International Journal of Infectious Diseases 26 (2014) 37–43

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Unanticipated *Mycobacterium tuberculosis* complex culture inhibition by immune modulators, immune suppressants, a growth enhancer, and vitamins A and D: clinical implications

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ARTICLE INFO

Article history:
Received 4 November 2013
Received in revised form 26 December 2013
Accepted 28 January 2014

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Vitamin A
Vitamin D
Mycobacterium avium subspecies *paratuberculosis*
Tuberculosis
Thioguanine
Clofazimine

SUMMARY

Background: The development of novel antibiotics to treat multidrug-resistant (MDR) tuberculosis is time-consuming and expensive. Multiple immune modulators, immune suppressants, anti-inflammatories, and growth enhancers, and vitamins A and D, inhibit *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in culture. We studied the culture inhibition of *Mycobacterium tuberculosis* complex by these agents.

Methods: Biosafety level two *M. tuberculosis* complex (ATCC 19015 and ATCC 25177) was studied in radiometric Bactec or MGIT culture. Agents evaluated included clofazimine, methotrexate, 6-mercaptopurine, cyclosporine A, rapamycin, tacrolimus, monensin, and vitamins A and D.

Results: All the agents mentioned above caused dose-dependent inhibition of the *M. tuberculosis* complex. There was no inhibition by the anti-inflammatory 5-aminosalicylic acid, which causes bacteriostatic inhibition of MAP.

Conclusions: We conclude that, at a minimum, studies with virulent *M. tuberculosis* are indicated with the agents mentioned above, as well as with the thioamide 5-propothiouricil, which has previously been shown to inhibit the *M. tuberculosis* complex in culture. Our data additionally emphasize the importance of vitamins A and D in treating mycobacterial diseases.

1. Introduction

Multidrug-resistant (MDR) and total drug-resistant (TDR) tuberculosis is an increasing problem worldwide.[1–4] Amongst the multiple evolving strategies attempting to address this problem is the development of new antibiotics.[5–8] However, identifying, evaluating, obtaining regulatory approval, and marketing totally new antibiotics is time-consuming and expensive.[9–11] Existing approved pharmaceuticals that have heretofore unanticipated inhibition on *Mycobacterium tuberculosis* could more rapidly and less expensively proceed to ethically acceptable clinical evaluation.

There are increasing concerns that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be zoonotic,[12–14] and is responsible for, at a minimum, Crohn's disease.[15] We posit that the reason the pathogenesis of MAP has been missed is because, unknowingly, since 1942,[16] the medical profession has been treating MAP without understanding that was what they were doing. Multiple agents called 'immune suppressants', 'immune modulators',[17–22] and 'anti-inflammatories',[23] as well as vitamins,[24] exhibit dose-dependent inhibition of MAP in culture: they are anti-MAP antibiotics. As controls in these and other experiments,[25] we used *Mycobacterium avium* subspecies *avium* and two biosafety level 2 strains from the *M. tuberculosis* complex.

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http://dx.doi.org/10.1016/j.ijid.2014.01.026

We present herein unreported data on the dose-dependent inhibition, in culture, of the *M. tuberculosis* complex by multiple agents we have studied, and correlate these data with those from prior publications.[17–24] We compared the known anti-*M. tuberculosis* antibiotics *para*-aminosalicylic acid (PAS) and isoniazid with sulfapyridine and the anti-leprosy antibiotic clofazimine. We also evaluated the anti-inflammatory 5-aminosalicylic acid (5-ASA), the thiopurine immunomodulator 6-mercaptopurine (6-MP), and the immunosuppressants methotrexate, cyclosporine A, thalidomide, rapamycin, and tacrolimus. In addition, we studied two vitamins that inhibit mycobacteria in culture, vitamins A[24] and D.[24,26]

2. Methods

This study was approved by the Research and Development Committee at the Veterans Affairs Medical Center, Bronx NY (0720-06-038) and was conducted under the Institutional Radioactive Materials Permit (#31-00636-07).

2.1. Bacterial culture

The purpose of this study was to evaluate inhibition on the *M. tuberculosis* complex. We used two biosafety level 2 strains, bacillus Calmette–Guérin (BCG) *Mycobacterium bovis* Karlson and Lessel (ATCC 19015) and an avirulent *M. tuberculosis* strain (ATCC 25177).[27]

When indicated, comparisons of inhibition of MAP are included; the MAP was mostly that isolated from humans with Crohn's disease ('Dominic' ATCC 43545; 'Ben' ATCC 43544; 'Linda' ATCC 43015; ATCC 700535; '303' ATCC # PTA 7788[28]) and UCF-4 (gift of Saleh Naser, Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL,USA).[29] All ATCC were from ATCC Rockville, MD, USA.

All agents studied were purchased and prepared as described in previous publications.[17–19,23,24,30] The solvent in which the chemical was dissolved is identified in each table in the Results section.

Our Bactec 460 (Becton Dickinson, Franklin Lakes, NJ, USA) ^{14}C radiometric culture inhibition methods have been published in detail previously.[17–19,23,24,30] This system quantifies bacterial growth, or the lack thereof, by providing ^{14}C in palmitate, an energy source for mycobacterial growth.[31] Vials are assayed on a daily basis, quantifying the amount of ^{14}C released as $^{14}CO_2$, by the integral detector in the Bactec 460. The data are obtained as manufacturer-determined arbitrary 'growth units' (GU) of 0–999. Because the Bactec 460 is only semi-automatic and because of the onerous regulatory requirements of using radionucleotides, this exquisitely sensitive[23] system is being phased out. The Bactec 460 radiometric system has been replaced by the fully automatic, oxygen consumption detecting fluorescent probe-based MGIT 960 system (Becton Dickinson).[32,33]

In this study we performed a parallel Bactec/MGIT comparison. For this comparison, both components of the study were set up on the same day, using the same pre-culture for the bacterial inoculum. For the Bactec component, we used our previously described methods.[17–19,23,24,30] The final volume in the Bactec system was always 5 ml, and the concentration of the dissolving liquid was identical in each tube, irrespective of the concentration of the agent being tested. In this Bactec/MGIT comparison experiment, the agents were dissolved in dimethyl sulfoxide (DMSO), and the final concentration was always 3.2% DMSO. In the MGIT system the final volume was 7 ml. Accordingly, we increased the volume of the inoculum, test agent, and DMSO so that the concentration was the same for each component in the final solution.

To minimize possible confounding variables in the Bactec/MGIT comparison, mycobactin J, Oleic Acid, Albumin, Dextrose & Catalase (OADC) (Cat # BD-237510), and Tween 80 were not added to either the Bactec or the MGIT cultures. Neither OADC nor Albumin, Dextrose & Catalase (ADC) (Cat # BD-212352) nor mycobactin J is required for the growth of the *M. tuberculosis* strain.[17–19,23,24,30] MGIT computer-declared 'positivity' occurred by day 7 of the control *M. tuberculosis* inoculum. We did not use Tween 80, recommended to minimize mycobacterial clumping,[31] because we,[19] and others,[34] have found that it interferes with inhibition.

Bactec quantifies growth as the 'growth index' (GI). Sequential days of data are added together and presented as the cumulative GI (cGI). The data are then mathematically manipulated to indicate the amount of inhibition from the control as the percentage change from control cGI (inhibition as %−ΔcGI; see Greenstein et al.[23] for calculation).

MGIT data are provided by the integral MGIT computer as either growth units or as the day when the computer determines an individual inoculum has reached log phase growth and is declared 'positive.' In our Bactec/MGIT *M. tuberculosis* comparison, we present the MGIT data in both ways. Agents being tested were added at the beginning of the experiment. The calculation for MGIT 'cumulative growth units' (cGU) was made by adding the growth units from the MGIT printout until an arbitrary day post inoculation; in this particular experiment we terminated the experiment on day 16 because the controls had passed log phase growth and showed no further increase in the control growth units. The calculation for cGU was as described for cGI for Bactec data.[23] The effect (or lack thereof) of each agent in the MGIT is presented as the percentage decrease in cGU units (%−ΔcGU). The calculation of %−ΔcGI was performed in two stages (using Excel) using the following formula: step one = [(A − B)/A] = C, step two = −C × % = final result of %−ΔcGU, where A = the cGU of the control inoculum for the given diluent (in these experiments DMSO see above and in each table), B = the cGU for the particular chemical at a particular dose being tested, incubated for the same number of days as A, and C = the product of [(A − B)/A]. Days to positivity are also presented in the tables and figure (see Figure 1 legend for details).

3. Results

The inhibitory control used was PAS. There was a marked dose-dependent inhibition (>95%−ΔcGI at 1 μg/ml) of *M. tuberculosis* (Table 1). This was not as pronounced with BCG, particularly when PAS was dissolved in 7H9 (18%−ΔcGI at 1 μg/ml) or water (−86%−ΔcGI at 1 μg/ml; Table 1). Isoniazid was an additional inhibitory control. It was found to be bactericidal against *M. tuberculosis* whether dissolved in NaOH or water (99%−ΔcGI at 1 μg/ml; Table 2). BCG was best inhibited when the dissolving solution was NaOH (99%−ΔcGI at 1 μg/ml; Table 2).

Our non-inhibitory control was the intact molecule of sulfasalazine (comprising sulfapyridine coupled to 5-ASA). There was no dose-dependent inhibition of either *M. tuberculosis* complex strain studied (Table 3).

Sulfapyridine, alone or with 5-ASA (the two component molecules of our non-inhibitory control sulfasalazine), showed poor dose-dependent inhibition of *M. tuberculosis* (63%−ΔcGI at 64 μg/ml; Table 4). BCG was more susceptible to sulfapyridine (≥89%−ΔcGI at 16 μg/ml; Table 4). There was no synergy of sulfapyridine with 5-ASA on BCG (Table 4).

Alone, 5-ASA showed no dose-dependent inhibition on the *M. tuberculosis* complex (Table 5). This is in contrast to the weak, but consistent and replicable, bacteriostatic dose-dependent