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Immunological response to *Mycobacterium tuberculosis* infection in blood from type 2 diabetes patients

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ABSTRACT

The convergence of tuberculosis and diabetes represents a co-epidemic that threatens progress against tuberculosis. We have investigated type 2 diabetes as a risk factor for tuberculosis susceptibility, and have used as experimental model whole blood infected in vitro with *Mycobacterium tuberculosis*. Blood samples from diabetic patients were found to have a higher absolute neutrophil count that non-diabetic controls, but their immune functionality seemed impaired because they displayed a lower capacity to phagocytose *M. tuberculosis*, a finding that had been previously reported only for monocytes. In contrast, an increased production of TNFα was detected in infected blood from diabetic patients. Despite the altered phagocytic capacity showed by cells from these patients, the antimicrobial activity measured in both whole blood and monocyte derived macrophages was similar to that of controls. This unexpected result prompts further improvements in the whole blood model to analyze the immune response of diabetes patients to tuberculosis.

1. Introduction

Many evidence show that diabetes is an important risk factor for active tuberculosis disease. Patients with diabetes have about a three-fold increase risk of developing tuberculosis compared with individuals without diabetes. Furthermore, a 1.89 times increase in the risk of death has been calculated for tuberculosis patients with diabetes [1]. It is also possible that tuberculosis may cause diabetes [2]. Tuberculosis and diabetes represent two of the main global health challenges of our time, and their convergence represents a co-epidemic that threatens progress against tuberculosis [3]. It is necessary to understand how diabetes and the immune response participate in a harmful interaction. We have known for some time that activated innate immunity is a factor in the pathogenesis of type 2 diabetes [4]. On the other hand, diabetes might change the appropriate coordination of immunological factors that maintain host defenses to infectious agents like cellular and humoral immunity, production of cytokines and chemokines, and production of reactive oxygen species [5]. Nevertheless, relatively few studies have addressed the immunological mechanisms against tuberculosis affected by diabetes.

The human innate immune response to tuberculosis in type 2 diabetes patients is altered because their monocytes show a reduced binding and/or phagocytosis of *Mycobacterium tuberculosis* [6]. Furthermore, there is also a lower expression of genes encoding for products that contribute to anti-mycobacterial activities like the vacuolar A-TPase, the hexokinase 2 or CD28 [7]. Regarding the adaptive immune response, the reported data is not consistent. Some authors have detected higher plasma levels of IFNγ, IL2, IL5 or IL17A cytokines in diabetes patients and an increased production of IFNγ, IL2, TNFα and GM-CSF after stimulation of peripheral blood mononuclear cells from diabetes mellitus patients with *Mycobacterium tuberculosis* antigens. Others found no difference in the cytokine production in whole blood from tuberculosis patients with or without diabetes mellitus after activation with lipopolysaccharide, phytohemagglutinin or an *M.*

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Table 1
Characteristics of type 2 diabetes patients and controls.

	Type 2 Diabetes (n = 41)	Control (n = 40)	*P* value
Age (yr)	73.1 SD 11.2	68.4 SD 16.2	0.234
Sex M/F	24/17	19/21	0.377
BMI (kg/m^2)	28.1 SD 5.3	26.4 SD 3.8	0.113
HbA1c (%)	9.6 SD 1.9	5.7 SD 0.4	**< 0.001**
Glucose (mg/dL)	248.0 IR 266	89.0 IR 17	**< 0.001**
Total cholesterol (mg/dL)	155.8 SD 46.2	175.5 SD 42.5	0.060
HDL cholesterol (mg/dL)	40.8 SD 17.0	52.2 SD 16.5	**0.005**
LDL cholesterol (mg/dL)	85.9 SD 39.7	105.5 SD 34.6	**0.028**
Triglycerides (mg/dL)	106.0 IR 82	82.0 IR 39	**0.003**
Statin treatment Y/N	20/21	5/35	**< 0.001**
Chronic kidney disease Y/N	22/19	13/27	0.073
Interferonγ-release assay P/N	17/24	9/31	0.096

Definition of abbreviations: F = Female; M = Male; BMI = Body Mass Index; HbA1c = Glycated hemoglobin; HDL = High Density Lipoprotein; LDL = Low Density Lipoprotein; Y = Yes; N = No; P = Positive; N = Negative. Values represent means and standard deviations (SD), except for glucose and triglycerides in which they represent median and interquartile range (IR). *P* values were calculated by Student's *t*-test except for glucose and triglycerides (Mann-Whitney) and sex, statin treatment, chronic kidney disease and interferonγ-release assay (Fisher's exact test). $^{*}P < 0.05$ was considered significant, indicated in boldface.

tuberculosis sonicate [8].

To investigate whether diabetes mellitus may influence anti-mycobacterial activity, we have infected blood from type 2 diabetes patients with *M. tuberculosis* and analyzed immunological parameters like phagocytosis and cytokine production.

2. Subjects and methods

2.1. Study population

This study was carried out at the Complejo Asistencial Universitario de León in accordance with the principles of the Declaration of Helsinki (2013), following approval of the protocol by the Hospital of León Clinical Research Ethics Board and collection of informed consent from all of the participants. The identification of diabetes was based on the diagnostic criteria recommended by the American Diabetes Association in 2014 [9]. A total of 41 consecutive patients with type 2 diabetes and 40 subjects without diabetes (control group) participated in the study (Table 1). The adherence of patients with diabetes to treatment was inadequate, and their blood glucose levels were not well controlled. Exclusion criteria were: recent antibiotic or glucocorticoid treatments, oncologic disease patients, individuals less than 18 years old and those who did not provide informed consent. All volunteers were tested by an interferonγ release assay (QuantiFERON TB Gold In-Tube, Cellestis, Australia).

2.2. Bacterial strains

M. tuberculosis HL186T was isolated at the Hospital de León (Servicio de Microbiología) and kindly provided by Julio Blanco and Manuela Caño. *Mycobacterium bovis* BCG was the RIVM strain derived from the 1173-P2 strain. Bacteria were grown on 7H11 agar supplemented with 0.2% glycerol and 10% Middlebrook enrichment OADC (oleic acid, albumin, dextrose, catalase, Becton Dickinson Microbiology Systems, USA). For detection of bacteria by flow cytometry *M. bovis* BCG was transformed with the expression plasmid pMDsRed, constructed as described elsewhere [10] that contains the gene of the fluorescent protein DsRed. T3_S4T. Flow cytometry with *M. tuberculosis* was precluded for biosafety reasons. Bacteria were individualized as described elsewhere [10]. After addition of glycerol to 20%, single use aliquots were frozen at −80 °C.

2.3. Phagocytosis and absolute count of cells by flow cytometry

Infections with *M. bovis* BCG transformed with pMDsRed (5×10^5 bacteria/ml) were performed in 40% blood, and volume was adjusted with RPMI-1640 medium to 300 µl in 2 ml tubes. They were incubated in a rotary shaker at 37 °C for two hours. Cell lineages were stained with appropriate monoclonal antibodies conjugated directly to fluorochromes: fluorescein isothiocyanate (FITC)-conjugated anti-CD66b (for neutrophils) and peridinin chlorophyll–protein complex (PerCP)-conjugated anti-CD14 (for monocytes, Becton Dickinson, USA). Phagocytosis was measured as a phagocytic index, determined by the number of neutrophils or monocytes associated with fluorescent bacteria divided by the total number of neutrophils or monocytes. Absolute count of neutrophils and monocytes was performed using the Stepcount kit (Immunostep, Spain) following manufacturer's instructions. Samples were analyzed on a FACScan flow cytometer using CellQuest software (Becton Dickinson, USA). Simultaneous count of leukocytes, neutrophils, lymphocytes and monocytes were performed using a CELL-DYN 3700 instrument system (Abbott Diagnostics, USA).

2.4. Quantification of cytokines in blood

After blood was infected as described above and incubated for two days, samples were centrifuged for 5 min at 16,000 × *g* and plasma was recovered. To remove remaining bacteria from plasma, samples were centrifuged for 3 min at 10,000 × *g* at room temperature in ultrafree MC filter units (Millipore Iberica, Spain) of 0.45 µm pore size and frozen at −80 °C. TNFα was quantified by ELISA using BD OptEIA Human TNF ELISA Set or BD OptEIA GM-CSF ELISA Set (Becton Dickinson, USA), following manufacturer's instructions.

2.5. Antimycobacterial activity

Blood was infected with *M. tuberculosis* HL186T (5×10^5 bacteria/ml) as described above. IFNγ (25 ng/ml; $\geq 2 \times 10^7$ units/mg, Peprotech, UK) was added when indicated. After two days, 10 µl was diluted in water (390 µl) to lyse cells. After 2 min, 50 µl of Middlebrook enrichment supplement ADC (albumin, dextrose, catalase, Becton Dickinson Microbiology Systems, USA) and 50 µl of concentrated 7H9 medium (×10, Biolife italiana, Italy) with 2% glycerol were added.

For the purification of monocytes, mononuclear cells were isolated by Ficoll-Paque Plus density gradient sedimentation (GE Healthcare, USA), and CD14$^+$ cells (monocytes) were purified by magnetic cell separation (Miltenyi Biotec, Germany). 10^5 monocytes per well in RPMI-1640 medium/10% autologous serum were seeded in 96-well plates and infected with 10^3 bacteria in the presence of IFNγ when indicated, and incubated at 37 °C in 95% air/5% CO_2 for two days while they differentiated to monocyte derived macrophages. Infected cells were lysed by sonication with a Branson S-450 digital ultrasonic cell disruptor microtip (Branson, Emerson, USA), at 10% amplitude (2 W) for 3 s. In both cases (lysed blood and monocyte derived macrophages), decimal dilutions in 7H9 complete medium were inoculated into 96-well plates and incubated at 37 °C for 7 days. Colony-forming units (CFU) were counted under an inverted microscope at ×100 magnification [11].

2.6. Statistical analysis

Log transformed CFU followed a normal distribution (Shapiro Wilks test), and were analyzed by paired Student's *t*-test. The rest of data were not normally distributed and were analyzed using the non-parametric Mann-Whitney test. Analysis was performed with PASW Statistics 18 (International Business Machines, USA). In all statistical analysis, a *P* value < 0.05 was considered significant.

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