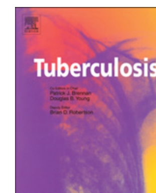
Tuberculosis 93 (2013) 167–178

Contents lists available at SciVerse ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



IMMUNOLOGICAL ASPECTS

A novel assay of antimycobacterial activity and phagocytosis by human neutrophils

David M. Lowe [a,b,*], Nonzwakazi Bangani [b], Meera R. Mehta [b], Dirk M. Lang [c], Adriano G. Rossi [d], Katalin A. Wilkinson [b,e], Robert J. Wilkinson [a,b,e], Adrian R. Martineau [a,e,f]

[a] Department of Medicine, Imperial College London, W2 1PG, UK
[b] Clinical Infectious Disease Research Initiative, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Observatory 7925, South Africa
[c] Department of Human Biology, Faculty of Health Sciences, University of Cape Town, Observatory 7925, South Africa
[d] Medical Research Council Centre for Inflammation Research, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, Scotland, UK
[e] Division of Mycobacterial Research, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
[f] Queen Mary University of London, Blizard Institute, London E1 2AB, UK

ARTICLE INFO

Article history:
Received 25 July 2012
Received in revised form
24 November 2012
Accepted 28 November 2012

Keywords:
Tuberculosis
Granulocytes
Bio-luminescence
Flow cytometry

SUMMARY

Despite abundant evidence that neutrophils arrive early at sites of mycobacterial disease and phagocytose organisms, techniques to assay phagocytosis or killing of mycobacteria by these cells are lacking. Existing assays for measuring the antimycobacterial activity of human leukocytes require cell lysis which introduces new bioactive substances and may be incomplete. They are also time-consuming and carry multiple risks of inaccuracy due to serial dilution and organism clumping. Flow cytometric techniques for measuring phagocytosis of mycobacteria by human cells have failed to adequately address the effects of organism clumping, quenching agents and culture conditions on readouts.

Here we present a novel in-tube bioluminescence-based assay of antimycobacterial activity by human neutrophils. The assay yields intuitive results, with improving restriction of mycobacterial bioluminescence as the ratio of cells to organisms increases. We show that lysis of human cells is not required to measure luminescence accurately.

We also present a phagocytosis assay in which we have minimised the impact of mycobacterial clumping, investigated the effect of various opsonisation techniques and established the correct usage of trypan blue to identify surface-bound organisms without counting dead cells. The same multiplicity of infection and serum conditions are optimal to demonstrate both internalisation and restriction of mycobacterial growth.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Tuberculosis is a major threat to humanity, but the host response to *Mycobacterium tuberculosis* remains incompletely understood. The role of neutrophils is particularly controversial, as these cells may contribute to both protection and pathology.[1,2] It has been proposed that control of mycobacteria by neutrophils, especially virulent *M. tuberculosis*, demonstrates inter-individual variability,[3] which may help to explain inter-individual differences in the ability of the innate immune system to control infection or dissemination, as well as differences in reported experimental results.[3,4] However, techniques to assess growth restriction by human neutrophils are not well established. Assessment of neutrophil antimycobacterial activity using a classical colony-forming unit (CFU) assay has multiple limitations. First, cell-pathogen cultures are necessarily disturbed in the process of pathogen enumeration. Cell lysis to release organisms introduces new bio-active reagents and may be incomplete. Serial dilutions before plating CFU may be inaccurate, due to clumping and pipetting error, and the process is time-consuming. Furthermore, existing techniques for mycobacterial quantification (including radiometric assays such as Bactec®) require prolonged culture of organisms in growth medium after the end of the restriction assay. This not only increases the risk of contamination and represents a safety issue for the laboratory but also deviates the *in vitro* assay further from *in vivo* reality, where organisms would not have the opportunity to recover and replicate in the absence of immune challenge.

* Corresponding author. Clinical Infectious Disease Research Initiative, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Anzio Road, Cape Town 8005, South Africa. Tel.: +27 21 406 6389; fax: +27 21 406 6796.

E-mail addresses: d.lowe@imperial.ac.uk (D.M. Lowe), nonzwakazi.bangani@uct.ac.za (N. Bangani), meera.r.mehta@gmail.com (M.R. Mehta), dirk.lang@gmail.com (D.M. Lang), a.g.rossi@ed.ac.uk (A.G. Rossi), katalin.wilkinson@uct.ac.za (K.A. Wilkinson), r.j.wilkinson@imperial.ac.uk (R.J. Wilkinson), a.martineau@qmul.ac.uk (A.R. Martineau).

1472-9792/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tube.2012.11.014

Another crucial aspect of host leucocyte function in addition to killing is phagocytosis of mycobacteria: this is either a prerequisite for elimination of the organisms or an essential stage in disseminating viable organisms to distant sites. However, assessment of this process is also challenging. Flow cytometric techniques[5–10] avoid labour-intensive microscopy but suffer a number of potential pitfalls. Specifically, it has been little appreciated that vital dyes such as trypan blue, used to 'quench' extracellular fluorescence and to identify surface-bound organisms, also stain dead cells and will enter fixed cells.[7] Furthermore, mycobacterial clumping in culture can significantly interfere with flow cytometry assays.[6] Although various techniques can minimise this at the point of inoculation, the organisms tend to re-aggregate during incubation.[6]

Here we describe a novel, luminescence-based, in-tube assay of antimycobacterial activity for human neutrophils infected with either *Mycobacterium bovis* BCG (BCG) or *M. tuberculosis (M. tb)* together with a flow cytometric phagocytosis assay. These assays utilise mycobacteria whose bioluminescence is conferred by a plasmid encoding the AB segment of the *Vibrio harveyi* lux operon, as previously described.[11] Light is emitted after addition of a substrate (1% n-decylaldehyde in ethanol), and this adenosine triphosphate (ATP)-dependent process reflects the metabolic activity of the organisms. Of note, transformation with this plasmid does not appear to negatively impact bacterial fitness or virulence, as previously demonstrated in an animal model.[11]

2. Materials and methods

2.1. Organisms and labelling

The plasmid construction and electroporation of organisms has been described previously.[11] 1.5 ml vials of mycobacteria stored at −80 °C were defrosted and added to 15 mls (*M. tb*) or 20 mls (BCG) liquid 7H9 (Becton Dickinson)/ADC (Becton Dickinson) growth medium enriched with 0.05% Tween 80 (Sigma) and 1 mcl/ml hygromycin B (Roche diagnostics). Organisms were grown to mid-log phase (72 h) before use in these assays.

For Fluorescein isothiocyanate (FITC) labelling, 5 ml of mid-log phase organisms in 7H9 were centrifuged at 2000 × g for 5 min and resuspended in 1 ml carbonate-bicarbonate buffer (pH 9.6) containing 0.05% Tween 80. 5 mcl of FITC stock (Sigma), previously made to 100 mg/ml with Dimethyl sulfoxide (DMSO) and stored at −20 °C until use, was added and the suspension was incubated at 37 °C for 15 min. The labelled organisms were spun in a microcentrifuge at 4000 × g for 2 min, the supernatant was aspirated and the pellet was resuspended in 1 ml Phosphate Buffered Saline (PBS) containing 0.05% Tween 80. This washing step was repeated twice and the organisms were then resuspended in 7H9.

Luminescence of stock was measured in a Berthold AutoLumat LB953 luminometer (for BCG) or in a Berthold Sirius single-tube luminometer (for *M. tb*, inside a Biosafety Cabinet) on duplicate samples of 100 mcl organisms added to 900 mcl PBS in 5 ml test tubes (Becton Dickinson). Both luminometers automatically inject 100 mcl of the substrate 1% n-decylaldehyde (Sigma) in ethanol.

The relative light unit (RLU) to colony forming unit (CFU) ratio for both stocks (established by contemporaneous RLU measurement and plating for CFU of mycobacteria growing in 7H9 medium) was determined to be approximately 3:1. Organisms were diluted in a standard volume of PBS to reach the required number of RLU for the experiments.

2.2. Neutrophil isolation

Human neutrophils were isolated either by magnetic beads or Percoll gradient from the peripheral blood of healthy consenting donors. For bead separation 4 ml freshly drawn heparinised blood was incubated for 15 min with 200 mcl magnetic human CD15 MicroBeads (Miltenyi Biotec) at 4 °C. During this time an LS column in a MidiMACS separation unit (Miltenyi Biotec) was 'primed' with 3 ml MACS buffer (0.5% bovine serum albumin + 20 mM Ethylene diamine tetra acetic acid (EDTA) in PBS). After incubation with beads the blood was diluted 1:1 with Roswell Park Memorial Institute-1640 medium (RPMI-1640) and pipetted onto the top of the LS column.

Once the blood had percolated through the column, 6 ml RPMI-1640 was added to wash out residual erythrocytes and loosely adherent cells. The column was then removed from the magnet and placed into a 15 ml Falcon tube, 2 ml RPMI-1640 was added to the top and plunged briskly through the column using the supplied syringe driver. The collected CD15 + cells were counted using a Beckman Coulter Ac.T Diff haematology analyser and diluted if necessary with further RPMI-1640 to reach the required final concentration.

For Percoll isolation, 30 ml heparinised blood was sedimented using 4 ml 6% Dextran (Sigma). The leucocyte-rich upper layer was then aspirated, transferred to a new 50 ml Falcon tube and centrifuged at 350 × g for 6 min. Pelleted cells were resuspended in 3 ml 55% Percoll and layered onto a discontinuous gradient of 3 ml 81% Percoll and 3 ml 70% Percoll. The gradient was centrifuged at 700 × g for 20 min with no deceleration. Granulocytes were harvested from the 71/80 interface.[12,13] Both techniques consistently yield >95% purity granulocytes by Coulter counting.

2.3. Serum generation, inactivation and pre-opsonisation

Freshly drawn non-anticoagulated blood was centrifuged in a 15 ml Falcon tube at 500 × g for 15 min with minimal deceleration. Separated plasma was transferred to another Falcon tube and incubated in a water bath at 37 °C. After the platelets had plugged serum was aspirated for use.

Heat inactivation of serum was performed in a water bath at 56 °C for 30 min or at 90 °C for 2 min, as indicated. Heat inactivated fetal calf serum was obtained from Biochrom AG (Berlin, Germany).

For pre-opsonisation, 200 mcl BCG-lux suspension in 7H9 at a concentration of 5 million RLU (1.7 million CFU)/100 mcl was incubated at a 1:1 volume ratio with autologous serum for 20 min at 37 °C.

2.4. Mycobacterial restriction assay

Granulocytes were resuspended to an appropriate concentration (usually 1×10^6 cells/ml) in RPMI-1640. 400 mcl of cell suspension was pipetted into a 5 ml Falcon flow cytometry tube (Becton Dickinson). 50 mcl autologous serum was added (giving a final concentration of 10% serum) followed by 50 mcl organism suspension, appropriately diluted to reach the required multiplicity of infection (MOI). The tubes were capped and then rolled to ensure that all organisms were mixed with the cell suspension before being incubated on their sides on a rocking plate (20 revolutions per minute (rpm)) at 37 °C. After the required time had elapsed, samples were allowed to cool to room temperature for five minutes (*V. harveyi* luciferase-induced luminescence is maximal at room temperature and relatively inhibited at 37 °C[14]), briefly vortexed, caps were removed and the tubes were placed in a luminometer for measurement.

2.5. Lysis

To lyse human cells, 1 ml 0.1% Saponin was added, samples were vortexed, incubated for 30 min and vortexed again. 1 ml PBS was added to control samples, which were otherwise treated identically. Permeabilisation was confirmed by microscopy of cells stained with trypan blue and by flow cytometry after addition of propidium iodide (see below).

Download English Version:

https://daneshyari.com/en/article/10962088

Download Persian Version:

https://daneshyari.com/article/10962088

Daneshyari.com