Early loss of neurogenic inflammation in the human diabetic foot

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SUMMARY

1. Neurogenic inflammation, mediated by nociceptor C fibres, is part of the acute neurovascular response to injury producing the axon reflex flare. Laser Doppler flowmetry was used to measure the flare response induced by the electrophoresis, at various current strengths, of a ring of acetylcholine solution into dorsal foot skin.

2. Nineteen control subjects and 52 long-duration insulin-dependent (Type 1) diabetic patients of similar age (20 without complications; 19 with laser-treated retinopathy; 13 with reduced vibration perception and retinopathy) were studied in order to investigate the possible attenuation of this defence mechanism in diabetes.

3. The maximal (1 mA) flare response [control median (interquartile range): 1.55 (1.16-2.06) arbitrary units] was reduced greatly in neuropathic patients to 0.37 (0.24-0.66) arbitrary units; \( P<0.001 \) with respect to all other groups, especially those with a previous history of foot ulceration. The flare was also reduced in some patients with retinopathy alone [1.06 (0.56-1.27) arbitrary units; \( P<0.005 \) with respect to control subjects].

4. No rightward shift of the curve of hyperaemic response plotted against current strength was found, suggesting that the abnormal response was due to axonal loss rather than to dysfunction.

5. Neurogenic inflammation, mediated by small pain fibres, was markedly impaired in a group of diabetic patients at risk of foot ulceration. Furthermore, impairment of this nociceptor C fibre response can develop before clinical large-fibre neuropathy and could itself predispose to foot complications.

Key words: hyperaemia, injury, insulin-dependent (Type 1) diabetes mellitus, laser Doppler flowmetry, neuropathy.

INTRODUCTION

Neuropathic diabetic subjects are at risk of developing foot ulceration even in the absence of macrovascular disease [1, 2]. Large-fibre sensory neuropathy, particularly loss of vibration perception, is thought to be one of the best clinical indicators of risk [3–6]. However, there are many patients with severe loss of vibration perception who do not ulcerate and, conversely, some patients with only mild loss who do. Other risk factors must therefore contribute to the presentation of ulceration in non-ischaemic diabetic feet.

Recently, the possible pathogenic role of small-fibre sensory and sympathetic neuropathy has been stressed. However, controversy persists as to whether this is more severe in patients with trophic ulceration than in those with other forms of diabetic neuropathy [6, 7–12]. This controversy is partly due to the frequent use of cardiac autonomic function tests, which may not accurately reflect peripheral function [13], and to the poor discriminatory power of many peripheral small-nerve-fibre function tests [10].

Inflammatory hyperaemic responses to injury can also be impaired in diabetes [14–16]. Furthermore, the local, neurologically independent part of Lewis’ triple response is impaired in the presence of retinopathy [16], perhaps explaining how this complication can predispose neuropathic patients to foot ulceration.

Accordingly, the purpose of the present study was to examine the peripheral small-fibre response of neurogenic inflammation in the feet of long-duration insulin-dependent (Type 1) diabetic patients with different complications. Neurogenic inflammation is mediated by small nociceptor C fibres that also subserve pain sensation. An antidromic axon reflex causes a spreading vasodilatation or flare response, probably through the action of substance P on microvessels and mast cells [17]. Until recently, neurogenic inflammation has been difficult to
induces a flare response, which can be measured by laser Doppler flowmetry. A modification of their technique has been developed for this study [19].

METHODS

Flare response

Nociceptor C fibres were stimulated to give the flare response of neurogenic inflammation by using the technique of acetylcholine electrophoresis, modified as previously described [19]. In brief, the skin was lightly cleansed with 70% (v/v) propan-2-ol while the subject underwent acclimatization, supine, in a constant temperature room at 24°C for 30 min. An electrophoretic capsule was attached to the dorsal surface of the foot by using double-sided sticking tape. The circular outer well, containing the anode of a constant current generator (SD <1% of the selected current output), was filled with a 0.55 mol/l solution of acetylcholine. During electrophoresis, a typical spreading flare response develops, which, because of the circumferential stimulation, is uniform within the capsule. The intensity of the hyperaemia was measured by a PF1d laser Doppler flowmeter (Perimed, Stockholm, Sweden) [20] at the capsule centre, 9 mm distant from the acetylcholine solution.

Laser Doppler flowmetry is a continuous and non-invasive technique of cutaneous blood flow measurement that has been validated using synchronous dynamic capillaroscopy [21]. However, the laser Doppler signal can be affected by factors such as skin thickness, pigmentation and vessel geometry. Furthermore, blood flow in the subpapillary plexus and arteriovenous anastomoses is measured as well as that in nutritional capillaries [22, 23]. Therefore the laser probe was positioned on the mid-dorsal foot, where there are few, if any, arteriovenous anastomoses [24] and no callus. Thus the readings represent only capillary and subpapillary plexus flows.

The local skin temperature influences the cutaneous capillary blood flow independently of the sympathetic nervous system [25, 26]. Therefore the skin, the electrophoretic capsule and the 0.55 mol/l solution of acetylcholine were gradually warmed, using local radiant heat when necessary, to the standard temperature range of 32–33°C, as measured by electronic thermometer (Comark, Rustington, West Sussex, U.K.). This temperature is near the top of the normal physiological range but below that at which rapid temperature-dependent vasodilatation occurs [27, 28] and does not interfere with neurovascular responses [29]. Other variables were limited by recruitment criteria.

The normal flare response, which is clearly dependent on intact nervous tissue [18], increases with increasing current strength, showing a clear-cut difference between unstimulated and stimulated skin [19]. The shape of the curve of hyperaemic response plotted against current strength was used to determine whether a reversible dysfunction or a loss of this neurovascular response had occurred. Therefore mean blood flow was measured sequentially from a stable 2 min period before and during acetylcholine electrophoresis at 0.2 mA (10 min) and 1.0 mA (5 min), currents at which the normal flare response approaches half-maximal and maximal intensities, respectively. An inhibition or dysfunction of the neurovascular unit would be expected to increase the current threshold at which hyperaemia occurs, shifting the response curve to the right and reducing the percentage of maximal (1.0 mA) hyperaemia attained during electrophoresis at 0.2 mA (i.e. the ratio of the increase in blood flow over basal at 0.2 mA compared with the increase over basal at 1.0 mA, expressed as a percentage). Conversely, a loss of function should reduce the maximal response, but leave the percentage of maximal hyperaemia attained at 0.2 mA unchanged or slightly increased. Intra-individual coefficients of variation were 21.5% and 6.7% for the hyperaemic responses at 0.2 and 1.0 mA, respectively, and 16.1% for the percentage of maximal hyperaemia during 0.2 mA electrophoresis.

Subjects

The hyperaemic response was assessed in 19 normal volunteers and 52 long-duration insulin-dependent (Type 1) diabetic patients grouped according to their complications (Table 1): (1) normal volunteers; (2) diabetic patients without complications; (3) diabetic patients with proliferative retinopathy that had required laser therapy, but asymptomatic for neuropathy and with normal vibration perception; and (4) diabetic patients with impaired vibration perception and retinopathy. Eleven subjects in group 4 had received laser therapy, and one was blind from proliferative disease. Seven had previously suffered from neuropathic foot ulceration.

Subjects were excluded from the study if they were over 65 years of age, non-Caucasian, were smokers, were taking drugs other than insulin, or had hypertension (diastolic blood pressure >90 mmHg), Raynaud's phenomenon, macrovascular disease (intermittent claudication or an ankle to brachial systolic pressure ratio <1 [30]), clinical oedema, active foot ulceration, a raised serum creatinine level or abnormal liver function tests. No patient had a blood glucose concentration below 4.0 mmol/l at the start of the study, or suffered symptomatic hypoglycaemia beforehand.

Insulin-dependent (Type 1) diabetes was defined by ketoacidosis or by an onset before 30 years of age that required insulin therapy within a few weeks of diagnosis. Retinopathy was assessed by an ophthalmologist using direct and indirect ophthalmoscopy or fundal photography. No microaneurysms, exudates or haemorrhages were seen in the uncomplicated diabetic group. Neuropathy was defined by the 90th centile of vibration perception threshold for age [31] using a biothesiometer (Biomedical Instrument Co., Newbury, OH, U.S.A.) on the hallux of both feet. Ankle reflexes were normal in all non-neuropathic patients but were absent from the neuropathic group except in three feet (two patients) when
Loss of neurogenic inflammation in diabetes 607

augmentation was required. Cardiac autonomic function was assessed by heart rate variation with deep breathing [32]. Glycaemic control was assessed from the venous blood glucose concentration and glycated haemoglobin (HbA1c) was measured by isoelectric focusing.

The studies were approved by the Hospital Ethics Committees and informed consent was obtained from each subject.

Statistical analyses

Results are expressed as medians and interquartile ranges. Groups were compared by using the Kruskal–Wallis test. If a significant difference between the groups was found (P<0.05), the two-tailed Mann–Whitney U-test was used to define where this occurred. Rank correlation was assessed by using Spearman’s test.

RESULTS

Skin temperatures before and during electrophoresis were comparable among the four groups (Table 2) and did not correlate with blood flow in any group. Resting blood flow levels were also similar (Table 2). There were positive correlations between the resting blood flow and the 0.2 mA hyperaemic response only in the retinopathic and neuropathic diabetic groups (rs=0.48 and 0.70, P=0.036 and 0.008, respectively).

The maximal 1.0 mA flare response to acetylcholine electrophoresis (Fig. 1) was greatly impaired in the neuropathic group compared with all other groups (P<0.001). Furthermore, subgroup analysis showed that the lowest responses in this group occurred in patients with a past history of foot ulceration [median (range) hyperaemia in ulcerated and non-ulcerated neuropathic patients: 0.27 (0.17–0.91) and 0.64 (0.36–0.95) arbitrary units, respectively; P<0.02]. A single patient in the ulcerated group had a good maximal flare response (0.91 arbitrary units), but his vibration perception threshold was unrecordable on the ipsilateral hallux. Some retinopathic patients with normal (large-fibre) vibration perception thresholds had impaired maximal flare responses (P<0.005 compared with control subjects).

The percentage of maximal (1.0 mA) hyperaemia attained during acetylcholine electrophoresis at 0.2 mA was slightly greater in the groups of diabetic patients with complications, suggesting that a loss of neurovascular function had occurred (Table 2). There was no correlation of the hyperaemic response with the blood glucose concentration, the level of HbA1c, the duration of diabetes, or the cardiac autonomic function in any group.

DISCUSSION

This study demonstrates that maximal nociceptor C (small) fibre inflammatory responses are impaired in diabetic patients with clinical large-fibre neuropathy. Earlier reports of distal small-fibre sensory neuropathy and of reduced neurogenic inflammation in diabetic patients with neuropathy [7, 12, 14, 33, 34] have therefore been corroborated. Our results extend these observations in two ways. First, maximal flare responses were more severely impaired in neuropathic patients with a history of trophic ulceration than in those without such a history, supporting studies that suggest that small-fibre neuropathy is important in ulcer development [7, 8]. Secondly, impaired flare responses were also found in some patients before the development of clinical large-fibre neuropathy.

Neither altered capillary density nor age-related neurovascular changes are likely to have caused the observed reduction in the flare response. Capillary density in the feet of diabetic patients with complications is reported to

Table 1. Characteristics of 19 normal volunteers and of 52 patients with insulin-dependent (Type 1) diabetes grouped according to complications

Data are expressed as medians with interquartile ranges in parentheses, or number. The E/I ratio is the mean of (the longest R-wave interval on an ECG during expiration/the shortest R-wave interval during inspiration) from six deep breaths performed in 1 min.

<table>
<thead>
<tr>
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<th>Normal volunteers</th>
<th>Insulin-dependent (Type 1) diabetic patients</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>No complications</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>10:9</td>
<td>11:9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45</td>
<td>43</td>
</tr>
<tr>
<td>(37–54)</td>
<td></td>
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<tr>
<td>Duration of diabetes (years)</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(19–27)</td>
</tr>
<tr>
<td>Blood glucose concn. (mmol/l)</td>
<td>5.1</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>(4.0–5.5)</td>
<td>(6.8–13.0)</td>
</tr>
<tr>
<td>HbA1c(%)</td>
<td>6.2</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>(5.9–6.6)</td>
<td>(8.6–10.3)</td>
</tr>
<tr>
<td>Mean vibration perception threshold (V)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(5–10)</td>
<td>(6–9)</td>
</tr>
<tr>
<td>E/I ratio</td>
<td>1.44</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>(1.25–1.49)</td>
<td>(1.17–1.45)</td>
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be unchanged, both in vivo [35] and on histological examination [36]. Resting blood flow levels at a standard skin temperature were similar for the study groups both in this work and in comparable earlier studies [16, 37]. An appropriate non-shunt supine skin blood flow therefore appears to be maintained in diabetic subjects with or without microvascular complications. Furthermore, the effects of ageing on capillary density [38] and neuropathy [18, 31] were minimized by using groups which were similar in both age and duration of diabetes, with an age limit of 65 years. This has not always been achieved in earlier studies [33, 34]. Other factors, including significant macrovascular disease, were accounted for by the exclusion criteria. The dorsal foot is not the usual site for neuropathic foot ulceration. However, the sensitivity of the technique is greatly improved by using the dorsal rather than the plantar surface of the foot [19] and nerve function is unlikely to be affected differently at the two sites.

Neurogenic inflammation depends on both intact nociceptor C fibres and the ability of the microvasculature to respond. The distinction between the two parts of the response is difficult, although intact nerves are essential for its initiation [17, 18, 33, 34]. This problem was addressed by Parkhouse & Le Quene [12], who demonstrated reduced flare responses and normal red reactions (the first part of the triple response) in diabetic patients with neuropathic foot lesions. The reduced ratio of the two responses does suggest that neuropathy has an important role in reducing the flare response. Unfortunately, their technique may have been too insensitive to conclude that the vascular component of the response was normal because there was overlap between the resting blood flow and both hyperaemic reactions. Many other workers have found reduced hyperaemic responses that are independent of the nervous system [15, 16, 37], as well as finding abnormal microvascular structure and function [36, 39, 40], in such patients. We have therefore assumed that the vascular component of the flare response could also be reduced (especially in the presence of retinopathy [16]) and so have not measured it.

Several observations have been made which would be consistent with the presence of small-nerve-fibre loss in our patients with reduced vibration perception and in some with retinopathy alone. Non-neurogenic responses do not seem to be abolished in diabetes. All diabetic and non-diabetic subjects are able to increase cutaneous blood flow substantially above resting levels whether post-ischaemic hyperaemia [37], vasodilatation during local heating [15] or the first part of Lewis' triple response [15, 16] are measured. Similarly, in all our normal subjects there was a very clear rise in cutaneous blood flow after stimulation by acetylcholine electrophoresis (Table 2 and Fig. 1) [19]. However, some diabetic subjects in both the retinopathic and neuropathic groups showed no flare response to acetylcholine electrophoresis. This strongly suggests that a loss of nervous tissue, on which the response depends, has occurred. Consistent with this is the progressive deterioration of flare response through the retinopathic to the neuropathic diabetic groups, in contrast to the first part of Lewis' triple response, which is similarly impaired in retinopathic diabetic patients with or without large-fibre neuropathy [16]. The slightly increased percentage of maximal (1.0 mA) hyperaemia

### Table 2. Dorsal foot skin temperatures and blood flow before and during acetylcholine electrophoresis in normal volunteers and in insulin-dependent (Type 1) diabetic patients grouped according to complications

Data are expressed as medians with interquartile ranges in parentheses. The percentage of maximal hyperaemia during acetylcholine electrophoresis at 0.2 mA was calculated as (the increase in blood flow from rest at 0.2 mA×100/increase in blood flow from rest at 1.0 mA). Statistical significance: *P < 0.01 compared with all other groups.

<table>
<thead>
<tr>
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<th>Normal volunteers</th>
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<tbody>
<tr>
<td></td>
<td>No complications</td>
<td>Retinopathy</td>
</tr>
<tr>
<td><strong>Skin temperature (°C)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unheated</td>
<td>30.8 (29.2–31.8)</td>
<td>30.0 (28.8–30.9)</td>
</tr>
<tr>
<td>Rest</td>
<td>32.5 (32.3–32.7)</td>
<td>32.6 (32.4–32.8)</td>
</tr>
<tr>
<td>Electrophoresis at 0.2 mA</td>
<td>32.5 (32.1–32.7)</td>
<td>32.5 (32.1–32.6)</td>
</tr>
<tr>
<td>Electrophoresis at 1.0 mA</td>
<td>32.6 (32.3–32.7)</td>
<td>32.6 (32.3–32.8)</td>
</tr>
<tr>
<td><strong>Skin blood flow (arbitrary units)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>0.13 (0.10–0.16)</td>
<td>0.14 (0.08–0.17)</td>
</tr>
<tr>
<td>Electrophoresis at 0.2 mA</td>
<td>0.60 (0.34–0.99)</td>
<td>0.52 (0.36–1.34)</td>
</tr>
<tr>
<td>Electrophoresis at 1.0 mA</td>
<td>0.52 (0.36–0.99)</td>
<td>0.58 (0.33–1.34)</td>
</tr>
<tr>
<td><strong>Percentage of maximal hyperaemia during electrophoresis at 0.2 mA</strong></td>
<td>35 (19–55)</td>
<td>42 (26–76)</td>
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</table>
patients with reduced vibration perception and previous trophic ulceration had more severely impaired maximal flare responses than similar patients without a history of ulceration. The good maximal flare response and unrecordable vibration perception seen in one patient with ulceration suggests that there is a relationship between the severity of small- and of large-fibre neuropathy which determines the risk of ulceration. Although microvascular pathology may have contributed to the reduction in neurogenic inflammation, we believe that an early loss of small nociceptor C fibres, preceding clinical large-fibre neuropathy, has been demonstrated. These findings could explain some of the apparent discrepancies seen between the presentation of foot ulceration and the severity of clinical sensory neuropathy in non-ischaemic diabetic feet.

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