Tuberculosis 92 (2012) 505–512

Contents lists available at SciVerse ScienceDirect

Tuberculosis

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

IMMUNOLOGICAL ASPECTS

Helminths and skewed cytokine profiles increase tuberculin skin test positivity in Warao Amerindians

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ARTICLE INFO

Article history:
Received 15 May 2012
Received in revised form
14 July 2012
Accepted 16 July 2012

Keywords:
Tuberculin skin test
Indigenous children
Cytokine profiles
QuantiFERON-TB gold in-tube test
Intestinal parasites

SUMMARY

The immune regulatory mechanisms involved in the acquisition of *Mycobacterium tuberculosis* infection in children are largely unknown. We investigated the influence of parasitic infections, malnutrition and plasma cytokine profiles on tuberculin skin test (TST) positivity in Warao Amerindians in Venezuela. Pediatric household contacts of sputum smear-positive tuberculosis (TB) cases were enrolled for TST, chest radiograph, plasma cytokine analyses, QuantiFERON-TB Gold In-Tube (QFT-GIT) testing and stool examinations. Factors associated with TST positivity were studied using generalized estimation equations logistic regression models. Of the 141 asymptomatic contacts, 39% was TST-positive. After adjusting for age, gender and nutritional status, TST positivity was associated with *Trichuris trichiura* infections (OR 3.5, 95% CI 1.1–11.6) and low circulating levels of T helper 1 (Th1) cytokines (OR 0.51, 95% CI 0.33–0.79). *Ascaris lumbricoides* infections in interaction with Th2- and interleukin (IL)-10-dominated cytokine profiles were positively associated with TST positivity (OR 3.1, 95% CI 1.1–8.9 and OR 2.4, 95% CI 1.04–5.7, respectively). A negative correlation of QFT-GIT mitogen responses with Th1 and Th2 levels and a positive correlation with age were observed (all $p < 0.01$). We conclude that helminth infections and low Th1 cytokine plasma levels are significantly associated with TST positivity in indigenous Venezuelan pediatric TB contacts.

1. Introduction

It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis* and that each year about 9 million people develop tuberculosis (TB), 1 million (11%) of whom are children under 15 years of age.[1] Children who are in close contact with adult pulmonary TB have a high risk of being infected and developing active TB disease. It is generally accepted that 30–50% of household contacts of adults with infectious forms of pulmonary TB will have a positive tuberculin skin test (TST).[2] Age, proximity of exposure, malnutrition, household crowding and the contact's Bacille Calmette-Guérin (BCG) vaccination scar status have been found to influence TST positivity among childhood

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http://dx.doi.org/10.1016/j.tube.2012.07.004

contacts of sputum smear-positive index cases.[3–6] A unique aspect of TB in children is the rapid progression to disease, typically within the first year following infection.[7] The risk of developing disease is determined by a combination of factors, including age, virulence of the TB strain, genetic factors, magnitude of the initial infection and host immunity.[8] The cytokine-mediated immune response to *M. tuberculosis* infection is an important determinant for the development of TB: the cell-mediated immunity, involving activation of macrophages and T helper 1 (Th1) cells, plays a protective role, whereas a Th2 response undermines the efficacy of immunity and contributes to immunopathologic conditions.[9–11]

Parasitic infections share the same 'developing world niche' as TB. One-third of the world's population harbors at least one species of intestinal parasite with children between 5 and 15 years of age bearing the greatest burden in terms of morbidity and mortality.[12,13] The Th2 skewing or immunomodulation induced by helminth infections can decrease the development of a Th1 response after infection with *M. tuberculosis* which may favor persistence of the *M. tuberculosis* infection.[14] Furthermore, helminth infections affect the efficacy of BCG vaccination[15] and helminth infections are associated with undernutrition in endemic populations.[16] As the cell-mediated immunity is influenced by nutritional status of the host,[17] the negative effect of helminth infections on cell-mediated immunity against *M. tuberculosis* may be partly explained by malnutrition accompanying helminth infections.

While many investigators have evaluated cytokine production by stimulated whole blood or peripheral blood mononuclear cells (PBMCs) recovered from patients with *M. tuberculosis* infection or disease,[18–20] few of them have measured circulating serum cytokine concentrations. As the *ex vivo* antigen-stimulated production of cytokines only reflects the cytokine response to a specific stimulus, it may not provide sufficient insight into the actual status of the cytokine network *in vivo*, as this is the result of many different antigen stimulations. Most studies examining cytokine responses have focussed on the comparison of TB patients with healthy controls[21,22] and cytokine plasma levels have been considered as biosignatures of TB disease.[23] Understanding the immune regulatory mechanisms in *M. tuberculosis*-infected children may provide novel insights into their increased susceptibility to progression to disease compared with adults.[8,24]

In Venezuela, the average annual national incidence rate of TB is moderate (25–50 per 100,000 inhabitants[25]), but an extraordinary high incidence rate (3190 per 100,000) has been reported in children among Warao Amerindians.[26] The Warao people, one of the largest indigenous populations in the South American lowland, live in the Orinoco Delta in northeastern Venezuela. Warao Amerindians live in extreme poverty and high prevalence rates of intestinal helminth and protozoan infections and malnutrition have been described in Warao children.[27,28] To explore whether parasitic infections and malnutrition influence the risk of *M. tuberculosis* infection and to what extent this risk is modulated by an immunological shift in Th1/Th2 profiles, we investigated circulatory plasma cytokine levels, intestinal parasitic infections and nutritional status in asymptomatic TST-positive and TST-negative Warao pediatric TB contacts.

2. Methods

2.1. Study design and setting

This study was conducted over a period of 12 months, from May 2010 to May 2011, in the Warao Amerindians, an indigenous population living in wooden houses raised on stilts along the Orinoco river banks. Household contacts living with sputum smear-positive pulmonary TB patients in the municipalities Antonio Diaz and Pedernales were asked to enroll in the study. All household contacts aged between 1 and 15 years were eligible. Contacts of patients who registered for TB treatment in the Venezuelan National TB Control Program in the study period were included within one month after registration of the index case. We defined household contacts as children who slept in the same home as the index case, ate with the index case and whose parents identified a common household head. Children who had previously been treated for TB were excluded ($n = 1$).

2.2. Sample collection

Blood was collected from each participant into 1 EDTA tube and 3 heparin-containing tubes for the QuantiFERON-TB Gold In-Tube assay (QFT-GIT), including a positive control (mitogen), a negative control (heparin), and a TB-antigen tube (containing antigens ESAT-6, CFP-10, and TB-7.7). The QFT-GIT assay was performed using QFT-GIT kits (Cellestis) according to manufacturer's instructions by a single experienced laboratory technician who was blinded to all clinical information.[29] The Hemocue Hb201+ and Hemocue WBC (Hemocue AB, Ängelholm, Sweden) were used to assess hemoglobin levels and total white blood cell (WBC) count, respectively. A peripheral blood smear was stored for microscopic leukocyte differentiation. Human immunodeficiency virus (HIV) antibody testing was done after appropriate counseling using the Determine (Abbott Laboratories) HIV 1/2 rapid test. Plasma was stored at −70 °C until cytokine analysis. Subsequently, TST was performed by following standard procedures: 0.1 mL of tuberculin purified protein derivative of *M. tuberculosis* strain RT-23 (Statens Seruminstitut, Copenhagen) was injected intradermally into the volar surface of the left forearm. A palpable transverse induration with a diameter of ≥10 mm measured 48–72 h after injection was considered positive.

In addition, a stool sample was requested from all participants. Stool samples were preserved in sodium acetate–acetic acid–formalin (SAF) preservative[30] and stored at 4 °C until examination by experienced laboratory technicians for the presence of helminths and intestinal protozoa. An aliquot of the unpreserved sample was mixed with ethanol 96%. Feces samples in ethanol were stored at −70 °C until DNA isolation with the High Pure PCR template preparation kit (Roche, Germany). Real-time polymerase chain reaction (PCR) of fecal samples was conducted on the Roche LightCycler® 480 system for detection of *Entamoeba histolytica*, *Dientamoeba fragilis*, *Giardia lamblia* and *Cryptosporidium parvum*.[31,32] The diagnosis of parasitic infections was based on both microscopy and PCR.

Standard antero-posterior and lateral chest radiographs (CXRs) were taken. Two independent experts, blinded to all clinical information, evaluated the CXRs and documented their findings on a standard report form. Where the two experts disagreed, a third expert was consulted and final consensus was achieved. A sputum sample was collected from all children who could expectorate with gastric aspirates taken from all children under 6 years of age. Specimens were cultured on Middlebrook (7H9) liquid broth-based media and on Ogawa solid media. Confirmed TB was defined as isolation of *M. tuberculosis* on culture. PCR-restriction analysis of the hsp65 gene (PRA) was performed to differentiate *M. tuberculosis* from nontuberculous mycobacteria. Probable TB was defined as clinical and radiographic findings consistent with intrathoracic TB as defined by Marais et al.[33] and either (1) a positive TST or QFT-GIT or (2) histopathologic findings compatible with TB, without positive mycobacterial culture results. Possible TB was defined as the presence of abnormal CXR findings not consistent with intrathoracic TB[33] and either (1) a positive TST or QFT-GIT or (2)