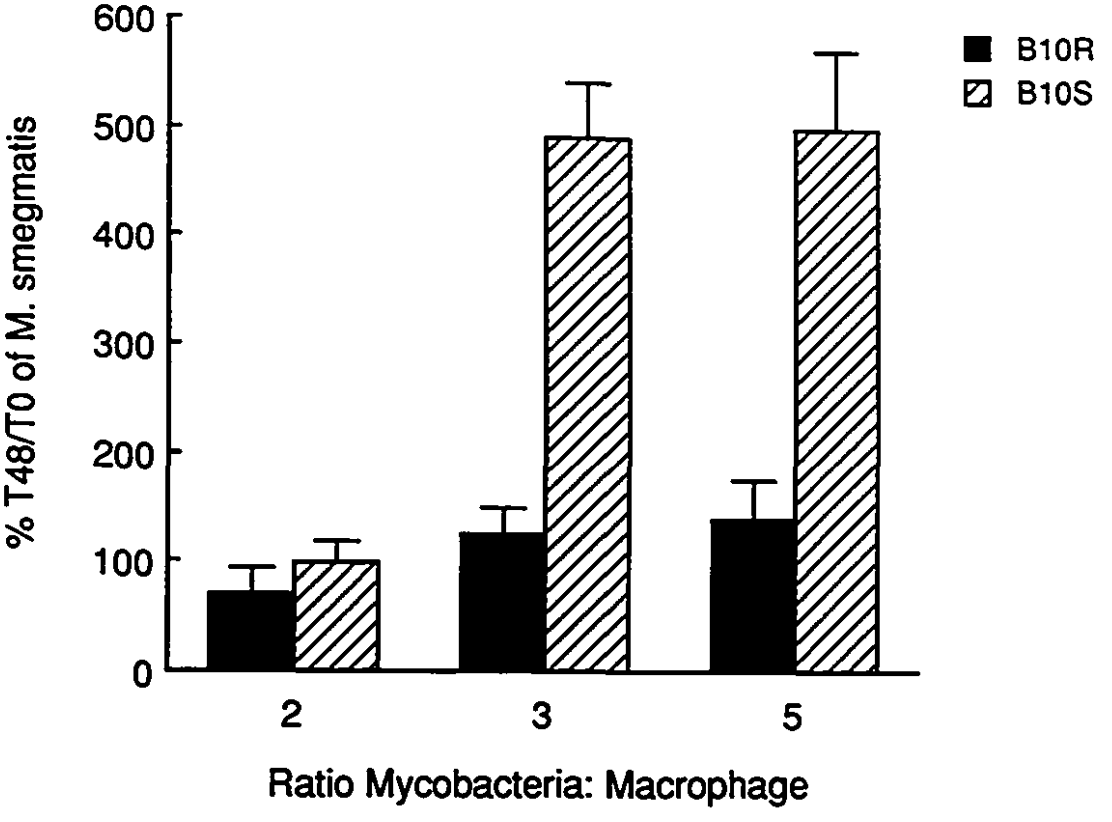

FIGURE 4. Effect of B10S and B10R macrophages on proliferative activities of *M. smegmatis*. $B10R_4$ and $B10S_4$ macrophages were infected with the ratios 2:1, 3:1 or 5:1 of viable mycobacteria per cell and mycobacteria uptake was evaluated of time 0 and of 48 hrs. The number of CFU of intracellular mycobacteria was then calculated. Vertical bars represent standard errors for four experiments.

infected with viable mycobacteria at ratios ranging from 2:1 to 5:1 of viable mycobacteria per macrophage and incubated for 6 hours, washed and tested for mycobacteria content immediately after or after additional 48 hours of incubation. The number of CFU of intracellular mycobacteria calculated for the $B10R_4$ cell line was consistently lower than for the $B10S_4$ cell line.

Bactericidal Activity of B10S and B10R Macrophages against *M.smegmatis*.

We have designed a new method to measure the intracellular destruction of bacilli. We have studied the mycobactericidal activity of B10R and B10S cells by a hybridization method using an oligonucleotide probe specific for mycobacterial ribosomal RNA. As shown in Fig.5 the probe was specific for mycobacteria and hybridized neither to eukaryotic RNA such as macrophage total RNA, nor to *E. coli* ribosomal RNA. The cpm recovered after the hybridization were proportional to the amount of *M. smegmatis* rRNA spotted on the nitrocellulose.

As illustrated in Fig.6 $B10R_4$ macrophages but not $B10S_4$ macrophages were able to eliminate 55-61% of *M. smegmatis* within 48 hrs. The results also indicate that the uptake of mycobacteria at time 0 was similar for B10S and B10R macrophages. Overall these data show that $B10R_4$ macrophages are more effective at bactericidal and bacteriostatic activities than $B10S_4$ macrophages.

DISCUSSION

The resistance of mice to BCG, *Salmonella typhimurium* and *Leishmania donovani* is genetically controlled by closely linked or identical genes *Bcg*, *Ity* and *Lsh* (5,16,23,33). Studies on macrophages isolated from genetically-resistant and genetically-susceptible

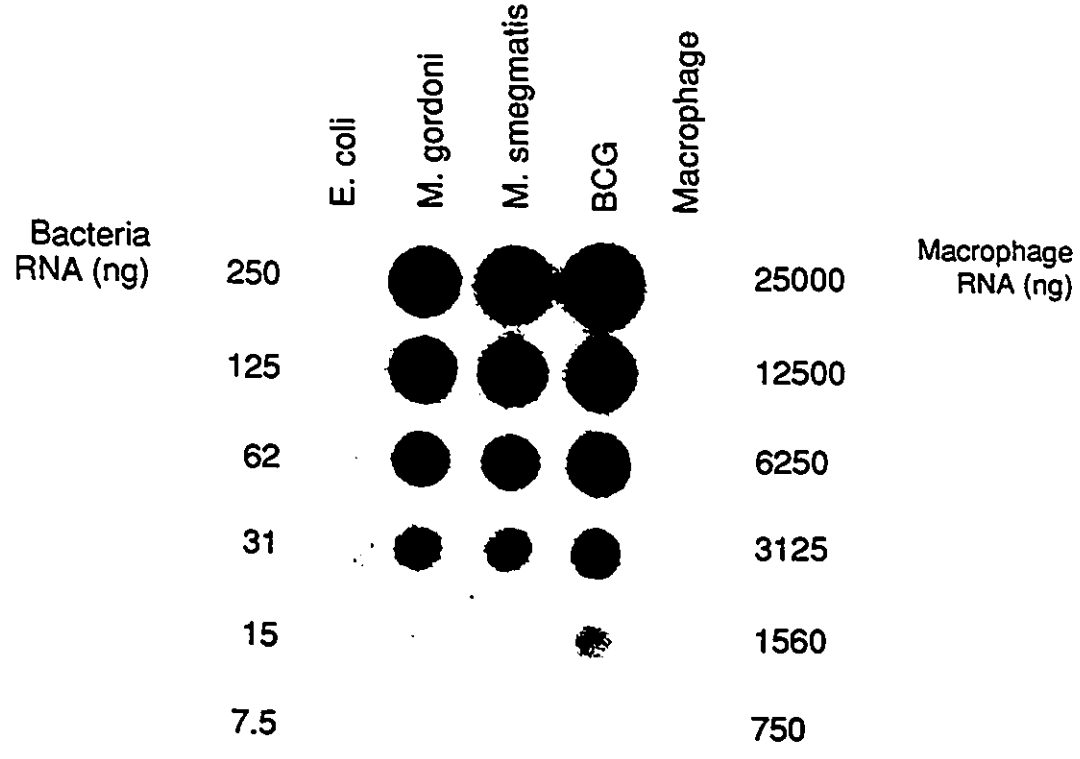

FIGURE 5. Specificity and sensitivity of oligonucleotide probe used for a detection of Mycobacteria. Dot blot analysis of bacterial and eucaryotic RNA was performed to establish specificity of hybridization to oligonucleotide probe. RNA isolated from M. gordoni, *M. smegmatis*, BCG, E. coli and macrophages was purified, spotted on the nitrocellulose ranging from 250ng to 7.5 ng for RNA from bacteria and ranging from 25μg to 0.75 μg for the macrophage total RNA. The dot blot was then prehybridized for 1.5 hr and hybridized with the ^{32}P-labelled oligonucleotide probe for 15 hours at 43°C followed by washing at 50°C. The blot was exposed for 4 hours to XAR film.

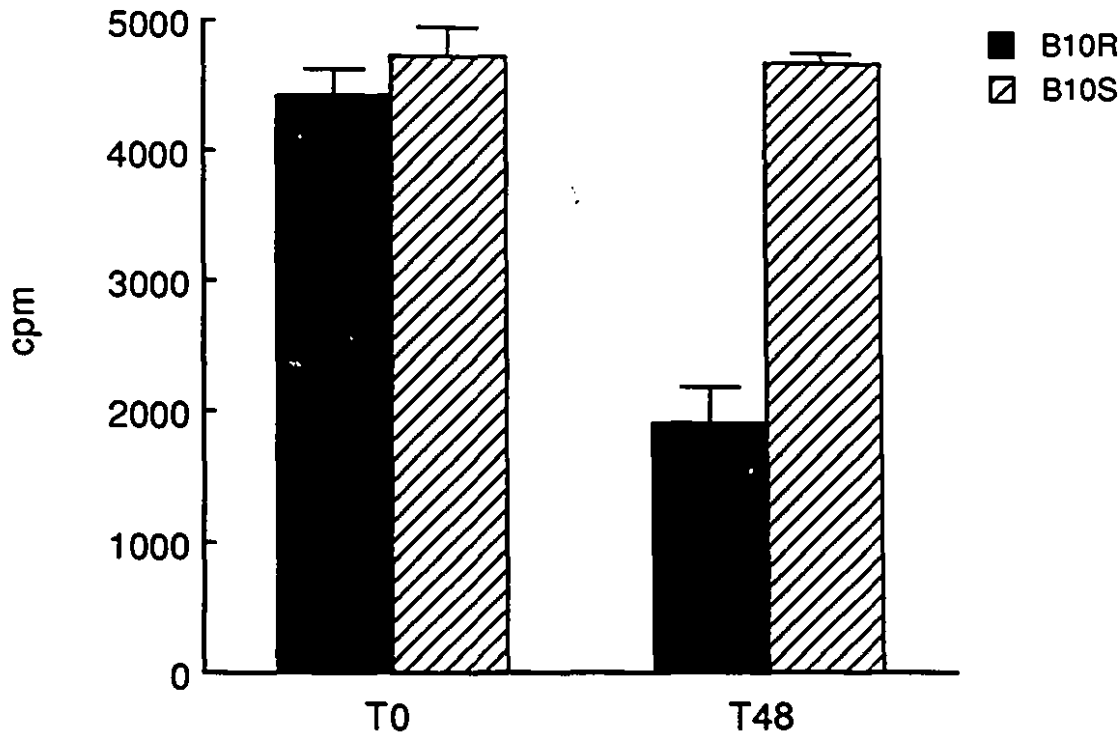

FIGURE 6. Bactericidal activity of B10S and B10R macrophages. Bactericidal activities of $B10S_4$ and $B10R_4$ macrophages were evaluated by the hybridization method described in detail in Materials and Methods. The results represent mean values calculated from 6 experiments.

strains had shown that macrophages express this gene (11,18,21,32). *In vivo* studies with a temperature-sensitive *Salmonella typhimurium* suggested that the gene is controlling bactericidal mechanism(s) (19). This finding was further supported by in vitro studies of peritoneal macrophages from *Salmonella*-resistant and susceptible mice (36). It has also been demonstrated that splenic and resident peritoneal macrophages from the *Bcg*^{r} congenic mice reduced the proliferative activity of mycobacteria significantly more than the macrophages from *Bcg*^{s} mice (11,30,32). Both the ^{3}H-uracil uptake assay as well as CFU measurement method used in those studies evaluated the ability of macrophages to modulate the proliferative capability of bacilli. The studies to determine the intracellular mechanisms triggering antimicrobial activities of murine macrophages have been complicated because of the low yield of macrophages isolated from tissues and serous cavities and because of their heterogeneity.

We report the establishment and comparative analysis of homogenous populations of macrophages immortalized from the bone marrow of congenic B10A.*Bcg*^{r} and B10A.*Bcg*^{s} mice. Both B10R and B10S cell lines morphologically resemble typical macrophages. Moreover, both lines exhibit macrophage surface phenotypes. Both macrophage cell lines responded to IFN-γ and LPS with similar levels of tumoricidal activity. We have not found any correlation between cytocidal activity and lysozyme secretion in the macrophage subclones which we tested. Interestingly, the B10S macrophages secreted more lysozyme than the B10R subclones. Whether or not the infection with mycobacteria would affect the ability of macrophages to produce, accumulate and secrete lysozyme remains to be established.

We have also established that B10R and B10S cell lines expressed comparable levels of macrophage specific mRNAs. Since the c-fms protooncogene is expressed in mononuclear phagocytes and their precursors (25,28,29,31,40), we tested its expression in the B10R and B10S cell lines. Both lines constitutively expressed high levels of c-fms

mRNA. We did not observe modulation of c-fms mRNA expression by IFN-γ in either macrophage line.

One of the consistent features of macrophages is the augmentation of membrane Ia antigen following IFN-γ treatment (13,14,26,38,39). Applying fluorescence-activated cell sorter analysis (FACS), Denis et al. (10) have shown that uninfected splenic macrophage populations of *Bcg*^{r} congenic mice contained more Ia positive cells that splenic macrophage population of *Bcg*^{s} mice. Furthermore, Zwilling et al. have shown, by indirect fluorescence methods, that IFN-γ treatment or BCG infection induced a prolonged expression of Ia antigen on the membrane of *Bcg*^{r} but not *Bcg*^{s} macrophages (12,37,41). Interestingly, we have observed that B10R macrophages showed a 3-4 fold higher induction of I-A_s mRNA when stimulated with IFN-γ as compared to B10S macrophages. These data indicated that the higher expression of cell surface Ia antigen on macrophages derived from resistant strains of mice may be due to the augmented expression of I-A_s mRNA. Our data do not however, exclude the possibility proposed by Zwilling et. al. that Ia antigen of *Bcg*^{r} macrophages is more stable than that of *Bcg*^{s} macrophages. The same RNAs were tested for the expression of TGF-β mRNA and we have found that both lines express constitutively high levels of TGF-β mRNA and we have not observed modulation of the TGF-β expression by IFN-γ.

In order to evaluate further functional capacities of both cell lines, macrophages were tested for their ability to phagocytose mycobacteria, to inhibit their proliferation and eventually to kill mycobacteria. B10S macrophages phagocytized mycobacteria better at 0.5, 1 and 2 hr but not at 3, or 6 hours. We have observed clear differences between B10R and B10S macrophages in their abilities to inhibit proliferation of *M. smegmatis* using the CFU assay. This analysis does not, however show whether the bacilli were killed or their proliferative abilities were impaired. To determine and compare the mycobactericidal activity of B10R and B10S macrophages, we developed a new hybridization assay which uses an oligonucleotide probe specific for ribosomal RNA of

mycobacteria. Since mycobacteria proliferation was deliberately blocked during the assay by the addition of chloramphenicol, we were able to test directly whether the bacilli were destroyed by macrophages. Our data have shown, for the first time, that resistant macrophages (B10R) but not susceptible macrophages (B10S) are able to destroy *M. smegmatis*. The antibiotic used in the assay did not affect the integrity of mycobacteria. We can not however exclude its influence on the innate ability to survive inside the macrophage.

Studies on the ability of cytokines and other biological response modifiers (BRMs) to modulate the bactericidal and bacteriostatic activities of B10S and B10R macrophage lines are in progress, and the availability of these cell lines will provide an important tool to evaluate the molecular bases of the macrophage-mediated, genetically-determined host defenses against mycobacterial infections. The development of these congenic lines also represents a crucial step in our efforts to clone the *Bcg* gene using subtraction hybridization, and to initiate the structure/function studies in a transfection model.

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CHAPTER VI

CLAIMS FOR ORIGINAL RESEARCH

1. The application of the PCR technology to assess mycobacterial infection and multiplication in macrophages, may constitute a major improvement for the study of the mechanisms that governs mycobacterial growth in cells of the MPS system. I have developed the method that correlates with other widely accepted techniques used for the estimation of mycobacterial proliferation and viability, such as ^{3}H-uracil incorporation and CFU, respectively. This method provides a reliable, simple, reproducible and rapid tool for the assessment of mycobacterial content in macrophages.

2. I studied the production of nitric oxide by B10R and B10S macrophages in response to IFN-γ and BCG infection. It was found that B10R macrophages produced significantly more nitric oxide and expressed more iNOS mRNA than B10S macrophages in response to stimulation with IFN-γ. The mycobacteriostatic activity of B10R and B10S macrophages correlated with their nitric oxide production in response to IFN-γ. Therefore, the *Bcg* gene encoded ability of BCG-resistant mice to control faster intracellular mycobacterial growth may relate to their superior capacity to produce nitric oxide early after infection.

3. It has been reported that macrophages from BCG-resistant mice express more MHC class II antigens than macrophages from BCG-susceptible mice. We used B10R and B10S macrophages as a model to study the molecular mechanism(s) underlying this difference. It was found that differences in MHC class II I-A surface expression between B10R and B10S macrophages in response to IFN-γ correlated with differences in I-A_β mRNA expression. This difference in I-A_β mRNA expression by B10R over B10S macrophages was observed in dose-response as well as in kinetic studies.

4. The molecular mechanism(s) underlying the I-A_β mRNA difference between B10R and B10S macrophages were further investigated. It was found that both

transcription of the I-A_β gene, and I-A_β and I-A_α mRNA stability differed between B10R and B10S macrophages. It was also observed a modest difference in the transcriptional activity of isolated nuclei from B10R and B10S macrophages stimulated with IFN-γ. Furthermore, the I-A_α and I-A_β mRNA were significantly more stable in B10R compared to B10S macrophages. These results suggest that both transcriptional and post-transcriptional events are responsible for the difference in I-A surface antigen expression between B10R and B10S macrophages.

5. The studies performed on the transcriptional activation of the I-A_β gene of B10R and B10S macrophages indicated that S, X, and Y motifs of the I-A_β promoter bound proteins present in nuclear extract preparations from unstimulated and IFN-γ-stimulated macrophages. An increase in protein binding to the X and Y cis-acting elements of the I-A_β gene promoter of B10R and B10S macrophages at the early time points after IFN-γ stimulation was observed. This increase in protein binding in response to IFN-γ suggests that an association between increase in protein binding activity and transcription of the I-A_β gene may exist.