Impaired cytokine production by peripheral blood mononuclear cells in type 1 diabetic patients

N.T. Fossa, M.C. Foss-Freitas, M.A.N. Ferreira, R.N. Cardili, C.M.C. Barbosa, M.C. Foss

Division of Dermatology, Department of Medicine, Ribeirão Preto Medical School, São Paulo University, Av. Bandeirantes, 3900. Monte Alegre, 14049-900 Monte Alegre, Brazil

Division of Endocrinology, Department of Medicine, Ribeirão Preto Medical School, São Paulo University, Av. Bandeirantes, 3900. Monte Alegre, 14049-900 Monte Alegre, Brazil

Received 19 October 2006; accepted 19 May 2007
Available online 07 November 2007

Abstract

Aims. – The objective of the present investigation was to study the production of IL-1, IL-6, IL-10, IFNγ and TNFα in cultures of peripheral blood mononuclear cells (PBMC) taken from type 1 diabetic patients with inadequate metabolic control.

Methods. – Seventeen type 1 diabetic patients and a gender- and age-matched group of 17 healthy individuals were studied. PBMC cultures were stimulated with phytohemagglutinin (PHA; 20 μg/ml) and lipopolysaccharide (LPS; 10 μg/ml), and enzyme immunoassay (Elisa) was used to measure IL-1, IL-6, IL-10, IFNγ and TNFα in the cell-culture supernatants.

Results. – IFNγ levels in PHA-stimulated cultures were lower in the type 1 diabetics than in the non-diabetic controls (P < 0.0001) while, in contrast, IL-10 levels were increased in the PHA-stimulated culture supernatants of the diabetics compared with the controls (P < 0.0001). In addition, supernatant levels of the cytokines IL-1, IL-6 and TNFα released in the presence of LPS in the cell cultures from the diabetic patients were significantly lower than in the non-diabetic subjects (P < 0.0001, P < 0.0001 and P < 0.03, respectively).

Conclusions. – The impaired production of IL-1, IL-6, TNFα and IFNγ, and the increased production of IL-10, in PBMC cultures from type 1 diabetics with inadequate metabolic control compared with healthy subjects may be an indication of a deficiency in mononuclear cell activation and, consequently, a deficient immune cellular adaptive response that, in turn, may be the cause of the increased incidence of infections in people with type 1 diabetes.

Résumé

Altération de la production de cytokines par les cellules mononucléées du sang périphérique des patients atteints de diabète type 1

Objet. – L’objectif de cette étude était d’évaluer la production de IL-1, IL-6, IL-10, IFNγ et TNFα dans les cultures de cellules mononucléées obtenues du sang périphérique des patients porteurs de diabète type 1 non équilibrés.

Méthode. – Dix-sept patients diabétiques de type 1 et 17 témoins, appariés selon le sexe et l’âge, ont été étudiés. Des cellules mononucléées du sang périphérique ont été stimulées en culture par phytohémaglutinine (PHA ; 20 μg/ml) et lipopolysaccharide (LPS ; 10 μg/ml). La technique Elisa (essai d’un immunosorbent lié aux enzymes) a été utilisée pour mesurer les taux d’IL-1, IL-6, IL-10, IFNγ et TNFα dans le surnageant des milieux de culture.

Résultats. – Après la stimulation par PHA, les taux d’IFNγ étaient plus bas dans le groupe diabétique par comparaison au groupe témoin non diabétique (P < 0.0001). En revanche, les taux d’IL-10 étaient plus élevés chez les diabétiques que chez les témoins (P < 0.0001). Par ailleurs, les taux des cytokines IL-1, IL-6 et TNFα étaient également plus bas chez les diabétiques que chez les témoins (P < 0.0001, P < 0.0001 et P < 0.03, respectivement).

Conclusions. – Les anomalies de la production d’IL-1, IL-6, TNFα et IFNγ ainsi que l’augmentation de la production d’IL-10 dans les cultures de cellules mononucléées du sang périphérique des diabétiques de type 1 insuffisamment équilibrés pourraient indiquer un déficit d’activation des cellules mononucléées, et par conséquent un déficit cellulaire adaptatif qui pourrait constituer une des raisons de l’incidence plus élevée d’infections chez le patient diabétique.

© 2007 Elsevier Masson SAS. All rights reserved.

E-mail address: mcfoss@fmrp.usp.br (N.T. Foss).
1. Introduction

The increased incidence of infectious diseases in diabetic patients has been reported in several studies [1–8], and is probably due to impairment of the immunological defense system of these patients. In previous studies, we found a higher prevalence of skin lesions (59%), especially dermatophytoses, in type 1 diabetes patients who are inadequately metabolically controlled [9]. Chronic hyperglycemia, causing an increase in glycated proteins, can stimulate the production of cytokines, which are involved in the activation of the immune system [10]. It appears that metabolic control is important not only in the pathogenesis and progression of the macro- or microvascular and neurological complications of diabetes [11–13], but also in the high susceptibility of these patients to infectious diseases [9].

The most likely damage that occurs in diabetes is to the cellular immune response — more specifically, T-cell function [14–16]. Moreover, it has been demonstrated that polymorphonuclear cells from diabetic patients have impaired chemotaxis and phagocytosis capability [17–20], defects that can be reversed by insulin therapy. In fact, recent studies have shown an anti-inflammatory effect of insulin [21].

Therefore, it may be important to determine immune-cell behavior in diabetic patients, focusing on mononuclear cells and especially the interaction between macrophages and T cells, to assess the mechanisms underlying the high susceptibility of diabetic patients to infections. On this basis, we have studied the production of interleukin (IL)-1, IL-6, IL-10, IFNγ and TNFα in cultures of peripheral-blood mononuclear cells (PBMC) from type 1 diabetic patients with inadequate metabolic control, and compared them with cell cultures from healthy volunteers.

2. Subjects and methods

Seventeen type 1 diabetic patients selected from the Outpatient Clinics at the University Hospital of the School of Medicine of Ribeirão Preto (USP), and 17 healthy, non-smoking volunteers were included in our study (Table 1). The patients presented with inadequate metabolic control (fasting plasma glucose and glycated hemoglobin levels higher than 200 mg/dl and 11%, respectively), had no infectious diseases and were not using immunosuppressive drugs. In addition to undergoing neurological examination, the patients were evaluated for the presence of microvascular complications using ophthalmological examination and urine-protein measurement. The presence of macrovascular complications was determined by clinical and electrocardiographic evaluation. Body mass index (BMI) was calculated as weight (kg)/height (m²). The Ethics Committee of our institution approved the study protocol, and patients gave their written informed consent to participate in the study. A whole blood (12 ml) sample was taken from each study participant and placed in a sterile tube containing lithium heparin as anticoagulant (Vacutainer, BD) for the cell proliferation test and cytokine quantification. Additional blood samples were taken for the measurement of fasting plasma glucose and glycated hemoglobin (Labtest Diagnostica) [22]. The cell cultures were based on a previously described protocol [23], using a Ficoll-Hypaque® density gradient for PBMC separation. The adherent cells (1.0 × 10⁶) were exposed to lipopolysaccharide (LPS; 10 μg/ml) and the supernatant collected after 24 h of culture for quantification of IL-1, IL-6 and TNFα. The non-adherent cells, at a concentration of 2.5 × 10⁵ cells/ml, were cultivated in triplicate and stimulated with 20 μg/ml of phytohemagglutinin (PHA). After 72 hours of culture at 37 °C, in a humid environment with approximately 5% CO₂, the supernant was collected for cytokine measurement (IL-10 and IFNγ), and 0.5 μCi of ³H-thymidine was added to each well for the cell-proliferation assay. The cells were maintained under the same conditions as described above for an additional 16 h. The cells were then collected using an automated cell harvester (Cambridge Technology Inc., Cambridge, UK) and radioactivity measured for a period of 10 min using a scintillation spectrometer (Beckman Coulter Inc., Fullerton CA, USA).

Monoclonal antihuman IL-1, IL-6, IL-10 IFNγ and TNFα antibody was used (Pharmingen International (Life Science Research), San Diego, CA, USA) as the capture antibody, and biotinylated antihuman antibodies for the five analyzed cytokines (Pharmingen) as the detecting antibody. Binding was detected with peroxidase-labeled streptavidin (DAKO, Glostrup, Denmark) and α-phenylenediamine-2HCl/substrate (OPD, Sigma, St Louis, MO, USA). The intra- and interassay variations were below 10%. Detection limits of cytokine Elisas were 31 pg/ml for IL-1, 78 pg/ml for IFNγ and 39 pg/ml for IL-10, IL-6 and TNFα.

The results are reported as median (M), mean (X) and standard error of the mean (SEM). The GraphPad Prism program (San Diego, CA, USA) was used for the statistical analysis. Comparisons between groups were made by the Mann–Whitney test and P < 0.05 was considered to be statistically significant.

3. Results

The age and BMI of the diabetic group were similar to those of the non-diabetic group (Table 1). All of the diabetic patients were diagnosed during a ketoadiabetes episode, and none had any chronic diabetic macro- or microvascular or neurological complications. Inadequate metabolic control among the diabetics was defined by high levels of glycated hemoglobin (11.7 ± 2.0% SEM) and by fasting plasma-glucose concentration (249.3 mg/dl ± 67.8 SEM).

The PBMC proliferation index of the PHA-stimulated cultures was higher in the non-diabetic controls (M = 97.5) than
in the type 1 diabetics ($M = 42.4; P = 0.001$) (Fig. 1). The supernatant levels of IFNγ in the PHA-stimulated cultures from type 1 diabetic patients were decreased ($M = 1592$ pg/ml) compared with levels in those from non-diabetic subjects ($M = 5606$ pg/ml; $P = 0.0001$) (Fig. 1). In contrast, IL-10 levels were increased in the PHA-stimulated culture superna-

tants from type 1 diabetics ($M = 3931$ pg/ml) compared with non-diabetics ($M = 1767$ pg/ml; $P < 0.03$) (Fig. 2).

The cytokines IL-1, IL-6 and TNFα levels detected in the supernatants of LPS-stimulated cultures of PBMC taken from type 1 diabetics were significantly lower compared with the levels detected in non-diabetic subjects (IL-1: $M = 1722$ and 7840 pg/ml, $P < 0.0001$; IL-6: $M = 444$ and 1644 pg/ml, $P < 0.0001$; TNFα: $M = 670$ and 1708 pg/ml, $P < 0.03$; in patients and controls, respectively) as shown in Fig. 3.

### 4. Discussion

Cytokines are produced in response to microorganisms or other antigens and act as important immune modulators in host defense against aggressors [16,24]. They also regulate cellular function in other systems, and may play a role in the development of chronic complications of diabetes mellitus involving neurological and vascular pathological processes [10,25,26].

Previous studies have demonstrated that plasma levels of cytokines in type 1 diabetic patients can be different from those of non-diabetic patients [27,28]. It is also well known that plasma concentrations of cytokines are influenced by many factors and that several types of cells could produce cytokines. Thus, we used PBMC cultures to evaluate cells of the immune system, focusing on the susceptibility to infections seen in diabetic patients. In our study, the mononuclear cells in type 1 diabetics showed impaired in vitro proliferation, probably due to an immunological abnormality of the disease itself and to chronic activation of the immune system, leading to a diminished response of PBMC to the stimulus. This chronic lymphocytic activation may be triggered by superantigen infections [29,30] and/or be related to immunological alterations brought about by the type 1 autoimmune process.

IFNγ is a cytokine produced by antigen-activated T cells that acts on the clones of T cells, stimulating cell proliferation using an autocrine mechanism to regulate the proliferation and differentiation of immune effector cells, including natural-killer

### Table 1

**Clinical and biochemical characteristics of type 1 diabetic patients and non-diabetic control subjects**

<table>
<thead>
<tr>
<th></th>
<th>Type 1 diabetic patients ($N = 17$)</th>
<th>Control subjects ($N = 17$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (m/f)</td>
<td>7/10</td>
<td>8/9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>$21.3 \pm 5.4$</td>
<td>$23.6 \pm 1.1$</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>$23.6 \pm 3.0$</td>
<td>$23.0 \pm 1.7$</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>$10.2 \pm 3.8$</td>
<td>–</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>$249 \pm 68$</td>
<td>$95 \pm 4$</td>
</tr>
<tr>
<td>Glycated haemoglobin (%)</td>
<td>$11.7 \pm 2.0$</td>
<td>$5.5 \pm 0.5$</td>
</tr>
</tbody>
</table>

![Fig. 1. Proliferation index of PHA-stimulated cultures from type 1 diabetic patients and non-diabetic subjects. The horizontal lines represent the median of each group.](image)

![Fig. 2. IL-10 (pg/ml) and IFNγ (pg/ml) levels in PHA (20 μg/ml)-stimulated PBMC-culture supernatants from type 1 diabetic patients (DM1) and non-diabetic controls (C). The horizontal lines represent the median of each group.](image)
(NK) cells [31]. Decreased levels of this cytokine in the PHA-stimulated cultures from type 1 diabetics who are inadequately metabolically controlled reflect impaired activation of the adaptive immune system in such patients. In addition, we found lower levels of IL-1, IL-6 and TNFα in the supernatants of LPS-stimulated PBMC cultures from the diabetic patients compared with those produced in non-diabetics, findings that confirm the hypothesis of an impaired adaptive immune response in type 1 diabetics. Finally, it must be borne in mind that ‘hyperglycaemic memory’ confounds the relationship between cell function and blood glucose concentration: hyperglycaemia-induced changes in gene expression and biological reactions such as advanced glycation end-products in long-lived macromolecules may not be reversed when blood glucose returns to normal and, as these changes are progressively ‘imprinted’ on cells [32], the result is impaired immune-cell activity.

On the other hand, IL-10 levels were increased in the culture supernatant from the diabetics, reflecting enhancement of the Th2 subset of CD4 helper T cells, with stimulation of immunoglobulin (Ig) production by B cells and suppression of the Th2 subset of CD4 helper T cells, with stimulation of IFN-γ-dependent macrophage function. These results suggest depressed macrophage activity, as determined by IL-1, IL-6 and TNFα cytokine release in non-adherent PBMC cell cultures from diabetic patients compared with that from non-diabetic subjects. The higher production of IL-10 may be a regulatory response to IFN-γ production.

Despite the small number of patients in our study, the data show significant differences in cytokine levels between type 1 diabetic patients and healthy subjects. The impaired production of IFN-γ and the inflammatory cytokines IL-1, IL-6 and TNFα, and the increased production of the regulatory cytokine IL-10, in PBMC cell cultures of type 1 diabetic patients suggest a deficiency in mononuclear cell activation and, consequently, a deficient immune response, which may be one of the reasons that type 1 diabetic patients have an increased incidence of, in particular, extracellular infections.

Acknowledgements

The authors are grateful to Maria Aparecida Nunes Ferreira and Sebastião L. Brandão Filho for their technical assistance, and to E. Greene for reviewing the manuscript in English.

References


