DOI: 10.1111/dme.14143

Research: Complications

Regional differences in cell-mediated immunity in people with diabetic peripheral neuropathy

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Accepted 23 September 2019

Abstract

Aim To study cell-mediated immunity in the feet of people with type 2 diabetes with polyneuropathy.

Methods In a cohort comprising people with type 2 diabetes with polyneuropathy (n = 17) and without polyneuropathy (n = 12) and a healthy control group (n = 12) indurations due to delayed-type hypersensitivity responses to intracutaneous *Candida albicans* antigen were determined in the foot and compared with those in the arm (an area relatively spared in diabetic polyneuropathy). The sizes of indurations on the foot were correlated with electromyographic measurements in the participants with diabetes.

Results No differences were observed in the median size of indurations between the foot and arm in healthy controls and participants without polyneuropathy; in participants with polyneuropathy, induration sizes on the foot were smaller than on the arm: 0 (95% CI 0 to 1) vs 5 (95% CI 2 to 6) mm ($P < 0.01$). In participants with diabetes, larger indurations correlated with better nerve function (Spearman's rho 0.35 to 0.39).

Conclusion Our findings suggest that diabetic peripheral polyneuropathy negatively affects cell-mediated immunity in the foot. (Clinical Trials registry no.: NCT01370837)

Diabet. Med. 37, 350–355 (2020)

Introduction

Foot infections are one of the most feared complications of diabetes mellitus; more than 25% of people with an infected diabetic foot ulcer underwent a lower extremity amputation in the Eurodiale study [1]. Several factors probably contribute to the aggressive course of these infections, including local factors such as ulcer characteristics and peripheral arterial disease [1–3], as well as systemic factors such as hyperglycaemia and end-stage renal disease [4,5]. Up to 50% of people with diabetes with a deep foot infection do not display signs of infection such as fever, increased erythrocyte sedimentation rate or leucocytosis [6], suggesting that these people may also have an impaired immune response to a foot infection. This defect does not appear to be generalized as C-reactive protein levels can increase up to 10- to 100-fold in people with diabetes with a systemic infection [7], while C-reactive protein levels are only modestly elevated (~10 to 20 mg/l) in people with an infected foot ulcer [8]. The vast majority of these foot infections occur in people with diabetic polyneuropathy, and a recent prospective study reported that polyneuropathy was a risk factor for postoperative infections in people with diabetes after foot and ankle surgery [9]. Interestingly, also in people without diabetes but with polyneuropathy the risk of postoperative foot infection was increased by nearly fivefold.

Several lines of evidence indicate that the peripheral nervous system closely interacts with the immune system. Activation of nociceptive C-nerve fibres by mechanical or chemical stimuli results in a local axon reflex, which in turn leads to the release of neuropeptides, such as substance P, neurokinin A, neuropeptide Y and tumour necrosis factor-α, that can initiate local inflammatory responses [10]. This neurogenic inflammation leads to vasodilation (the flare response), which is diminished in people with diabetic polyneuropathy [11–13]. Moreover, the enhanced production of neuropeptides increases vascular permeability and affects the function of several types of cells of the immune system [10], and experimental elimination of nerve fibres abolishes both induction and effector stages of a delayed-type

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What's new?

- Local immune responses can be impaired as a result of neurological deficits.
- Diabetic peripheral neuropathy could negatively affect cell-mediated immunity selectively in the foot, the site most frequently and severely affected by peripheral neuropathy.
- More severe impairment of cell-mediated immunity correlates with worse peripheral nerve function as measured by electromyography.
- Our study is the first to suggest the presence of locally diminished cell-mediated immunity in the foot in people with diabetic peripheral neuropathy.
- Impaired cellular immunity in the foot may explain the paucity of signs of inflammation in diabetic foot infections.

hypersensitivity response in mice [14]. In view of these findings, impaired cell-mediated immunity in the foot due to polyneuropathy might explain the paucity of signs of infection and might facilitate the extensive tissue destruction in infected diabetic foot ulcers [15].

Cell-mediated immunity can be evaluated *in vivo* by determining the delayed-type hypersensitivity response to intracutaneously injected *Candida albicans* antigen, which is analogous to the purified protein derivative skin test (also called the Mantoux test), used to establish a previous *Mycobacterium tuberculosis* infection [16–18]. In the presence of an intact immune system, the *C. albicans* antigen will induce a cutaneous delayed-type hypersensitivity response in most people as they have been in contact with this yeast. We hypothesized that people with diabetic polyneuropathy would have a diminished response to the *C. albicans* antigen on their foot (an area preferentially affected by polyneuropathy) compared to their upper arm (relatively spared in polyneuropathy), in contrast to people with diabetes without polyneuropathy and healthy controls.

Methods

Study population

We included 41 participants, aged 40–75 years, in the study: 12 healthy controls, 12 participants with type 2 diabetes without polyneuropathy and 17 participants with type 2 diabetes and diabetic polyneuropathy. Our exclusion criteria were: presence of a current diabetic foot ulcer; active Charcot disease or gout; estimated GFR (calculated with the Modification of Diet in Renal Disease formula) < 30 ml/min; any current active infection; immunodeficiency due to medication or comorbidities; history of anaphylaxis; peripheral arterial disease; a blood transfusion in the 2 months prior to study inclusion; capillary blood glucose < 4 mmol/l or > 15 mmol/l at the time of the study; skin disease of the dorsal aspect of the foot or of the inner upper arm; any bleeding disorder; peripheral oedema and pregnancy or breastfeeding. The absence of peripheral arterial disease was confirmed by determination of the ankle–brachial index using a handheld Doppler, with an ankle–brachial index > 0.9 considered normal. The diagnosis of peripheral polyneuropathy was made using the Valk score, a validated score based on a standardized clinical neurological examination to diagnose and grade clinical neuropathy [19]. All participants with diabetes subsequently underwent electromyography to confirm the findings of the clinical neurological examination. Before performing electromyography, the participants' extremities were warmed to at least 30 °C as necessary. The amplitudes, conduction velocities and F-waves of the (sensory and motor) median nerve, (sensory) ulnar nerve and (motor) peroneal nerve were measured on the right side; the same measurements of the (sensory) sural nerve and (motor) tibial nerve were acquired on the left side, and the H-reflex of the tibial nerve was measured at the musculus soleus. One clinical neurophysiologist, who was blinded to the results of the clinical neurological examination, evaluated all electromyography results.

Delayed-type hypersensitivity to *Candida albicans* antigen

The *C. albicans* antigen for intracutaneous administration was obtained from Allergopharma (Zeist, the Netherlands) at a concentration of 750 protein nitrogen units per vial; one protein nitrogen unit is equivalent to 0.01 μg of acid-precipitable protein nitrogen. The antigen was dissolved in 5 ml of concurrently supplied solvent. Subsequently, 1 ml of the solution was logarithmically diluted with, respectively, 0.7, 4 and 9 ml of NaCl 0.9%, to yield four concentrations of the *C. albicans* antigen; these concentrations were selected based on a pilot study. While in the supine position, participants were intracutaneously injected with 0.05 ml of each of the four concentrations of antigen on the dorsum of the right foot and the right inner upper arm. In all participants the different concentrations were injected at the same location on the foot and the arm, with a distance between the injection sites of ~2 cm. The exact location of each injection was marked with a surgical marker.

The maximum diameter of the largest induration, and the diameter perpendicular to this diameter, were measured by a researcher blinded to study group at 24, 48 and 72 h after the injections. The term 'induration' in the present paper refers to the mean of these two diameters.

Statistical analysis

Analyses were performed using IBM SPSS statistics (version 22). The number of participants needed to detect a significant difference in induration size with 80% power was 12 per

group based on a previously performed feasibility study. Because of the relatively small sample size and the skewed distribution of the data obtained in the arms and feet, we could not exclude non-normality and therefore used non-parametric tests according to the prespecified protocol. The differences in induration size on the arm and the foot were determined in each group using the Wilcoxon related samples test. Between-group analyses were performed with the Kruskal–Wallis test for independent samples. In participants with diabetes, the conduction velocities and the amplitudes of the peroneal, tibial and sural nerves were summed, after which the correlation between these sums and induration size at 48 h was described with Spearman correlation coefficients. A P value < 0.05 was considered to represent a statistically significant difference.

Ethics

All participants gave written informed consent before participating in the trial. The study was approved by the Ethics Committee of the University Hospital Maastricht/Maastricht University.

Results

Characteristics of study participants

Baseline characteristics of study participants are shown in Table 1. In general, we studied elderly men. The participants without polyneuropathy and with polyneuropathy were generally well matched except that the participants with polyneuropathy had (by definition) a higher score on the standardized clinical neurological examination (Valk score). Metabolic control was moderate in both groups. In comparison to the healthy controls, the participants with diabetes were somewhat older, had a higher BMI and more frequently had a history of cardiovascular disease.

Intracutaneous injection of the *C. albicans* antigen elicited erythema and induration in all participants. Among the four concentrations of *C. albicans* antigen, the highest concentration elicited the largest induration in both the arm and the foot in all three groups. As the induration on the arm was used as a benchmark, we first determined the time course of the *C. albicans* response on the arm in all participants. Because the induration on the arm was largest in all study groups at 48 h (data not shown), we chose this induration and the induration in response to the same antigen concentration on the foot at 48 hours, as the response measures for statistical analyses.

Differences in responses to *Candida albicans* antigen on the arm vs the foot

The induration in response to the intracutaneous injection of *C. albicans* was similar on the arm and the foot in healthy controls and in participants without polyneuropathy (Fig. 1). In healthy controls the median size in the arm was 4 mm (95% CI 1 to 13.5) and 4 mm (95% CI 0 to 9) in the foot; in

Table 1 Baseline characteristics of study participants

	Healthy controls (n = 12)	Type 2 diabetes without polyneuropathy (n = 12)	Type 2 diabetes with polyneuropathy (n = 17)	P
Age*, years	59.4 (8.8)	65.1 (6.7)	66.5 (5.2)	0.54
Men*, %	67	83	82	0.95
Current smoker*, %	8	8	18	0.49
BMI*	25.9 (3.9)	29.2 (3.0)	32.9 (4.7)	0.02
History of myocardial infarction*, %	0	17	24	0.67
History of TIA/CVA*, %	0	17	12	0.72
History of vascular surgery*, %	0	8	6	0.81
History of angina pectoris*, %	0	0	18	0.13
Ankle-brachial index*	1.1 (0.2)	1.2 (0.1)	1.2 (0.2)	0.82
Valk score*	2.7 (3.1)	4.1 (3.2)	18.5 (5.7)	< 0.001
Random glucose†, mmol/l	6 (0.9)	9.8 (2.7)	8.8 (3.4)	0.39
HbA_{1c}†, mmol/mol	34.1 (3.4)	57.0 (7.0)	62.0 (13.6)	0.21
HbA_{1c}†, %	5.3 (0.3)	7.4 (0.5)	7.8 (1.2)	0.21
Creatinine*, µmol/l	74 (12)	95 (15)	89 (28)	0.53
Microalbuminuria†, g/mol creatinine	0.18 (0.34)	4.03 (10.91)	8.78 (18.32)	0.43
Diabetic retinopathy*, %	na	25	50	0.18
RAAS inhibition*, %	25	83	88	0.72
Metformin therapy*, %	na	92	88	0.77
Sulfonylurea derivative therapy*, %	na	25	6	0.14
Insulin therapy*, %	na	42	77	0.06
Units of insulin per day†	na	109 (86)	90 (31)	0.69

CVA, cerebrovascular accident; RAAS, renin-angiotensin-aldosterone system; TIA, transient ischaemic attack. Values are presented as mean (SD) unless otherwise specified
ANOVA comparing all three groups.
*Pearson chi-squared test comparing type 2 diabetes and polyneuropathy groups.
†Independent samples t-test comparing groups with type 2 diabetes with and without polyneuropathy.

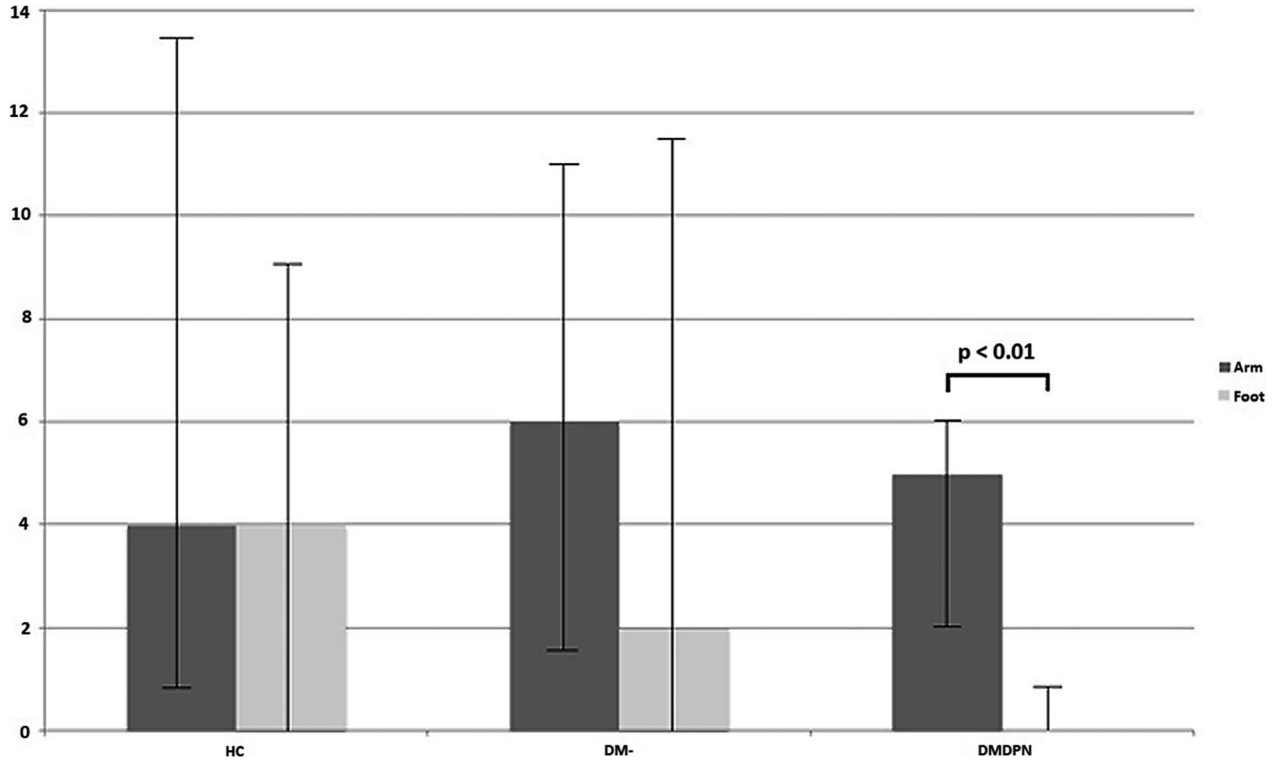

FIGURE 1 Median induration (mm) in response to intracutaneous *Candida albicans* antigen in the arm and foot. DM, diabetes without diabetic peripheral neuropathy; DMDPN, diabetes with diabetic peripheral neuropathy; HC, healthy controls. Error bars represent 95% CIs.

Table 2 Results of nerve conduction studies

	Type 2 diabetes without diabetic polyneuropathy	Type 2 diabetes with diabetic polyneuropathy	*P*
Conduction velocity, m/s			
Peroneus	45.2 (3.3)	40.4 (15.2)	0.34
Tibialis	45 (2.7)	28 (18)	< 0.01
Nervus suralis	43 (4.9)	2.5 (9.5)	< 0.01
Amplitude, mV			
Peroneus	4.8 (1.8)	1.7 (1.4)	< 0.01
Tibialis	10 (5.3)	4.4 (9.2)	0.09
Nervus suralis	7.1 (2.6)	0.8 (2.6)	< 0.01

Values are mean (SD).

the arm and foot of the participants without polyneuropathy these responses to *C. albicans* were 6 mm (95% CI 1.5 to 11) and 2 mm (95% CI 0 to 11.5), respectively. In contrast, in the participants with polyneuropathy, the induration was larger in the arm compared to the foot: 5 mm (95% CI 2 to 6) vs 0 mm (95% CI 0 to 1; $P < 0.01$). There was no significant difference in induration between the arm ($P = 0.86$) and the foot ($P = 0.10$) when the three groups were compared with each other.

Table 2 shows the results of nerve conduction studies in the participants with diabetes. Better nerve function correlates with larger induration on the foot in people with diabetes

In people with diabetes (without polyneuropathy and with polyneuropathy combined), a weak correlation (Spearman's rho 0.35) between the sum of the conduction velocities of the peroneal, tibial and sural nerves and induration size on the foot ($P = 0.07$) was observed. A moderate correlation (Spearman's rho 0.39) was observed between the induration on the foot and the sum of the amplitudes of these nerves ($P = 0.04$). Additional analyses showed that there was a statistically significant correlation between the induration on the foot and the conduction velocity of the nervus suralis (rho 0.44, $P = 0.03$), the amplitude of the nervus suralis (rho 0.42, $P = 0.04$) and the amplitude of the n. tibialis (rho 0.48, $P = 0.02$).

Discussion

Diabetic foot infections frequently display relatively few signs of inflammation but these infections often result in extensive tissue damage. As the majority of people with such infections have peripheral diabetic polyneuropathy, we explored whether diabetic polyneuropathy is associated with impaired cellular immunity. In contrast to healthy controls and participants without diabetic polyneuropathy, the participants with diabetic polyneuropathy had diminished delayed-type hypersensitivity responses to *C. albicans*

antigen in their foot compared to their arm. Moreover, diminished delayed-type hypersensitivity responses were correlated with more severely impaired lower leg nerve function, determined with electromyography. As participants were their own control, it is unlikely that systemic factors could have selectively affected cellular immunity in the foot but not in the arm.

Delayed-type hypersensitivity response to *C. albicans* assesses acquired immunity to this common antigen, similarly to the assessment of acquired immunity to *Mycobacterium tuberculosis* with a purified protein derivative skin test. Delayed-type hypersensitivity responses are a measure of cellular immunity: for instance, the delayed-type hypersensitivity response to *C. albicans* is impaired in patients with HIV infection and can be restored by anti-viral treatment [20,21]. Also, *C. albicans* is one of the suggested skin tests for the evaluation of purified protein derivative anergy in HIV-infected patients by the Centres for Disease Control and Prevention [22].

In healthy controls, the delayed-type hypersensitivity response to *C. albicans* was the same in the upper arm and the foot, suggesting that the anatomical region in which the response is elicited does not influence induration size. The presence of diabetes *per se* also does not appear to have a major effect on induration size, as we could not observe a difference in response between healthy controls and participants with diabetes but without polyneuropathy. Our data are in line with a previous study using multiple intradermal antigens, which concluded that cell-mediated immune responses are intact in people with diabetes. In this study, the delayed-type hypersensitivity response to *C. albicans* antigen was actually increased [23], probably due to frequent exposure to and colonization with *C. albicans* in these people with diabetes [24]. It has been suggested that poor metabolic control, leading to deficiencies in both the innate and adaptive immune systems, explains increased susceptibility to infections in people with diabetes [25–27]; however, in the present study, participants had moderate metabolic control and this level of hyperglycaemia did not seem to affect T-cell responses to *C. albicans* antigen.

There are several previous observations that support our hypothesis of a locally impaired immune response attributable to a neurological deficit. First, in people with rheumatoid arthritis, after a cerebrovascular accident the arthritis resolves on the paretic side [28,29]. Moreover, in experimental models of arthritis, development of arthritis can be prevented by surgical denervation of a joint or limb [28,30]. Second, Tarkowski *et al.* [31,32] demonstrated decreased delayed-type hypersensitivity to purified protein derivative and decreased axon reflex vasodilatation in people who sustain lower motor neuron damage or inhibition due to poliomyelitis and minor stroke, respectively.

The present study has several limitations and should be interpreted as a hypothesis-generating study in a new area of research. First, the number of participants was relatively small, although our power calculation based on a preliminary study, suggested that there was >80% power to detect a difference. Moreover, the sample size of the three groups was unbalanced, with more participants in the polyneuropathy group; however, this did not mitigate our observations in our opinion, as the power calculation was based on 12 participants per group. Second, the variation in induration sizes was large, increasing the chance of spurious findings or type II errors, and we only observed differences when each individual was analysed as his/her own control. The variation in induration size, in particular in the participants without diabetes, may be explained by differential previous exposure to *C. albicans*, as it is unlikely that a hypersensitivity response can be elicited without previous exposure. This difference in immunological memory could be addressed in future studies by using stimuli to which all participants have previously been exposed. Third, we used large-fibre measurements to define and quantify neuropathy status, but the inflammatory response to *C. albicans* is possibly more affected by small-fibre dysfunction than large-fibre dysfunction. Unfortunately, we did not have a direct measure of skin small-fibre function in arms and legs that was accurate and robust enough to be used in our experiments; therefore, some of the participants without polyneuropathy might have undetected small-fibre dysfunction. Nevertheless, the induration in the foot correlated with several electromyographic abnormalities in the lower extremity in the participants with diabetes (rho values between 0.35 to 0.48), in line with our hypothesis that regional nerve dysfunction impairs local cell-mediated immunity. Finally, Pence *et al.* [17] demonstrated decreased delayed-type hypersensitivity responses to *C. albicans* in older people with higher C-reactive protein levels. Theoretically, our participants with peripheral neuropathy could have had low-grade inflammation due to poorer metabolic control or more extensive subclinical atherosclerosis, but this does not explain the differentially decreased delayed-type hypersensitivity response in the foot compared to the upper arm. Moreover, age, sex and BMI were unbalanced between the study groups. It is not clear if and how this influences induration sizes; however, this is unlikely to explain a significant difference in induration size between the foot and the arm in the same participant.

Only one stimulus was used in this study; future research should be focused on alternative inflammatory stimuli to attempt to corroborate our findings and to determine the pathways that could be involved in the loss of the immune-regulatory function of the peripheral nervous system in diabetic polyneuropathy.

Funding sources

The Dutch Diabetes Research Foundation funded this study, project number 2012.11.1502.

Competing interests

None declared.

Acknowledgements

The authors thank Mr R. Sleegers, nurse practitioner diabetic foot; Mr J. Wilms, podiatrist; Mr R. Jeuken, Mr R. Verjans, Mr M. van Houten and Mr T. Zegers, medical students, for their help with recruiting participants for the study, Dr H. Yucel, internist-endocrinologist and Dr P. van Hirtum, MD for their help with the feasibility study, and Prof. Dr B. Lipsky, Emeritus Professor of Medicine, University of Washington, Visiting Professor, Infectious Diseases, University of Geneva, and Teaching Associate, Green Templeton College, University of Oxford for critically reviewing the manuscript.

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