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RANDOMISED CLINICAL TRIAL

Immunotherapy with *Mycobacterium vaccae* and Peripheral Blood Flow in Long-treated Leprosy Patients, a Randomised, Placebo-controlled Trial

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Objective: *to evaluate immunotherapy as a means of improving peripheral blood flow in chronic leprosy patients.*
Design: *this was a double-blind, randomised, placebo-controlled, clinical trial.*
Materials: *heat-killed* Mycobacterium vaccae *1 mg plus 0.02 μg Tuberculin protein per 0.1 ml dose in borate buffer, with saline as placebo. Those studied were 92 long-treated residents of a leprosy centre in Iran, 10 of their healthy children and 10 staff members. Evaluation employed the Perimed PF2, Laser–Doppler Flowmeter, a platinum skin thermistor, and a thermal sensibility tester.*
Methods: *single intradermal injections of test or placebo were given to 103 patients 18 months before the blinded evaluation. Fingerpulp blood flux was measured in controlled conditions and vasomotor reflexes and skin sensation to touch, pain and heat were evaluated in 45 and 47 patients in the placebo and* M. vaccae *groups, respectively, and in 20 healthy control persons.*
Results: *Laser–Doppler flux, skin temperature, vasomotor reflexes and sensation were impaired in leprosy patients. Immunotherapy improved ($p < 0.05$) Laser–Doppler flux, skin temperature and temperature sensation.*
Conclusions: *immunotherapy, given 18 months earlier, significantly improved blood flow and temperature sensation, in fully-treated, chronic, leprosy patients. The same principles might be employed in other conditions of reduced peripheral blood flow.*

Key Words: Immunotherapy; Mycobacterium vaccae*; Randomised clinical trial; Placebo-controlled; Blood-flow; Chronic leprosy.*

Introduction

An immunotherapeutic based on killed *Mycobacterium vaccae* is undergoing investigation in the treatment of leprosy[1–4] and of vascular diseases.[5,6] *M. vaccae* combines adjuvant activity regulating T helper cell maturation, with antigens important in immune regulation.[7] It is a rich source of group i, common, mycobacterial antigens,[8] including the mycobacterial 65 and 70 kiloDalton (kDa) heat-shock proteins (hsp). Like *M. leprae*, *M. vaccae* lacks both groups ii and iii antigens and has its own species-specific, group iv antigens.[8,9] Small studies of the efficacy of killed *M. vaccae* in immunotherapy of leprosy have been reported and it has been shown that the addition of one tenth of a skin test dose of new Tuberculin may improve its efficacy in this disease.[1] In a study of immunotherapy with *M. vaccae* in leprosy patients, improved resolution of pre-existing anterior uveitis has been observed,[10]

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indicating a regulatory effect on vasculitis. This, together with observations of improvements in Raynaud's disease in otherwise healthy persons, led to our study.

Chronic, long-treated, leprosy patients often show a progressive deterioration in peripheral microcirculation and in nerve function,[11] with serious consequences such as ulcers on the feet, progressive clawing of the hands[12] and destruction of bone[13] of face, hands and feet, as well as soft tissue lesions of the face and body. Microcirculatory impairment has been shown to be an important factor in pathology and to complicate prognosis, especially in patients of long-standing, resulting in disproportionately cold fingers at high ambient temperatures.[14,15]

Eighteen months after suitable patients had been randomized to receive *M. vaccae* or placebo,[16] their fingerpulp circulation and that of an healthy control group were compared by a number of parameters.

Materials and Methods

Immunotherapy and placebo

The immunotherapeutic consisted of an heat-killed (autoclaved for 15 min at 121 °C) suspension of *M. vaccae* strain NCTC 11659 grown on Sauton's medium, solidified with 1.3% agar. The suspension contained 10 mg wetweight of bacilli in 1 ml of borate buffered saline (pH 8) to which 0.1 ml new Tuberculin (2 μg/ml) was added shortly before administration.[1,16] Intradermal injection of 0.1 ml of the thoroughly shaken immunotherapeutic or of saline as placebo, were given over the left deltoid muscle.

Setting

The leprosy colony at Baba Baghi was set up some 80 years ago in a fertile, isolated and enclosed valley a few miles from the city of Tabriz in Iranian Azerbaijan. Modern Baba Baghi consists of a hospital with 60 beds, with an out-patient department, surrounded by a village of patients houses and flats with supporting facilities. Approximately 200–250 patients live there with their healthy children and grandchildren.

Subjects and study design

Eighteen months before our study 103 patient volunteers under the age of 65 were skin tested with 4 new tuberculins (Tuberculin, Leprosin A, Scrofulin and Vaccin).[17] On the basis of their responses to these and their clinical type of leprosy, they were randomised into two groups to receive an injection of the immunotherapeutic or of placebo.[16] In general, hospitalised patients with beds on the left sides of wards were given the immunotherapeutic and those on the right were given the placebo. Non-hospitalised patients were seen in the clinic and alternate patients received immunotherapy or placebo. This was done taking care that similar numbers of paucibacillary patients fell into each group and that patients making "Koch-type" responses to Tuberculin were evenly represented.[18] Patients did not know which injection they received and the injector (JLS) was not present at the time of follow-up. Figure 1 gives the participant flow in the study.

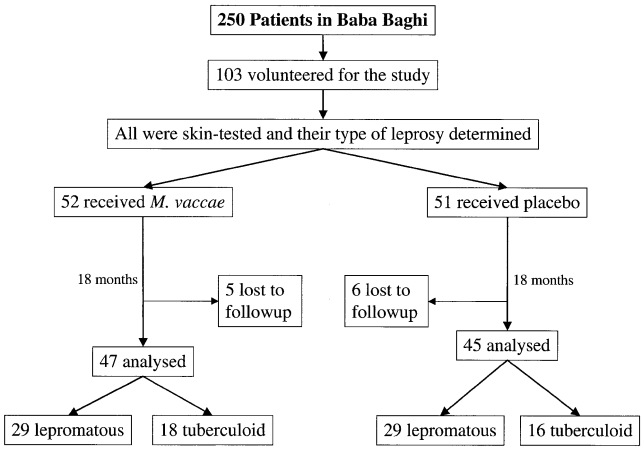

Fig. 1. Flow diagram showing the progression of patients through the study.

Of the original 103 patients, 47 *M. vaccae* and 45 placebo recipients were resident at the hospital and available for testing when our clinical measurements were carried out. All gave informed consent to have measurements with the Laser–Doppler Flowmeter and for their vasomotor reflexes and finger sensation to be assessed. Assessments were made by an operator (NCA) who was unaware of which study treatment the subjects had received. Twenty healthy persons volunteered to be controls; 10 were adult offspring of patients and resident at Baba Baghi and 10 were members of staff.

At the end of our investigation, an injection of *M. vaccae* was offered to the placebo recipients, and accepted by most.

Estimate of blood flow

The Laser–Doppler Flowmeter (Model PF2, Perimed, Stockholm, Sweden) measures the movement of erythrocytes in the most superficial 1 mm of skin

from changes in the frequency of coherent light reflected out of the tissue.[19] Internally standardised, the instument gives an integrated measurement of microvascular blood flow related to the product of the number of moving erythrocytes and their mean velocity: Laser–Doppler flux, expressed in volts.[20,21] The fibre-optic tip of the flowmeter was attached to the pulp of the distal phalanx by a probe holder fixed with double-sided adhesive tape ensuring optimal alignment between sensor head and skin surface. The output signal was recorded continuously during the period of observation on a chart recorder calibrated to a full scale deflection of 10 volts. Flowmeter settings were as described previously.[14] The fingerpulp was investigated because its abundant arteriovenous anastomoses are under strict autonomic control.[22] Skin sympathetic reflexes were assessed from the change in Laser–Doppler flux following a deep inspiratory gasp or a contralateral challenge of plunging the hand into cold water.[13,14,23]

Measurement of fingerpulp skin temperature

A platinum skin thermistor was used to measure skin surface temperature in the manner described previously.[14] The probe (Model 4098, 9 mm diameter, Yellow Springs Instrument Co. Inc., OH, U.S.A.) was held in close contact with the skin with a single strip of Millipore adhesive tape. A stable temperature was generally achieved after 5 minutes contact with the skin of the fingertip pulp. The same sensor was used to measure room temperature near the subject during experimentation.

Sensory testing

Sensation in the upper limb was tested for light touch with cotton wool, for sharp touch or pain by pin prick and for temperature sensation with a thermal sensibility tester.[14] Three sites were tested on the palmar aspect of each finger, including the fingerpulp. The results were recorded on a three-point scale: 1 = absent or consistently mistaken; 2 = partial sensation (i.e. variable positive and negative results from site to site on a finger); 3 = unimpaired sensation. For convenience of analysis the summary result on each subject was the mean score (out of 3) for each type of sensation tested.

Experimental protocol

Subjects were seated comfortably with the forearm and hand resting on a table at heart level at an ambient temperature of 25–29 °C maintained by a paraffin stove in the experimentation room. Ambient temperature was measured by the thermistor, described above, situated within 25 mm of the subject at the same level as the hands. These conditions induce near maximal peripheral vasodilatation and stable blood flow through the fingertips in healthy subjects. Each subject was allowed to equilibrate under these conditions for at least 15 min before measurements were started. All four fingers were studied on both the right and left hands. In some patients one or more fingers were missing, or too deformed for measurements to be carried out.

Measurements of sensation were made in the order; light touch, sharp touch and temperature discrimination. Skin temperature was then recorded and, lastly, Laser–Doppler flux was measured.

Outcome measures

The primary outcome measure was the Laser–Doppler flux measurement expressed in volts. Secondary outcome measures were the fingerpulp skin temperature, the vasomotor reflex responses and the measurements of sensation.

Statistical analysis

All analyses were performed using SPSS for Windows (ver 6.1, SPSS, Chicago). Differences between patients and control subjects and between patients receiving *M. vaccae* or placebo were assessed for each measured parameter using two-way ANOVA taking intra-individual differences between fingers into account. Differences in the responses to skin testing between groups were assessed using the Mann–Whitney *U*-test.

To detect a difference of 0.8 volts of Laser–Doppler flux (approx. 20% of mean flux in groups of leprosy patents[14,15]), and assuming a standard deviation of 1.5 volts,[15] it was calculated that at least 44 patients would be required for each group assuming a power of 80% at the 0.05 level of significance.

Results

There were no significant differences between the patient groups in age, sex, duration of stay in Baba Baghi, in the proportion with lepromatous disease or in their responses to skin tests prior to administration

of immunotherapy or placebo (Table 1). However, the patient groups were significantly older than the healthy volunteers in the control group ($p < 0.05$).

Table 2 shows the effect of leprosy, with or without immunotherapy, on Laser–Doppler flux, fingerpulp skin temperature, sympathetic vasomotor reflexes and touch, pain and temperature sensation. Since the 95% confidence limits for the 20 healthy control subjects were 3.1–10.0 volts for Laser–Doppler flux (mean 6.6) and 31.0–35.0 °C for skin temperature (mean 33.1 °C), leprosy patients fingers with mean flux measurements of less than 3.1 volts and fingertip skin temperature less than 31.0 °C were considered impaired for the purposes of analysis. Laser–Doppler flux, skin temperature and temperature sensation were greater ($p < 0.05$) in the immunotherapy group than in the placebo recipients. In the placebo group 45% of all fingers had impaired Laser–Doppler flux values (<3.1 volts) and 42% had impaired skin temperatures (<31.0 °C) whereas these values were 33 and 31% respectively for the immunotherapy group. All measurements were significantly impaired in the leprosy patient groups compared with the healthy control persons ($p < 0.05$).

Table 1. Basic characteristics of patients and controls, and the results of skin tests with Tuberculin, Leprosin A, Vaccin and Scrofulin carried out immediately before administration of immunotherapy or placebo. Data are shown as medians (range).

	Control	Placebo	*M. vaccae*
Subjects (*n*)	20	45	47
Age (years)	25.5 (16–70)	47 (25–65) †	47.5 (16–72) †
Years in Baba Baghi		23 (3–38)	23 (8–40)
Patients with lepromatous leprosy (%)		64	62
Skin test responses*			
Tuberculin			
+ve reponses/*n* (%)		30/44 (68)	26/46 (57)
median induration (mm)		10 (3–22)	6.5 (2–15)
Leprosin			
+ve reponses/*n* (%)		9/44 (21)	12/46 (26)
median induration (mm)		7 (3–15)	3 (2–14)
Vaccine			
+ve reponses/*n* (%)		12/44 (27)	15/46 (33)
median induration (mm)		6 (4–14)	9 (3–17)
Scrofulin			
+ve reponses/*n* (%)		14/44 (32)	15/46 (33)
median induration (mm)		4.5 (2–9)	4.5 (2–18)

* Median diameter of induration of 2 mm or more at 72 h.
† Difference from control group ($p < 0.05$).

Table 2. The effect of immunotherapy with *M. vaccae* or placebo on measurements (median [range]) of Laser–Doppler flux, temperature, vasomotor reflexes in response to inspiratory gasp and cold challenge, and estimates of sensory function.

	Control	Placebo	*M. vaccae*
No. of subjects	20	45	47
Fingers tested	160/160	356/360	369/376
Room temp in °C*	25.4 (23.1–28.9)	24.8 (22.1–29.6)	25.4 (23.1–28.9)
LDFlux in Volts	6.4 (3.1–12.3)	3.6 (0.1–12.6) †	4.5 (0.1–14.1) †‡
Percent fingers with impaired LDFlux	1	45 †	33 †‡
Percent fingers with LDFlux < 1 volt	0	25 †	13 †‡
Fingertip skin temperature in °C	33.1 (29.3–35.4)	31.8 (22.6–35.2) †	32.1 (20.6–34.9) †‡
Percent fingers with low skin temp	4	42 †	31 †‡
Inspiratory Gasp (%)	82 (0–100)	24 (0–90) †	27 (0–90) †
Cold Challenge (%)	81 (0–100)	0 (0–90) †	0 (0–90) †
Sensory Score			
Light Touch	3.0 (3–3)	3.0 (0–3) †	3.0 (0–3) †
Sharp Touch	3.0 (3–3)	3.0 (0–3) †	3.0 (0–3) †
Temperature	3.0 (3–3)	2.0 (0–3) †	3.0 (0–3) †‡

* Mean of the air temperatures when the members of each group were tested.
† Difference from control group assessed by ANOVA ($p < 0.05$, multiple range test).
‡ Difference between placebo and *M. vaccae* groups assessed by ANOVA ($p < 0.05$, multiple range test).

By disease classification, lepromatous patients receiving immunotherapy had significantly higher Laser–Doppler flux values than those receiving placebo (mean values 4.59 vs 3.91 volts, representing 27% vs 42% impaired fingers respectively in each group). Tuberculoid patients showed a similar difference between immunotherapy and saline groups but this did not reach a level of significance (mean values 4.06 vs 3.5 volts).

Discussion

Leprosy, a disease caused by infection with *Mycobacterium leprae*, is associated with major perturbation of the immune response. This results in a number of different clinical presentations ranging over a well-established immunopathological spectrum.[24] The disease predominantly affects skin and nerves, but many tissues are secondarily affected. At the multibacillary, or lepromatous, end of the spectrum large numbers of bacilli are demonstrable in the tissues and the cellular immune response to *M. leprae* antigens is suppressed, though high titres of antibodies are frequently present. At the paucibacillary, or tuberculoid, end of the spectrum, bacilli can be very hard to find and an abnormal cellular immune hyper-responsiveness is observed with lower antibody levels. Leprosy bacilli combine antigens sharing major amino-acid sequence homologies with human tissue antigens, including the stress proteins of mitochondrial origin, with potent mycobacterial adjuvants. Thus autoimmune phenomena involving many tissues complicate the disease, especially in its chronic forms. Despite modern chemotherapy with effective antibacterial agents eliminating living bacilli, tissue damage may continue for the rest of the patient's life, largely due to autoimmune consequences of the disease. The continued peripheral vascular deterioration is an important part of this post-infective leprosy syndrome.

The present study is the first to describe the effect of immunotherapy with *M. vaccae* on peripheral microcirculation. Our finding that this resulted in higher, though still abnormally low, fingerpulp blood flows was unexpected given a disease and treatment duration in the patients in excess of 20 years. There is also evidence that the effect occurs across disease classification, lepromatous patients receiving immunotherapy had significantly higher Laser–Doppler flux than those receiving placebo and tuberculoid patients showed a similar trend between treatment groups. The lack of pre-treatment measurements of microcirculation and sensation makes the results less impressive than they might have been, yet they agree with the reported reduction in anterior uveitis seen in these and similar patients after treatment with *M. vaccae*.[10]

Many studies have addressed the immune situation in active leprosy but very little has been done on long-treated chronic disease such as that of our study group. In active lepromatous disease there is an excess of T helper cell type 2 (Th2) activity with predominantly Th2-associated antibody production. Unlike the situation in pulmonary tuberculosis, the toxicity of tumour necrosis factor (TNF) is rarely apparent. Active tissue necrosis is not seen except during reactive episodes known as erythema nodosum leprosum (ENL) and Lucio's phenomenon of which local endarteritis is the major lesion. In tuberculoid leprosy both Th1 and Th2 lymphocytes infiltrate the lesions giving rise to granulomas which rarely necrose. Sudden changes in the intensity of cellular immunity are thought to be the cause of reversal reactions in which inflammation of peripheral nerves and their subsequent fibrosis is an important feature. Again, it may be that arteriolar and capillary inflammation is the underlying lesion.

The Laser–Doppler flux method of microcirculatory monitoring has been found reproducible for the detection of differences in fingerpulp blood flow and skin sympathetic reflexes between groups of leprosy patients (new vs treated; lepromatous vs tuberculoid) and also between patients and healthy subjects.[14] Iranian patients show a similarly reduced peripheral flow giving support to the use of this technique as a sensitive monitor of changes in their peripheral circulation.[12,14]

The cause of reduced peripheral blood flow in leprosy patients is uncertain, and regulating factors may include sympathetic vasoconstrictor activity, intrinsic vascular tone, circulating hormones and haemorheological factors. Histopathological evidence shows that the smaller blood vessels can be damaged[25–28] and that functional changes such as impaired blood flow in terminal vascular loops[13] or tortuosity of the arteries of the hands[26] occur. There is no evidence, however, of large vessel disease, such as atherosclerosis *per se*, in leprosy patients. The demonstration, in both Indian and Iranian leprosy patients,[14] that reduced flow often co-exists with local sensory loss suggests that nerve damage[11] is linked to chronic inflammation and functional deterioration of vasculature.

Vascular deterioration in leprosy may be due to continuing sub-chronic vasculitis, possibly associated with immune complex formation[29] during clearance of residual bacteria. It may also be due to persisting antibodies to stress proteins induced by the leprosy bacilli continuing to act as autoantibodies long after

the bacilli have gone. Such antibodies are known to be associated with vasculitis and the laying down of atheroma.[30,31] A significant reduction in the titre of antibodies to the 65 kDa hsp of BCG following immunotherapy with *M. vaccae* has been reported from our study group of patients.[32] Whatever the mechanism by which it acted, immunotherapy with *M. vaccae* has partly reversed the process or prevented further deterioration of blood flow in fully treated leprosy patients, an observation that may be applicable to many conditions other than leprosy.

Immunotherapy with *M. vaccae* has been investigated in a whole series of diseases in which dysregulation of T helper cell maturation has occurred. These include tuberculosis,[33–35] asthma,[36,37] atopic dermatitis,[38] psoriasis,[39] HIV infection[40] and a range of different cancers.[41–43] A very recent publication has shown that, in mice at least, an injection of heat-killed *M. vaccae* stimulates the production of regulatory T cells passageable between animals and capable of regulating specific allergen induced eosinophilia.[44] It is highly likely that many of the activities recorded for *M. vaccae* are related to the induction of these cells.

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