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Differences of gene expression in bovine alveolar macrophages infected with virulent and attenuated isogenic strains of *Mycobacterium bovis*

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Abstract

Infection with *Mycobacterium bovis* is a significant human and animal health problem in many parts of the world. The first stage of pulmonary tuberculosis occurs after inhalation of the bacilli into an alveolus where they are ingested by resident macrophages. DNA microarray analysis was used to detect genes expressed in bovine lung alveolar macrophages infected with two isogenic strains of *M. bovis*, a virulent strain, ATCC35723 and an attenuated strain, WAg520 derived from ATCC35723. Chemokines, interleukin-8 and monocyte chemotactic protein 1, were more strongly expressed in ATCC35723-infected macrophages compared to WAg520-infected macrophages. Conversely, a group of genes, including fibrinogen-like protein 2 and legumain, were expressed at a higher level in macrophages infected with WAg520 compared to ATCC35723. Quantitative real-time PCR of a selected group of these differentially expressed genes confirmed enhanced levels of IL-8 mRNA in ATCC35723-infected macrophages compared to WAg520-infected macrophages. Microarray analysis of gene expression in macrophages infected with attenuated isogenic strains of *M. bovis* may identify key genes involved in early and protective immune responses to tuberculosis.

Keywords: *Mycobacterium bovis*; DNA microarray; Alveolar macrophage; Interleukin-8

1. Introduction

Infection with *Mycobacterium bovis* is a significant human health problem, as well as a problem for the cattle industry, particularly in developing countries. The first stage of pulmonary tuberculosis occurs after inhalation of the bacilli into an alveolus where they are ingested by resident macrophages. The outcome of the interaction between *M. bovis* and macrophages has important consequences for development of immunity to tuberculosis.

DNA microarray analysis has been applied to defining the repertoire of genes expressed in macrophages infected with *Mycobacterium tuberculosis* [1,2]. In the current study, microarrays were used to compare gene expression in bovine alveolar macrophages infected with a virulent strain of *M. bovis*, ATCC35723 to those infected with the attenuated isogenic strain, WAg520. The latter strain was derived from ATCC35723 by illegitimate recombination, differs by only 2 base pairs in the gene coding for a putative undecaprenol kinase, and is avirulent in guinea pigs [3]. In a guinea pig model of tuberculosis, vaccination with *M. bovis* WAg520 conferred a similar level of protection against challenge

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with *M. bovis* to that achieved with the attenuated bacille Calmette Guérin (BCG) strain of *M. bovis* [4,5].

2. Materials and methods

2.1. Bacterial strains

M. bovis strains were cultured as described previously [3].

2.2. Macrophage cultures

Lung alveolar macrophages were obtained from two cattle and cultured according to previously described methods [6,7]. Macrophages (2×10^7) were infected with *M. bovis* ATCC35723 or WAg520 at a multiplicity of infection (MOI) of 5:1 according to methods described previously [6,7]. Briefly mycobacteria were added to macrophage cultures and macrophages were incubated for 2 h at 37 °C, 5% CO_2. Extracellular bacteria were removed by washing the monolayers twice with warm PBS and the infected macrophage cultures were incubated for a total of 24 h prior to isolation of RNA. A 24 h infection time was chosen, as this has been shown previously to highlight differences in cytokine expression between macrophages infected with a virulent strain of *M. bovis* and BCG [6,7]. In addition, our work, and the work of others have shown that substantial cell death starts occurring in mycobacteria-infected cells after this 24-h period. We therefore sought to measure the gene expression profile in cells before extensive cell necrosis and apoptosis took place in the infected cells. Growth of the mycobacterial strains within macrophages was assessed by metabolic labelling with [^{3}H]-uracil according to previously described methods [6,7].

2.3. DNA microarray analysis

DNA microarray slides each contained a total of 21419 ESTs, amplified bovine and cervine cytokines and control spots [8,9]. Total RNA was prepared from the infected macrophage cultures using TRIzol (Invitrogen, Carlsbad, CA, USA) with subsequent RNeasy kit (Qiagen, Maryland, USA) purification. Labelled cDNA (Cy5 or Cy3 dye (Amersham Biosciences, Little Chalfont, Bucks, England) was produced from 20 μg of total RNA using an Ambion labelling kit (Ambion, Austin, TX, USA). Microarrays (six slides including replicates for the two macrophage cultures) were competitively hybridised with the labelled probes and then scanned in a ScanArray 5000 (Packard BioScience, Meriden, CT, USA). Processing of the images was performed using the GenePix Pro software (Axon Instruments Inc. Union City, CA, USA).

2.4. Real-time PCR and quantification of gene expression

To confirm differential gene expression results from microarray analysis, quantitative real-time RT-PCR (qRT-PCR) was performed on RNA prepared from macrophages infected at a MOI of 5:1 for 24 h with *M. bovis* ATCC35723 ($n=7$), WAg520 ($n=7$) or BCG ($n=6$). qRT-PCR was performed on a GeneAmp 5700 Sequence Detection System with detector software (Applied Biosystems, Foster City, CA, USA) and SYBR Green 1 dye according to methods described previously [10]. Primers for qRT-PCR were: GAPDH, 5′-CA CCATCTTCCAGGAGCGAG-3′ (forward) and 5′-CCA GCATCACCCCACTTGAT-3′ (reverse); IL-8, 5′-CTCT CTTGGCAGCTTTCCT-3′ (forward) and 5′-GAACTGCAG CTTCACACAGAGC-3′ (reverse); MCP-1, 5′-CACCAGC AGCAAGTGTCCTA-3′ (forward and 5′-CCCAGGATG GTCTTGAAAAT-3′ (reverse); CDC-like kinase 3, 5′-CAGT GAAGAACACCAGCATCC-3′ (forward) and 5′-AAGGTAG CACTGCCGAAGTC-3′ (reverse); FGL2, 5′AACGTAGCC AAGTTTTCAAAATAAA-3′ (forward) and 5′-TGG GTAAAGCTGCATTTTTGT-3 (reverse); legumain, 5′-ATT ACAGGCACCAGGCAGAT-3′ (forward) and 5′-TTT CGGTGAACGATCTGGTA-3′ (reverse). The relative quantities of amplified gene products were determined by comparing expression in *M. bovis*-infected macrophages to expression in non-infected macrophages using the $2^{(-\Delta\Delta Ct)}$ method [10].

2.5. Statistical analysis

Data was analysed by ANOVA for statistical significance.

3. Results

M. bovis ATCC35723 and WAg520 grew in macrophages at a comparable rate and growth of both these strains in macrophages was significantly greater than BCG ($P<0.01$) (Fig. 1).

The microarray data obtained from the infected macrophages was normalised for each individual slide following the

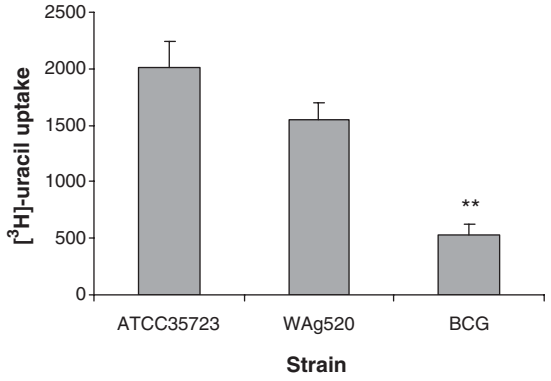

Fig. 1. Replication of *M. bovis* strains in bovine alveolar macrophages Bovine alveolar macrophages were infected with *M. bovis* strains at a MOI of 5:1 and the replication of bacilli was assessed by measuring incorporation of [^{3}H]-uracil at 24 h after infection. Results are mean uptake of [^{3}H]-uracil (cpm)±SE of triplicate cultures. Data is from the macrophages of one animal. Similar results were obtained with three separate animals. **Significantly lower than mean for ATCC35723- and WAg520-infected macrophages ($P<0.01$).