Tuberculosis 89 (2009) 91–94

Contents lists available at ScienceDirect

Tuberculosis

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

Prevention of the post-chemotherapy relapse of tuberculous infection by combined immunotherapy

Simona Buccheri [a], Rajko Reljic [b], Nadia Caccamo [a], Serena Meraviglia [a], Juraj Ivanyi [c], Alfredo Salerno [a], Francesco Dieli [a,*]

[a] Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo, Corso Tukory 211, Palermo, Italy
[b] Molecular Immunology Group, St. George's Hospital Medical School, London, UK
[c] Clinical and Diagnostic Sciences Group, Guy's Campus of Kings College London, UK

ARTICLE INFO

Article history:
Received 20 May 2008
Received in revised form 31 July 2008
Accepted 5 September 2008

Keywords:
Tuberculosis
Cytokines
Antibodies
Immunotherapy
Relapse

SUMMARY

We report that a recently developed combined immunotherapy (CIT) has the capacity to prevent a spontaneous relapse of replicating *Mycobacterium tuberculosis* bacilli in the lungs of BALB/c, C57Bl/6 or C3H/HeJ strains of mice, following 4 weeks of non-sterilising treatment with isoniazid and rifampicin. The CIT regimen, represented by recombinant IFNγ, anti-α crystalline monoclonal IgA antibody and IL-4 neutralizing polyclonal antibody, reduced the 8-week relapse of viable bacterial counts in the lungs most significantly, when CIT was inoculated during the 5th week post infection, i.e. during the 3rd week of chemotherapy. Although CIT enhanced lung granuloma area, nitric oxide, cytokine and chemokine levels in lung washings significantly, these could not be directly associated with the beneficial effect of CIT on the degree of relapse in the lungs. These results represent a proof-of-principle, that the described CIT, when combined with chemotherapy, could have potential for future development of a shorter regimen of tuberculosis treatment.

1. Introduction

Chemotherapy of tuberculosis (TB) can have a very high cure rate; however, poor patient compliance with the protracted regimen in areas with limited resources can be a significant problem, leading to relapse of active disease, transmission of infection and development of drug resistant strains. Attempts to eliminate latent persisters by non-specific (e.g. cytokine) or antigen-specific (e.g. vaccination) immunological agents,[1,2] i.e. 'immunotherapy as an adjunct to chemotherapy' have been made in a number of experimental models. Using a short regimen of incomplete (non-sterilizing) chemotherapy, muramyl dipeptide was reported to reduce the relapse partially in the lungs, but not in the spleens of mice.[3] Treatment of TB patients with recombinant IL-2[4] or *Mycobacterium vaccae*[5] failed to improve chemotherapy. Vaccination of mice reduced the post-chemotherapy relapse of *Mycobacterium tuberculosis* (Mtb) infection when using hsp65[6,7] or Ag85A[8] DNA plasmids, or a detoxified Mtb extract in liposomes (RUTI),[9] although negative results were reported by others.[10,11] Passive inoculation of a polyclonal antiserum against an Mtb extract reduced the post-chemotherapy relapse in SCID mice.[12] Considering the advances made and the importance of the potential clinical aims, further research on the concept of immunotherapy, as an adjunct to chemotherapy of TB, has been recommended.[13,14]

This study has been initiated under the direct influence of our preceding experiments which showed, that (i) passive vaccination with a mouse IgA monoclonal antibody (mAb) against the α-crystallin (Acr) antigen of Mtb together with IFNγ reduced the lung infection and pathology in BALB/c mice[15] and (ii) combining this regimen with the inoculation of IL-4 neutralizing antibody was even more protective.[16] The combined immunotherapy regimen composed of IFNγ + IgA + anti-IL-4 was evaluated in this study for its capacity to reduce the spontaneous relapse of active tuberculous infection following short-term (incomplete) chemotherapy.

2. Methods

2.1. Mice and infection

BALB/c and C57Bl/6 mice (OLAC Ltd. through Nossan, Correzzana, Italy) and C3H/HeJ mice (Jackson Laboratories USA) were kept under specific pathogen-free conditions. Six mice per group (matched for sex and age between 8 and 10 weeks) were infected

* Corresponding author. Tel.: +39 091 6555916; fax: +39 091 6555901.
E-mail address: dieli@unipa.it (F. Dieli).

1472-9792/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tube.2008.09.001

(under light anaesthesia) i.n. with 2.5×10^5 colony-forming units (CFUs) of mid-log-phase Mtb H37Rv in 0.02 ml of saline.

2.2. Chemotherapy

The 'short duration' chemotherapy entailed the delivery of 25 mg of isoniazid (INH) and 10 mg of rifampicin (R) (Sigma) per 100 ml of drinking water from day 14 post infection, for a period of 4 weeks (i.e. 2nd to 6th week). This regimen is known to abrogate almost completely CFU counts in the lungs and spleen, followed by a rapid spontaneous relapse ('regrowth') of tubercle bacilli within a period of 2–4 weeks.[3]

2.3. Combined immunotherapy (CIT) agents and regimen

Mouse IFNγ (Serotech, Oxford, UK) 1 μg (100,000 U)/mouse, intranasally (i.n.) goat polyclonal anti-mouse IL-4 antibody (R&D Systems, cat. no. AF-404-NA), 500 μg per mouse intravenously (i.v.); TBA61, affinity purified mouse anti-Acr IgA mAb (1.5 mg/ml, antigen-binding titre 100,000), 37 μg in 25 μl per mouse i.n.[17] The time schedule of the CIT is shown schematically in Figure 1. Inoculations during the 3rd, 5th or 7th week and re-inoculation on the 9th week post-infection were given on the following days of the respective weeks: day 1, IFNγ i.n.; day 2, anti-IL4 i.v.; days 3 and 7, IFNγ and TBA61 i.n.

2.4. CFU assay

Lungs and spleens harvested at different time intervals post infection were homogenised in 5 ml of sterile water and 10 μl aliquots of the serially diluted homogenates were plated on duplicate Middlebrook 7H11 (Difco, cat. no. 283810) agar plates. CFUs were counted after 2–4 weeks of incubation at 37 °C.

2.5. Nitrite, cytokine and chemokine assays

Bronchoalveolar lavage obtained by flushing 2 ml of PBS into the lungs of killed mice was used to determine the concentration of nitrite, using the Griess reagent (Sigma). ELISA kits (R & D Systems) were used to test cytokine (TNF-α, IL-1β) and chemokine (CCL3/MIP-1α, CCL4/MIP-1β) levels according to the manufacturer's recommendations.

2.6. Histology and morphometry

Lungs were fixed in 10% buffered formalin and embedded in paraffin. Haematoxylin–eosin stained sections (5–6 μm) were photographed (×6) using a Stereoscopic Zoom SMZ800 microscope (Nikon, Tokyo, Japan) and a Coolpix 990 digital camera (Nikon). Software programs Scion Image (Scion Corporation, Frederick, MD, USA) and Photoshop 5.0 (Adobe Systems Incorporated, San José, CA, USA) were used to determine the area with granuloma lesions.[18]

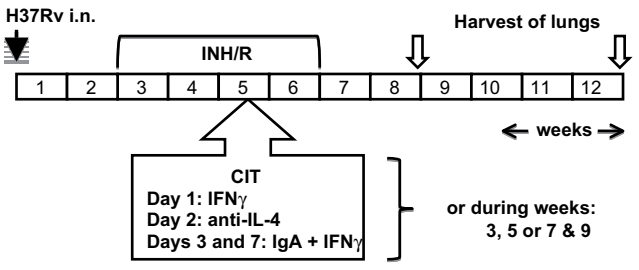

Figure 1. Schematic representation of the experimental design for analysing the effects of the combined immunotherapy (CIT) on the relapse of Mtb infection following INH/R chemotherapy.

The relative granuloma involving areas were calculated from two independents measurements.

2.7. Statistical analysis

The significance of differences in CFU counts between groups was determined by one-way analysis of variance (ANOVA) on the $\log_{10}$ values with Scheffe tests for the post-ANOVA individual comparisons. When calculating group mean values, samples without detectable colonies were assigned a value of 50 CFU, representing the threshold of detection sensitivity. Student's *t* test was used for the analysis of granuloma, NO, cytokine and chemokine values in the lungs.

3. Results

3.1. The effect of CIT timing

The results (Figure 2A, Table 1) showed that mean CFU counts in the lungs at the end of 4 weeks of INH/R treatment were below detection in 4 out of 6 mice (85 CFUs). A pronounced spontaneous relapse of infection was demonstrable on the 8th week post infection (11,220 CFUs) in all 6 mice in the group. We investigated the efficacy of the CIT, when given during the 3rd, 5th or 7th week post infection to prevent this relapse. The results showed that CIT given during the 5th week, reduced the mean CFU values of lung infection more effectively (78 CFUs, $p = 0.001$; CFUs undetectable in 5/6 mice) than CIT given either during the 3rd week (562 CFUs, $p = 0.006$) or the 7th week (1995 CFUs, $p = 0.393$). Nevertheless,

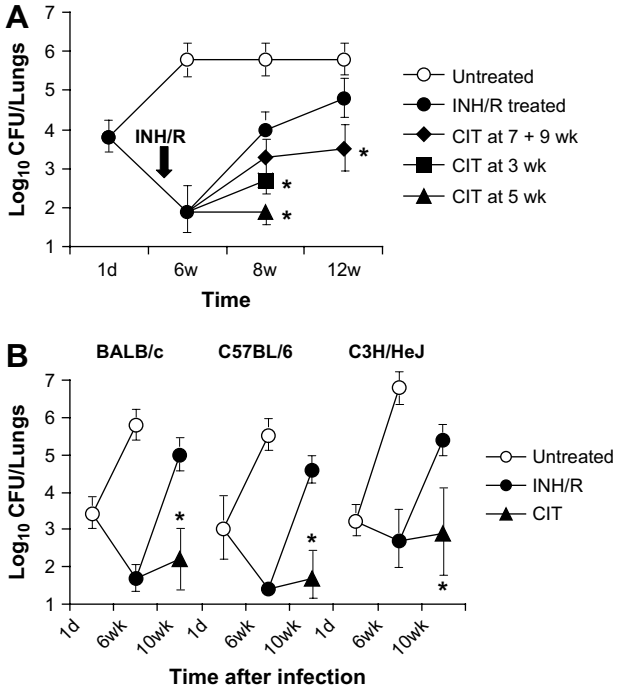

Figure 2. Influence of CIT on the post-chemotherapy relapse in the lungs of Mtb infected mice. (A) The influence of CIT timing. Isoniazid and rifampicin (INH/R arrow) were in the drinking water from the 2nd to 6th week after i.n. H37Rv infection and CIT was given at different weeks (wk) indicated in the figure (dosage: see Methods section). Symbols: mean $\log_{10}$ CFU values, vertical bars = SD; * indicates statistically significant difference when compared with the INH/R only treated group ($p < 0.05$). (B) The effect of CIT in three different strains of mice. BALB/c, C57BL/6 and C3H/HeJ mice received INH/R on weeks 2–6 and CIT during week 5 after i.n. H37Rv infection. Mean $\log_{10}$ CFU and SD (vertical bars) values from lungs harvested 10 weeks after infection. *Significant ($p < 0.005$) difference, when comparing CIT treated with INH/R only treated groups.