Circulating monocytes are activated in newly diagnosed type 1 diabetes mellitus patients

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SUMMARY

Investigations in the BB rat and the non-obese diabetic (NOD) mouse have provided substantial evidence for the involvement of the monocyte/macrophage system in the development of type 1 diabetes mellitus. However, it is not known whether monocytes play the same role in the pathogenesis of human type 1 diabetes. We investigated this problem in a longitudinal study of 29 recent-onset type 1 diabetes mellitus patients. Monocyte chemotaxis, phagocytosis and superoxide production as well as metabolic and haematological parameters were studied immediately after diagnosis and 6 months later. At diagnosis the patients had activated casein and C5a chemotaxis (casein 70 ± 9 *versus* 150 ± 5 (mean $\pm$ s.e.m.), $P < 0.001$; C5a 137 ± 10 *versus* 158 ± 5, $P < 0.05$ (activation immobilizes monocytes, reducing the measured values)), and activated superoxide production (3.6 ± 0.3 *versus* 3.0 ± 0.3, $P < 0.05$). After 6 months casein chemotaxis (115 ± 16 *versus* 150 ± 5, $P < 0.05$) and Candida phagocytosis (3.3 ± 0.1 *versus* 2.8 ± 0.2, $P < 0.001$) were still activated. There was no correlation with other clinical or paraclinical parameters. We conclude that the circulating monocytes in newly diagnosed type 1 diabetes patients are activated. It is reasonable to expect that monocytes at the local site of inflammation in pancreas are even further activated. This could play a pathogenic role in β cell destruction.

Keywords monocytes macrophages superoxide production phagocytosis chemotaxis type 1 diabetes mellitus newly diagnosed

INTRODUCTION

Animal models for type 1 diabetes have provided strong evidence for the involvement of macrophages in the development of the disease. Both in BB rats, non-obese diabetic (NOD) mice, the low-dose streptozotocin mouse and the EMC-D virus model cells of the monocyte line are the first to infiltrate the islets of Langerhans preceding the clinical onset of the disease [1–9]. Also, evidence exists that nitric oxide synthetase, found in monocytes, could be important [10,11] and data suggest that a general activation of the monocyte system takes place as glycolysis [12], macrophage killing [13], secretion of tumour necrosis factor-alpha (TNF-α) [14], phagocytosis [15], and spleen macrophage activity [16] was increased in various models. Finally, eradication of macrophages by silica or specific antibodies almost completely prevents diabetes in several models [1,3,9,17–25].

Human studies have mostly included patients with long-lasting disease in whom metabolic derangements may flaw the

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immune system functions, or relatives to patients in whom the disease may not yet be active. We undertook the present study to investigate the possible pathogenic role of macrophages in human type 1 diabetes. Ideally, macrophages present in the pancreas before the onset of the disease should be examined, but ethically only peripheral monocytes are acceptable. However, we limited study subjects to patients that could be examined immediately after diagnosis. This precisely defined group showed activation of several peripheral monocyte parameters at diagnosis and at the 6-months follow-up examination.

PATIENTS AND METHODS

Patients
Thirty consecutive patients (22 men and eight women, aged 30.1 ± 1.2 years and 23.1 ± 2.8 years, respectively) admitted to three different hospital wards were included in the study under the following criteria: age < 40 years, not overweight as estimated by body mass index, random blood glucose

> 12 mM, glucosuria, and ketonuria. One was excluded as he no longer received insulin after 6 months. Nine patients could not be reexamined. All patients, except one, were Scandinavians. Seven (four) patients had a family history of type 1 (type 2 or unspecified) diabetes. At admission the patients had experienced diabetes-related symptoms for 8.7 ± 2.1 weeks. None of the patients suffered other diseases at any of the examinations, in particular no infections or inflammations as reflected by their normal leucocyte counts. The control persons were healthy volunteers without any family history of diabetes. They were not age- or sex-matched, as the testing laboratory has previously established that the monocyte tests were independent of age and sex in adults. All had normal leucocyte counts. The study was conducted according to the Helsinki Declaration and was approved by the Ethics Committee for Copenhagen Community.

Design

Two blood samples were obtained: one as soon as possible after diagnosis (on average 4.6 ± 0.8 days) and one after 6.0 ± 0.3 months. Blood was collected before breakfast and insulin administration. Routine blood and urine parameters were recorded on both occasions. Blood from the various participating hospitals was brought by courier to the testing laboratory and tested within 2 h. Variances in transportation time did not affect the tests.

Isolation of blood monocytes

Peripheral venous blood was drawn into citrated polypropylene tubes. After dextran sedimentation the monocytes were harvested by metrizoate/polysucrose gradient centrifugation (Lymphoprep, Nyegaard, Oslo, Norway) [26]. The monocytes were washed twice in Eagle's minimal essential medium (MEM; GIBCO, Gaithersburg, MD) and suspended in MEM at 2×10^6 monocytes/ml. The percentage of monocytes, as assessed by morphology after Wright's stain and cytochemical identification of non-specific esterase [27], was 15–33%. The contamination with neutrophils was < 2%, and the viability of cells, assessed by nigrosin exclusion, was always > 95%. The cell number was adjusted accordingly, so that all assays used an equal number of monocytes.

Oxidative burst response

Reduction of cytochrome c was used as a measure of superoxide anion production [28]. Cells were suspended in Eagle's MEM and a reaction mixture of 10^6 monocytes and 2 mg/ml horse heart cytochrome c (Sigma, St Louis, MO) in Krebs–Ringer solution with 5 mM glucose was stimulated with phorbol myristate acetate (PMA; Sigma) at a concentration of 100 ng/ml. The reduction of cytochrome c was continuously measured with a double beam spectrophotometer (Shimadzu UV-190, Kyoto, Japan) corrected for background values in blanks without PMA. The slope of the absorbance curve was converted to nanomoles of cytochrome c reduced per minute using the extinction coefficient $E_{550} = 2.1 \times 10^4$ M/cm. The assay was performed in duplicate.

Phagocytosis

The phagocytic activity of monocytes was assessed by incubating opsonized *Candida albicans* with monocytes at a ratio of 5:1 for 30 min. The number of intracellular phagocytosed yeast cells was calculated by direct microscopy of 100 non-specific esterase-positive monocytes on a cyto-centrifuge spin preparation. The experiments were performed in duplicate.

Chemotaxis

The migration of monocytes towards standard chemo-attractant products was measured in modified Boyden chambers as previously described [29]. In brief, blood monocytes in MEM with 2% human serum albumin were separated from chemoattractant by a polycarbonate filter with 5-μm pores (Nuclepore, Pleasanton, CA). After incubation for 90 min at 37°C the filters were fixed, stained in haematoxylin and mounted on slides. The number of monocytes migrating completely through the filter was counted by direct microscopy ($\times$ 900) in 10 random fields on each of two duplicate filters and the activity expressed as mean number of monocytes per field. The number of cells were corrected for non-specific migration towards MEM.

Statistical analysis

All results are given as mean $\pm$ s.e.m. Student's t-test was used for testing differences between means. Multiple regression analysis was used to evaluate the influence of various clinical parameters upon chemotaxis. F statistics was used for testing equality of variance between groups. $P < 0.05$ was considered significant.

RESULTS

Chemotaxis, phagocytosis and oxidative burst responses

Casein and C5a chemotaxis were activated among the patients at diagnosis (70 ± 9 *versus* 150 ± 5, $P < 0.001$ and 137 ± 10 *versus* 158 ± 5, $P < 0.05$, respectively; Table 1). Casein chemotaxis was still activated at the follow-up examination (115 ± 16, $P = 0.047$), whereas C5a chemotaxis had normalized (note that activation immobilizes monocytes, reducing the measured value). The phagocytosis activity was normal at diagnosis (3.0 ± 0.1 *versus* 2.8 ± 0.2, $P > 0.1$) but stimulated at follow up (3.3 ± 0.1, $P < 0.001$), whereas superoxide production was moderately activated on both occasions (3.6 ± 0.3 and 3.9 ± 0.4 *versus* 3.0 ± 0.3, $P < 0.05$ and $P = 0.051$, respectively). The variance was smaller at diagnosis than at follow up for casein chemotaxis ($P < 0.05$).

Casein and C5a chemotaxis values were correlated, both at diagnosis and at follow up (correlation coefficients 0.503 and 0.750, respectively), and so were C5a chemotaxis and superoxide production (0.427 and 0.633), and casein chemotaxis and superoxide production (0.219 and 0.427).

To exclude that the monocyte activation was caused by factors following rather than leading to diabetes, such as impaired glycaemic control or ketoacidosis, the results from the monocyte tests were examined for correlation with a large number of parameters. These included: constitutive (sex, age, weight, height), disease-related (duration of symptoms, blood glucose at diagnosis and at the time of examinations, haemoglobin A_{1C}, insulin dosage, arginine-stimulated c-peptide secretion), electrolyte recordings (serum-K, -Na, -bicarbonate and haemoglobin) as well as blood cell counts (leucocytes,

Table 1. Summary of chemotactic responses, phagocytic activities and superoxide production by diabetes patients and control persons

| | Blood glucose (mM) | HbA$_{1c}$ (mM) | Chemotaxis (cells/filter) | | Phagocytosis (*Candida albicans*/cell) | Superoxide production $\left(\dfrac{\text{nmoles}}{\text{min} \times 10^6 \text{ cells}}\right)$ |
			Casein	C5a		
Diabetes patients						
Diagnosis (n = 29)	20·8 ± 1	12·1 ± 0·6	70 ± 9 (P < 0·001)	137 ± 10 (P < 0·05)	3·0 ± 0·1 (P > 0·1)	3·6 ± 0·3 (P < 0·05)
Six months follow up (n = 20)	8·0 ± 5	5·4 ± 0·6	115 ± 16 (P < 0·05)	178 ± 15 (P > 0·1)	3·3 ± 0·1 (P < 0·001)	3·9 ± 0·4 (P = 0·051)
Controls (n = 82, except superoxide production: n = 20)			150 ± 5	158 ± 5	2·8 ± 0·2	3·0 ± 0·3

Chemotaxis was measured as the number of monocytes per visual field that penetrated a polycarbonate filter separating the cells from a chemoattractant. Phagocytosis was measured as the average uptake per monocyte of opsonized *Candida albicans* during a 1-h incubation. Finally, superoxide production was measured as reduction of cytochrome c. Results are indicated as mean ± s.e.m.

polymorph nuclear leucocytes, lymphocytes, monocytes, eosinophils, basophils, thrombocytes, erythrocytes). No correlation with any of these parameters was found. Also, comparing poorly regulated patients or patients with long-lasting symptoms with well regulated patients or patients with short disease duration did not yield differences in monocyte function characteristics. Finally, multiple regression analysis, even when using up to four variables, could only account for less than 40% of the chemotaxis variation, and identified no factors with major impact on the chemotaxis activation.

Blood cell parameters

The patients had a lower number of circulating leucocytes at diagnosis than at the 6-month follow-up examination (6·2 ± 0·3 versus 6·8 ± 0·4 × 10^9/l, P < 0·01, n = 20) due to a reduced number of lymphocytes at this time (2·1 ± 0·1 versus 2·5 ± 0·2 × 10^9/l, P < 0·005, n = 20). No changes were seen in monocyte, leucocyte or eosinophil counts. Finally, the patients had slightly reduced haemoglobin values at diagnosis (8·3 ± 0·1 versus 8·7 ± 0·2 mM, P < 0·005, n = 20).

DISCUSSION

Two markers, chemotaxis and phagocytosis, indicated activation at diagnosis and normalization or less activation at the follow-up examination, whereas phagocytosis increased during the same period. It is likely that the activation of phagocytosis at the follow-up examination is important, and the question is therefore why phagocytosis was not activated in newly diabetic patients. A possible explanation for this could be that phagocytosis is a 'late' function in the autoimmune destruction of the islets. Whereas superoxide production and chemotaxis could be part of the pathogenic events, phagocytosis is possibly a scavenger function needed after cellular destruction. The variable correlation coefficients found between the various tests probably reflect heterogeneity in monocyte activation.

Inflammatory conditions *in vivo* and cytokine stimulation *in vitro* are known activators of the monocyte system. However, no intercurrent illnesses were present in the diabetes patients, and no clinical or paraclinical parameters were correlated with monocyte function. Monocyte activation therefore probably reflects the ongoing immunologic destruction of the pancreas.

During the development of diabetes phenotypical differences can be seen between resident tissue macrophages (ED2$^+$) and infiltrating macrophages (ED1$^+$) [30,31]. Furthermore, infiltrating monocytes could become activated by local factors such as cytokines, which are produced in islet cells [32]. It is therefore reasonable to expect that monocytes at the local site of inflammation in the islets are even further activated, and this could play an important role in β cell destruction.

Previous studies have particularly examined patients with long-lasting diabetes, in whom mostly decreased or normal functions were reported. These included: normal antigen presentation activity [33], reduced numbers of circulating monocytes and reduced phagocytosis [34], normal cell migration distance, but fewer migrating cells [35] and reduced number of phagocytic cells [36]. One study has reported increased chemiluminescence activity, but this was attributed to the increased levels of blood glucose [37].

Only recently the role of monocytes in newly diagnosed type 1 diabetes was investigated. Although lower percentages of adhesion molecule-expressing monocytes were found in patients compared with controls [38], no differences were found in cytokine secretion from cultured monocytes [39]. Phagocytosis was also normal, although decreased in probands at risk [40]. These studies are not directly comparable to the present work, but it is likely that changes in adhesion molecule expression and phagocytosis reflect various activation states.

Data from the animal models provide strong evidence for the involvement of monocytes in type 1 diabetes. We conclude from this study that peripheral monocytes are also activated in human diabetes patients. Since macrophages do not re-enter the blood stream after differentiating into tissue macrophages, the monocytes must become stimulated in the blood stream. The immune system is an obvious source for factors that could activate peripheral monocytes, but diffusible β cell factors could also play a role, as factors that could be responsible for the initial chemoattraction and subsequent activation of monocytes have recently been identified ([41] and Josefsen et al., manuscript in preparation). If the precise source of peripheral monocyte stimulation can be identified drugs interacting with

monocyte activity might become useful in the management of the disease.

ACKNOWLEDGMENTS

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