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SCIENCE DIRECT®
Microbes and Infection 8 (2006) 183–190
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Neutral-red reaction is related to virulence and cell wall methyl-branched lipids in *Mycobacterium tuberculosis*

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Received 5 April 2005; accepted 13 June 2005

Available online 10 August 2005

Abstract

Searching for virulence marking tests for *Mycobacterium tuberculosis*, Dubos and Middlebrook reported in 1948 that in an alkaline aqueous solution of neutral-red, the cells of the virulent H37Rv *M. tuberculosis* strain fixed the dye and became red in color, whereas the cells of the avirulent H37Ra *M. tuberculosis* strain remained unstained. In the 1950 and 1960s, fresh isolates of *M. tuberculosis* were tested for this neutral-red cytochemical reaction and it was reported that they were neutral-red positive, whereas other mycobacteria of diverse environmental origins that were non-pathogenic for guinea pigs were neutral-red negative. However, neutral-red has not really been proven to be a virulence marker. To test if virulence is in fact correlated to neutral-red, we studied a clinical isolate of *M. tuberculosis* that was originally neutral-red positive but, after more than 1 year passing through culture mediums, turned neutral-red negative. We found that, in comparison to the original neutral-red positive strain, this neutral-red negative variant was attenuated in two murine models of experimental tuberculosis. Lipid analysis showed that this neutral-red negative natural mutant lost the capacity to synthesize pthiocerol dimycocerosates, a cell wall methyl-branched lipid that has been related to virulence in *M. tuberculosis*. We also studied the neutral-red of different gene-targeted *M. tuberculosis* mutants unable to produce pthiocerol dimycocerosates or other cell wall methyl-branched lipids such as sulfolipids, and polyacyltrehaloses. We found a negative neutral-red reaction in mutants that were deficient in more than one type of methyl-branched lipids. We conclude that neutral-red is indeed a marker of virulence and it indicates important perturbations in the external surface of *M. tuberculosis* cells.

Keywords: *Mycobacterium tuberculosis*; Virulence; Neutral-red

1. Introduction

It is estimated that the etiologic agent of human tuberculosis, *Mycobacterium tuberculosis*, infects one-third of the world's population and kills 2 million people each year [1]. These data clearly indicate that new vaccines and drugs are needed to control tuberculosis. To develop these new antituberculosis agents, it is crucial to understand *M. tuberculosis–host* interaction. After about 100 years of research, knowledge of the cellular components responsible for *M. tuberculosis* pathogenicity is still limited. Throughout this time, the virulence of *M. tuberculosis* strains has been measured using experimental animal models, ascertaining which bacterial deficiencies or modifications affect the progression of the disease. The successful development of methods for creating mutations in specific genes combined with studying these mutants in animal models has enabled several viru-

Abbreviations: BAL, broncoalveolar liquid; DIM, pthiocerol dimycocerosates; HPRT, hypoxanthine guanine phosphoribosyl transferase; IFN-γ, interferon-gamma; iNOS, isoform of nitric oxide synthetase; NR, neutral-red cytochemical reaction; PAT, polyacyltrehaloses; RANTES, regulated upon activation normal T-cell expressed and secreted; SL, sulfolipids; TLC, thin-layer chromatography; TNF, tumor necrosis factor.

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doi:10.1016/j.micinf.2005.06.011

lence factors in *M. tuberculosis* to be described. An excellent review of the determinants of virulence in *M. tuberculosis* has recently been published [2]. On the other hand, interesting observations concerning the virulence of tubercle bacilli have also been carried out by studying natural mutants. One of the most analyzed natural mutant has been the H37Ra strain.

In 1934, Steeken et al. [3] observed that the H37 strain of *M. tuberculosis* produced two distinct forms of colonies on egg plates and that one of these forms was less virulent in guinea pigs. The experiments continued until two stable strains were obtained, the virulent (H37Rv) and the avirulent (H37Ra) [3–5]. Unlike H37Rv, H37Ra was unable to produce progressive disease either in guinea pigs or in mice. Many comparative analyses between H37Ra and H37Rv were undertaken in order to search for cellular structures of tubercle bacilli related to virulence. In 1948, Dubos and Middlebrook [6] described the neutral-red cytochemical reaction (NR) that clearly distinguishes H37Rv from H37Ra. They reported that, in an alkaline aqueous solution of neutral-red, the cells of the virulent H37Rv *M. tuberculosis* strain fixed the dye and became red in color (NR positive), whereas the cells of the avirulent strain H37Ra remained unstained in the same conditions (NR negative). In the following years, NR was evaluated and the resulting studies showed coinciding results, namely, that *M. tuberculosis* complex freshly isolated strains were NR positive, whereas other mycobacteria of diverse environmental origins that were non-pathogenic for guinea pigs were, with few exceptions, NR negative [7–9].

In 1959, Middlebrook et al. [10] studied the cell wall components responsible for NR in H37Ra and H37Rv, and established a positive correlation between the content of sulfolipids (SL) and a positive NR in H37Rv. Recently, a *pks2* mutant of *M. tuberculosis* H37Rv unable to produce SL has been generated [11]. We have analyzed this mutant for NR and it is NR positive; SL are therefore not responsible for the NR positive character in H37Rv [12]. Consequently, the cell wall components responsible for this striking difference between virulent H37Rv and avirulent H37Ra strains of *M. tuberculosis* remain unknown. It is not the objective of this work to identify these components; before undertaking research on the components related to NR, we believe more evidence on the relationship between NR and virulence in *M. tuberculosis* strains needs to be obtained. With this aim in mind, we have studied an *M. tuberculosis* strain (originally both deficient in SL and NR positive) isolated from an immunocompetent tuberculosis patient [13]. After more than 1 year of passages in culture media, this strain turned NR negative. We analyzed the virulence of the two NR variants of the *M. tuberculosis* strain in immunocompetent and immunodeficient mice. We also analyzed the content of the cell wall lipids of these two NR variants.

As cell wall methyl-branched lipids have been related to virulence in *M. tuberculosis* [14], we studied NR in different *M. tuberculosis*-constructed mutants unable to produce some of these lipids.

2. Materials and methods

2.1. Bacterial strains

The *M. tuberculosis* strains used in this study were the two reference strains H37Rv (ATCC 27294) and H37Ra (ATCC 25177); the MT103 *M. tuberculosis* strain, a clinical strain isolated from an immunocompetent patient [15]; an NR negative natural mutant of the MT103 strain, renamed MT103a; the MT103-derived mutants deficient in the expression of *fadD26, fadD28*, *mmpL7* and *drrC* genes and unable to synthesize pthiocerol dimycocerosates (DIM) or translocate DIM into the cell wall [13,15]; the complemented *drrC* deficient mutant with *drrC* gene (*drrCc*), this complementation restored the localization of DIM in the cell wall [13]; the H37Rv-mutants deficient in the expression of the *pks2, msl3* and *pks12* genes and unable to synthesize SL [11], polyacyltrehaloses (PAT) [16] and DIM [17], respectively.

2.2. Neutral-red staining

Neutral-red staining was performed in a test tube, as described previously [18]. Briefly, mycobacterial strains were grown on Middlebrook 7H10 medium at 37 °C. When required, the following antibiotics were used at the specified concentrations: kanamycin (25 μg/ml) and hygromycin B (100 μg/ml). Bacterial cells were placed in screw-cap tubes containing 5 ml of 50% aqueous methanol, and washed twice for 1 h, each time at 37 °C. Following this, a solution of 0.002% neutral-red in barbital buffer (1% sodium barbital in 5% NaCl, pH 9.8) was added to the washed cells; the results were evaluated after 1 h.

2.3. Mouse model

Our study was performed using specific pathogen-free C57BL/6 and CB-17 *scid/scid* (SCID) female mice, 6–8 weeks old, which were obtained from Charles River (Bagneux cedex, France). The mice were shipped in appropriate travel conditions with the corresponding certificate of health and origin. All the animals were kept under controlled conditions in a P3 high security facility with sterile food and water ad libitum.

2.3.1. Bacteria and infection

M. tuberculosis strains were grown in Proskauer Beck medium containing 0.01% Tween 80 to mid-log phase and stored at –70 °C in 2 ml aliquots. The mice were placed in the exposure chamber of an airborne infection apparatus (Glascol Inc., Terre Haute, IN, USA). The nebulizer compartment was filled with 7 ml of a *M. tuberculosis* suspension at a previously calculated concentration to provide an approximate uptake of 20 viable bacilli into the lungs. Four mice were used for every time-point in each experimental group. The numbers of viable bacteria in the left lung and spleen homogenates and broncoalveolar liquid (BAL) in weeks 3, 9, 18 and