Differential microbial clearance and immunoresponse of Balb/c (Nramp1 susceptible) and DBA2 (Nramp1 resistant) mice intracerebrally infected with *Mycobacterium bovis* BCG (BCG)

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Received 14 September 2001; received in revised form 30 October 2001; accepted 31 October 2001  
First published online 28 November 2001

Abstract

In mice, the gene encoding *Nramp1* (natural resistance-associated protein 1) exists in two allelic forms, differing for a point mutation. According to *Nramp1* genotype, extensive literature documents a clear-cut distinction of inbred strains in two non-overlapping groups that phenotypically express resistance (*Nramp1r*) and susceptibility (*Nramp1s*) to systemic infections. Here, we provide evidence that *Nramp1r* (DBA/2) and *Nramp1s* (Balb/c) mice differently handle intracerebral infection with *Mycobacterium bovis* BCG. Distinct trends of microbial clearance from the brain and also different patterns of local immune responses occur, thus arguing on the involvement of *Nramp1* gene product on the accomplishment of cerebral anti-mycobacterial defenses. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Intracerebral infection; *Nramp1* gene; *Mycobacterium bovis* BCG

1. Introduction

It is well known that some strains of mice show a higher level of natural resistance than others to infection with certain intracellular pathogens, including *Mycobacterium bovis* BCG (BCG) (for review, see [1,2]). The locus responsible for such resistance was initially mapped to a small region of the murine chromosome 1 and designed *Bcg* locus [3]. Subsequently, a candidate gene for *Bcg* locus has been isolated and cloned [4]. This gene, named *Nramp1* (natural resistance-associated macrophage protein 1), is present in two allelic forms in inbred strains, *Nramp1r* (resistant) and *Nramp1s* (susceptible), differing by a point mutation [4]. With respect to the wild-type, the mutated protein shows a single non-conservative glycine to acid aspartic substitution at position 169. Such a modification has been related with the loss of ability to resist microbial infections [4]. Evidence exists that *Bcg* and *Nramp1* are the same gene [5–8].

Among inbred strains of mice, a clear-cut distinction in two non-overlapping groups has been established by means of systemic-infection models employing BCG. In particular, an absolute association *Nramp1 Gly169* allelic form/resistant phenotype and *Nramp1 Asp169* allelic form/susceptible phenotype has been observed in 27 strains, since differential BCG growth rates have been observed in resistant and susceptible mice at spleen and liver during the early phases of infection (for review, see [9]).

To date, the large body of studies performed has dealt with the role of *Nramp1* on resistance or susceptibility to microbial insult by focusing exclusively on systemic-infection models, while no information is available on the involvement of *Nramp1* gene product on the accomplishment of cerebral anti-mycobacterial defenses. The aim of our study was to investigate the outcome of intracerebral BCG infection and the immunological events.
evoked locally by Nramp1 resistant and susceptible strains of mice.

2. Materials and methods

2.1. Mice

DBA/2 (Nramp1') and BALB/c (Nramp1') mice, 6 weeks old, were obtained from Charles River breeding Laboratories, Calco, Milan.

2.2. Microorganisms

*M. bovis* BCG (strain Montreal) was used. The microorganisms were cultured twice for 7 days in Dubos–TWEEN–albumin liquid medium, and the last culture was filtered through a 5-µm filter (Gelman Sciences, Ann Arbor, MI, USA) to remove aggregates. The resulting bacterial suspension was centrifuged at 10,000 × g at 4°C for 20 min, resuspended in complete medium without antibiotics, counted in a Thoma chamber, and used at the concentrations indicated below for the in vivo inoculation.

2.3. Intracerebral (i.c.) inoculation

i.c. inoculations were performed on anesthetized mice, as previously described [11]. Briefly, mice received the inoculum (30 µl) into the brain, 1 mm lateral and posterior to the bregma, at a depth of 2 mm with a 0.1-ml microsyringe and a 27-gauge disposable needle. Mice recovered from the trauma within 30–60 min. Surgical mortality was less than 3% and always occurred within 1–5 min after infection.

2.4. Quantitation of BCG in brains and spleens

Brains and spleens were aseptically removed and placed in a tissue homogenizer with 3 ml of sterile water. The number of colony forming units (cfu) was determined by a plate dilution method on Dubos agar. Plates were then sealed and colonies of BCG were counted after 3 weeks of incubation at 37°C. Results were expressed as number of cfu per organ.

2.5. Histological analysis

Brain specimens were fixed in Carnoy solution (ethanol:chloroform:acetic acid, 6:3:1), dehydrated and embedded in paraffin; sections (5 µm), were cut and stained with hematoxylin eosin by standard procedures. Modified Ziehl–Neelsen or Grocott staining were also performed. Adjacent sections were deparaffinized, incubated in methanol containing 0.3% H2O2 to block endogenous peroxidases and hydrated in 50 mM TBS (pH 7.6). Sections were stained with (i) antibody against glial fibrillary acidic protein (anti-GFAP; 1:200 polyclonal from Dako, Denmark) in TBS and secondary biotinylated antibody (1:40) to identify astrocytic immunoreactivity or with (ii) lectin *Ricinus communis* agglutinin-1 (RCA1; biotinylated compound from Vector), known to visualize human [12] as well as rodent [13] microglial cells. All sections were exposed to avidin–biotin–peroxidase complex (Vectastain ABC reagent, Vector). The peroxidase reaction was carried out by incubation with Tris–HCl, pH 7.6, containing diaminobenzidine tetrahydrochloride and hydrogen peroxide.

2.6. RNA extraction

Total RNA was isolated from a pool of three brains per experimental group by solubilization with guanidine isothiocyanate, as detailed by Chomczynski and Sacchi [14]. Following digestion in DNase, a 15-µg aliquot of RNA was electrophoresed on a 1% agarose formaldehyde denaturing gel containing ethidium bromide to detect the intact 18S and 28S rRNA and to confirm the integrity of the isolated RNA. The amount of RNA was calculated by measuring the optical density at 260 nm in a spectrophotometer (Beckman). Three aliquots of total RNA were made from each sample and processed separately for cDNA synthesis and polymerase chain reaction (PCR) amplification assay.

2.7. Reverse transcription (RT)

Following heating at 65°C for 3 min and subsequent chilling on ice, a 5-µg aliquot of total RNA in 13.66 µl of diethylpyrocarbonate-treated water was used in each RT reaction. RT buffer for each sample contained the following: 1.1 µl of 1 M Tris–HCl (pH 8.3) (Bethesda Research Laboratories, BRL, Gaithersburg, MD, USA), 0.13 µl of 1 M MgCl2 (BRL), 0.5 µl of 1 M KCl (BRL), 0.22 µl of 150 mM dithiothreitol (Promega, Madison, WI, USA), 1.33 µl of deoxynucleoside triphosphate (25 mM each, Promega), 1.4 µl of oligo (dT)15 primer (0.8 µg µl⁻¹; Promega) 0.33 µl of RNasin (40 U µl⁻¹; Promega) and 1.33 µl avian myeloblastosis virus reverse transcriptase (7.5 U µl⁻¹; Promega). The total reaction was stopped by the addition of 980 µl of TE buffer (final volume of each sample of cDNA, 1 ml).

2.8. PCR

Each cycle of amplification consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min (for tumor necrosis factor (TNF)γ, interleukin (IL)-6 and interferon (IFN)γ), 65°C for 1 min (for IL-1β, and GAPDH) or 45 s (for MIP-2), or 52°C (for IL-12) for 30 s, and extension at 72°C for 1 min. Before each cycle, the samples were heated to 100°C for 2 min and then cooled to 80°C before being added to the reaction mixture. Amplification was repeated
for 30 cycles in a Perkin Elmer Cetus DNA thermal cycler. The rate of amplification with all the primer sets used was exponential up to 32 cycles. Ten microliters of the PCR amplification products were separated on an ethidium bromide-stained 1.5% agarose gel, visualized by UV transillumination. Densitometric analysis was performed using a Gel Doc 1000 densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and relative peak areas were expressed in densitometric arbitrary units. For each sample, the densitometric units of the cytokine band were normalized to that found in the GAPDH band. Such bands showed comparable peak areas among the experimental samples tested, the range being 220\textsuperscript{250} counts mm\textsuperscript{2}.

Aliquots of 0.05 µg of φX174 replicative-form DNA-HaeIII fragments (New England BioLabs, Beverly, MA, USA) were run in parallel as molecular size markers (providing bands at 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp). The amplified bands showed their predicted sizes. Cytokine-specific primers were DNA-specific and non-reactive with RNA. The following oligonucleotide 5’- and 3’-primer sequences (synthesized in our laboratory) were used: IL-12 p40, CGTGCTCATGGCTGGTGCAAAG and GCCCAAGAAGTTGCAGATGAAG; TNFα, AGCACCACCTGAGCAACCACCA and ACACCATTCTCCACAGAGAAC; IL-1β, TGAAGGGCTGCTTCCAAACCTTTGACC and TGTCCATTGAGGAGAGCTTTCAGC; IL-6, GTGACAACCACGGCTTTCCCTACT and GGTAGCTATGGTACTCCA; IFNγ, TGAACGCTACACACTGCATTTG and CGACTCCTTTCTCGTCTCTGAG; MIP-2, GTGATGAGCTGTCTTAC and GCCTGGGGCATCCTTCTAC; GAPDH, CCTTCATTGACCTCAACTACATG and AGTCTTCTGGGTGCGATG.

Positive control DNAs for each cytokine were obtained from Clontech Laboratories, Palo Alto, CA, USA, while negative controls consisted of samples in which (i) RNA was replaced by diethylpyrocarbonate plus distilled water, (ii) the RT was omitted to detect any contamination by previously amplified cDNA, and (iii) primers were not added.

2.9. Statistical analysis

The significance of the data was evaluated using the Student’s t-test. The results depicted in the figures are the mean of three independent experiments.

3. Results

To investigate the onset and outcome of BCG cerebral infection in mice with Nramp\textsuperscript{1} resistant or susceptible genotype, DBA/2 and Balb/c mice were i.c. infected with two different doses of the microorganism and then assessed for microbial load (cfu organ\textsuperscript{1}) at different times post-infection. As shown in Fig. 1A,B, the kinetic profiles of microbial growth in brains of Balb/c mice were similar to those observed in the brains of DBA/2 mice. In fact, the number of cfu increased during the first 2 weeks after infection and then started to decline. Nevertheless, major differences were observed between Nramp\textsuperscript{1} and Nramp\textsuperscript{1}s mice in terms of total load. Balb/c mice were more heavily colonized in the brain, with four- to eight-fold more BCG in their brains than the resistant DBA/2 mice. Particularly, when 10\textsuperscript{5} BCG mouse\textsuperscript{1} was injected, DBA/2 mice showed little significant colonization that reached the maximal peak of 22\times10\textsuperscript{3} cfu brain\textsuperscript{1} on day 14. In contrast, at the same time point, BALB/c mice showed as much as...
Fig. 3. Photomicrographs of parasagittal brain sections from DBA/2 (Nramp1r) or Balb/c (Nramp1s) BCG-infected mice. Mice were i.c. infected at day 0, and histopathological analysis was performed on day 14 after challenge as detailed in Section 2. Ziehl-Neelsen-stained brain sections are shown in panels A (×75, DBA/2 mice) and B (×75, Balb/c mice); Grocott-stained brain sections are shown in panels C (×37.8, DBA/2 mice) and D (×37.8, Balb/c mice); hematoxylin eosin-stained brain sections are shown in panels E (×87.5, DBA/2 mice) and F (×87.5, Balb/c mice).
Fig. 4. Photomicrographs of parasagittal brain sections from DBA/2 (Nramp1r) or Balb/c (Nramp1s) BCG-infected mice. Mice were i.c. infected at day 0, and histopathological analysis was performed on day 14 after challenge as detailed in Section 2. GFAP-stained brain sections, identifying astrocytes, are shown in panels A (175×, DBA/2 mice) and B (175×, Balb/c mice); RCA1-stained sections, visualizing microglial cells, are shown in panels C (175×, DBA/2 mice) and D (175×, Balb/c mice).
In both groups, the microbial load decreased to minimal comparable levels at 28–35 days post-infection. When the dose of $10^6$ BCG mouse was employed (Fig. 1B), both strains of mice were colonized to a higher extent with respect to the low infecting dose. Again, significant differences between BALB/c and DBA/2 mice were observed up to day 28 post-infection.

Figure 2 shows the kinetic of spleen colonization in BALB/c and DBA/2 mice infected with $10^5$ and $10^6$ BCG mouse. In these organs, massive BCG multiplication was observed only in susceptible mice, with maximum spleen load at day 21 post-infection (Fig. 2A,B). In contrast, the cfu from DBA/2 mice remained unchanged regardless of the time-points tested. By prolonging the observation period, we found that both brains and spleens were still colonized as far as 70 days post-infection; little but consistent mycobacterial load was detected regardless of the mouse strain and the initial challenging doses used (data not shown).

Histopathological analysis of serial sections was performed in brains from Balb/c and DBA/2 mice infected with $10^6$ BCG mouse on day 14 post-infection. Ziehl–Neelsen staining showed the presence of acid-fast bacilli in both sets of slides (Fig. 3A,B). By using Grocott staining, differences in mycobacterial load were observed between DBA/2 and Balb/c mice (Fig. 3C,D); in fact, only the latter showed abundant and large-size clusters of bacteria, likely representing independent colonies, while brains from DBA/2 mice were characterized by scattered and isolated bacteria. As depicted in Fig. 3E,F, the hematoxylin eosine staining of sequential slides showed that, in both strains of mice, infection did not result in granulomas, giant-cell formation or necrotic areas, that were never detectable even at later time points (data not shown). Interestingly, a major difference between Balb/c and DBA/2 response was observed, since a massive infiltrate of inflammatory cells was evident in Balb/c but not in DBA/2 brains. A similar picture, with an even more pronounced cell recruitment, was observed on day 21 post-infection (data not shown). In all animals, cerebellum was never affected by mycobacterial infection (data not shown).

In order to establish the possible involvement of local immune cells in the outcome of BCG i.c. infection, astrocytes and microglia were identified by specific staining with anti-GFAP and RCA1, respectively. As depicted in Fig. 4, brain sections from BCG-infected mice showed a marked positivity to both detection systems. In particular, GFAP-positive cells appeared localized mainly in the peri-ventricular areas and the pictures were comparable in the two strains of mice (Fig. 4A,B); as expected, the infiltrate cells remained unstained. In contrast, appreciable differences were observed following RCA1 staining, since DBA/2 brains showed positive cells detectable in the peri-ventricular areas where they were abundantly recruited, and also in the adjacent parenchyma (Fig. 4C). Brains from Balb/c mice showed reactive cells homogeneously dispersed around the parenchyma or close to blood vessels (Fig. 4D).

To investigate the immunological events locally evoked by BCG, molecular studies were performed by semi-quantitative RT-PCR analysis. The kinetic curves of gene expression were established for MIP-2, IL-12, IL-1β, IFNγ, IL-6 and TNFα. As shown in Fig. 5, time-dependent differences were observed between Balb/c and DBA/2 mice.
In particular, unlike DBA/2 mice, Balb/c mice showed early and pronounced increase in MIP-2 mRNA levels. Similarly, IL-1β gene transcripts were also detected at higher levels in Balb/c mice with respect to DBA/2 mice, at least at early stages of infection. In contrast, DBA/2 mice showed consistently high levels of IL-12 gene transcripts already detectable as early as day 1 post-infection, whereas such transcripts in Balb/c mice were detectable only from day 28 on, and remained consistently below those observed in DBA/2 mice. Differences were also observed with regard to IFNγ mRNA levels, in that early but rapidly decreasing transcripts appeared in Balb/c mice, while late (from day 14 on) and long-lasting transcript levels were observed in DBA/2 mice. A rapid increase in IL-6 mRNA levels, followed by a gradual decrease, was observed in DBA/2 mice, while such levels appeared later (from day 7 on) and remained at steady-state in Balb/c mice. Finally, a similar kinetic of appearance of TNFα gene transcripts was found in both strains of mice.

Overall, we provide evidence that BCG cerebral infection evolves differently in Balb/c Nramp11r and DBA/2 Nramp11s mice, and that distinct patterns of immune responses are locally evoked by the infecting microorganism in these two genetically non-overlapping hosts.

4. Discussion

Inbred strains of mice segregate in two non-overlapping groups with respect to natural resistance to several mycobacterial species (as reviewed in [1,2,9,15]). The studies so far performed, exclusively dealing with systemic infection, have allowed the identification of Nramp1 gene, that maps to chromosome 1 and controls host resistance to disseminated mycobacterial infections [3,4]. Here, we give the first evidence on the contribution of Nramp1 gene also in the outcome of BCG infection at the cerebral level. In fact, differential patterns of BCG growth are observed in brains from Nramp11s Balb/c and Nramp11r DBA/2 mice during the early times post-infection, closely recalling previously described data on systemic-infection models [3,16,17]. The control of early mycobacterial growth occurs only in Nramp11s mice and the phenomenon is evident in both brain and spleen. The innate greater ability to contain brain BCG growth observed in the Nramp11s mice with respect to Nramp11r mice is documented at both inoculum sizes employed, although more evident at the low than at the high challenging dose. The high inoculum size allows a prolonged colonization of the brain, with statistically significant differences in bacterial counts observed between the two strains as late as 28 days post-infection. It is conceivable that the innate anti-BCG mechanisms are involved in the control of cerebral BCG infection to a different extent, depending on the Nramp1 genotype. The peculiarity of this compartment, where astrocytes and microglia are the first line of defense against microbial insult [18,19], allows us to conclude that Nramp1 gene influences the functional status of brain macrophages in vivo. In this respect, in vitro studies on microglial cell lines established from Nramp11s, Nramp11r and Nramp1-knock out (Nramp1KO) mice provide formal proof that Nramp1 genotype influences microglial cell-mediated anti-microbial functions. In particular, killing and secretory activities, but not phagocytosis, are impaired in Nramp11r [10] and Nramp11s KO microglial cells (submitted for publication).

The growth of bacilli in the brains of Nramp11r Balb/c mice results in dissemination and massive spleen colonization, while resistant mice are able to efficiently control such growth in the periphery. These results are in line with previous data on tissue distribution of mycobacteria following systemic infection [3,16,17], thus implying that the immunological devises employed against BCG remain unmodified, independently on the initial site of infection.

The relevant differences observed in terms of microbial counts between Nramp11s and Nramp11r mice have been confirmed by histopathological analysis of brain tissues, providing direct evidence on BCG presence and distribution following i.c. infection. It is worth noting that Grocott staining, known to detect mainly fungi [20], appears a particularly useful approach to emphasize the presence of mycobacteria within tissues, as well as microbe-to-microbe aggregations. Furthermore, hematoxylin eosin staining indicates that, differently from that observed using other pathogens, such as Candida albicans [21] or Cryptococcus neoformans [22], no necrotizing granulomatous reaction or giant cells occur at the cerebral level in response to local BCG infection in both Balb/c and DBA/2 mice. Interestingly, a consistent difference between the two strains is observed in terms of the inflammatory response, that is significantly more pronounced and widely distributed in the ventricular and peri-ventricular areas of Balb/c mice. Such a different picture between Nramp11s and Nramp11r mice is detectable also at the meningeal level and at different time points (data not shown).

Many studies stress the role of immunocompetent brain elements, such as microglia and astrocytes, in the course of cerebral infections [18,19,23,24]. Soluble factors, such as cytokines or nitric oxide, are known to be locally produced by microglia and astrocytes, and to play an important role in anti-microbial processes [25–28]. For example, we have previously shown that i.c. inoculation with a low-virulent strain of C. albicans causes a peculiar trend of cytokine gene expression, that accounts for the resistance to a subsequent lethal challenge with a high-virulent strain of Candida [21] or with C. neoformans [29]. Here, we show that, as assessed by GFAP staining, astrogliosis occurs to a comparable extent in both strains of mice, while appreciable differences are detected in terms of RCA1-reactive cells, that are more abundant and clustered in DBA/2 mice with respect to Balb/c mice. Moreover, BCG infection allows differential levels of cytokine gene expression between Nramp11s and Nramp11r mice. In particular, the high and/or...
early levels of MIP-2, IL-1β and IFNγ observed in Balb/c mice indicate that the BCG insult is capable of evoking a cytokine-related immune reaction. In particular, the early presence of MIP-2 gene transcripts implies the elaboration of a potent chemoattractant signal by Balb/c brains, where a more pronounced inflammatory reaction is documented.

Nevertheless, such a reaction happens to be unable of counteracting both mycobacterial local growth and subsequent dissemination. DBA/2 mice react to i.c. infection with BCG via early induction of IL-6 and TNFα and, even more important, with (i) early and pronounced levels of IL-12 transcripts, and (ii) late but highly consistent enhancement of IFNγ mRNA levels. Given the pivotal role of both IL-12 and IFNγ in driving the outcome of a protective immune response to mycobacterial infections [30–33], we favor the conclusion that the brain compartment responds to BCG with different patterns of cytokine gene expression. Thus, depending upon the Nramp1 genotype, either containment or colonization and dissemination is allowed.

Overall, the present data add insights on the issue of host resistance/susceptibility to pathogens, by focusing to the i.c.-infection model. The most feasible conclusion is that Nramp1 genotype affects brain macrophages, as well as any other macrophages. The histological and molecular data argue for the hypothesis that not only the direct cerebral anti-mycobacterial effector systems, but also the secretory immune reaction depend on the Nramp1 genotype. Although aware that only the use of Nramp1-disrupted and -reconstituted mice will eventually allow the formal proof for Nramp1 gene involvement in the phenomenon described, we are confident on the usefulness and accuracy of data obtained with strains segregating in non-overlapping groups, with respect to natural resistance to mycobacterial infections.

Acknowledgements

The authors wish to thank Dr. Paolo Mosci and personnel of the Animal Facility (University of Perugia) for their excellent support. This work was supported by Progetto Tuberculosis ISS Contract No. 96/D/T16, Rome, Italy.

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