RESPIRATORY MEDICINE (1999) 93, 557–562

# Clinical and serological studies of tuberculosis patients in Argentina receiving immunotherapy with *Mycobacterium vaccae* (SRL 172)

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Two small, placebo-controlled studies of immunotherapy with heat killed *Mycobacterium vaccae* added to routine chemotherapy for pulmonary tuberculosis, together involving 40 HIV seronegative patients, were carried out in Argentina. The immunotherapy was associated with reduced sputum smear positivity of AFB at 1 month and a greater reduction in ESR at 2 months. In the first study radiological improvement was better ($P<0{\cdot}05$) among immunotherapy recipients. In the second study, weight regain and time to become apyrexial were measured and were found to be improved amongst immunotherapy recipients ($P<0{\cdot}05$).

In the first month of treatment the levels of IgG to the 65 kDa and 70 kDa heat-shock proteins showed greater falls following immunotherapy ($P<0{\cdot}05$ and $P<0{\cdot}001$, respectively). On admission serum cytokine levels of interleukins 4 and 10 (IL-4, IL-10), interferon gamma (IFN-$\gamma$) and tumour necrosis factor alpha (TNF-$\alpha$) were grossly raised in comparison with a matched control group ($P<0{\cdot}001$). After 1 month. Levels of IL-4, IL-10 and TNF-$\alpha$ fell ($P<0.001$, $P<0.01$ and $P<0.01$, respectively) and levels of IFN-$\gamma$ rose more ($P=0{\cdot}005$) in immunotherapy recipients than in those receiving chemotherapy alone. The results are in accord with a switch towards a TH1 immunological status and clinical benefit for immunotherapy recipients.

RESPIR. MED. (1999) 93, 557–562


## Introduction

Two small studies of immunotherapy for tuberculosis with *Mycobacterium vaccae* (SRL 172) have been carried out at Carrasco Hospital in Rosario, Argentina. Both were pilot studies in small numbers of volunteer patients, but they illustrate well the potential value of immunotherapy added to chemotherapy for the disease and have led to some new findings. Both studies were placebo-controlled, randomized and partially blinded. It is the practice at Carrasco Hospital to keep patients in the wards for the first 1–2 months of treatment and then to treat them on an outpatient basis. Patients have clinic appointments for the end of the second month and at intermediate times to 6 months when chemotherapy is stopped. Out of hospital, their chemotherapy is not directly observed, though this changed to a full DOTS programme after our study was completed. Those failing treatment, or subsequently relapsing, usually return to the same hospital which is the major one dealing with tuberculosis in the city. Thus most patients with recurring disease are discovered.

As well as clinical benefits, previous studies have shown an accelerated return to normal values of serum agalactosyl immunoglobulin (Gal [0]) following immunotherapy with *M. vaccae* in those in whom this marker of disease is initially raised (1). We have carried out a blinded study of the levels of IgG antibodies to the 65 kDa and 70 kDa heat shock proteins (hsp) of BCG, and of levels of tumour necrosis factor alpha (TNF-$\alpha$), gamma interferon (IFN-$\gamma$), interleukin 4 (IL-4) and interleukin 10 (IL-10) in the serum of patients at the start of treatment and 1 month later.

Received 23 February 1999 and accepted in revised form 21 April 1999.
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0954-6111/99/080557+06 $12·00/0

# Materials and Methods

## SUBJECTS

These were 40 newly diagnosed HIV seronegative patients without previous histories of tuberculosis. Thirty-seven patients had pulmonary disease. One with pleural effusion, and three had miliary tuberculosis. In both studies the patients in the two treatment groups had very similar disease based on the amount of lung involvement and their clinical condition. All had acid-fast bacilli (AFB) demonstrable in their sputum by culture with the exception of one patient with miliary tuberculosis in whom the diagnosis was confirmed by biopsy and culture. All patients had the studies explained to them and agreed to participate. Except for the single injection of intervention and the saving of sera from routine venipunctures from some patients for immunological studies, procedures were those in standard use in the hospital.

## ETHICAL APPROVAL

The studies were approved by the Ethical Committee of the Facultad de Ciencias Medicas, Universidad Nacional de Rosario (on 1 July 1991 and 2 March 1994, respectively).

## INTERVENTION

This consisted of a single intradermal injection of 0·1 ml of either immunotherapy or placebo given over the left deltoid muscle. The immunotherapeutic consisted of a suspension of heat-killed *M. vaccae* NCTC 11659 (SRL 172) batch A4 containing 10 mg wet-weight of bacilli per ml of M/15 borate-buffered saline at pH 8.0, equivalent to $10^9$ bacilli per dose; the placebo was tetanus toxoid in the first study and physiological saline in the second study. In the first study (2) the injections were given 15 days after starting chemotherapy and in the second study they were given on the first day of chemotherapy. Injections were given by a physician who played no further part in the treatment of the patients. For the first study, 20 patients were allocated to receive the intervention in the ratio of one placebo to two immunotherapy. For the second study, in which 20 patients received intervention on day 1 of chemotherapy, patients were paired according to the severity and nature of their disease and a sealed envelope was opened to determine which intervention should be given to the first of each pair presenting for treatment.

## CHEMOTHERAPY

Short-course chemotherapy was employed with daily isoniazid (H), rifampicin (R) and streptomycin (S) for 2 months, followed by 4 months of H and R alone. In the first study three of 13 patients given immunotherapy and one of seven given placebo and all patients in the second study received pyrazinamide (Z) in addition. In both studies the prescribed continuation phase was 4 months of H and R alone (2HRS[Z]/4HR). Therapy was directly observed during the time that patients were in hospital but not thereafter.

## ROUTINE INVESTIGATIONS

Symptomatic patients were admitted to hospital on production of a sputum sample with acid-fast bacilli visible on microscopy, or with a biopsy showing acid fast bacilli, and from which they could be cultured.

Susceptibility tests were carried out on all initial cultures to the four drugs used and to ethambutol. Subsequently sputum samples were examined by microscopy after 1 month and, if sputum could be produced, at outpatient visits. Blood samples were taken for routine heamatology and biochemistry at diagnosis and after 1 and 2 months. Laboratory staff did not know which patients had received which intervention, or even that the patients were in a study. Chest x-rays were routinely performed on admission, after 2 months and at the end of chemotherapy. Clinical observations of fever (37·5°C or more), cough, bodyweight etc. were recorded in the clinical notes, and data was extracted at the end of the studies. Recovery from pyrexia was followed by different parameters in the two studies. In the first study, time for pyrexia to resolve was measured and in the second study, numbers of patients showing resolution of fever at 2 weeks was the end-point.

## SPECIAL INVESTIGATIONS

Serum samples were saved from venipunctures performed for routine investigations in the second study and samples taken at these times were available from the first study. The samples were stored at −20°C and analysed together at the end of the study by an immunologist who did not know which intervention patients had received. Similarly, sets of x-rays were presented for analysis to an experienced chest physician blinded as to the intervention given.

## IMMUNOLOGICAL INVESTIGATIONS

Serum samples taken at diagnosis and after 30 days of treatment were tested by the enzyme-lined immunosorbent assay (ELISA) for IgG antibodies to the 65 kDa and 70 kDa hsp of BCG; results were recorded as absorption values. The same serum samples were tested for IFN-$\gamma$, TNF-$\alpha$, IL-4 and IL-10, by ELISA using commercial kits (R&D Systems Ltd, Abingdon, U.K.) and results were calculated to pg per $cm^3$ of serum. All samples were tested in duplicate at the same time, and following the manufacturers instructions. Twelve healthy people of the same age range and living in the same area but not known to be in contact with tuberculosis patients provided control sera.

## STATISTICAL ANALYSES

These were by student's *t*-test the paired *t*-test and Wilcoxon's test.

TABLE 1. Initial parameters of the patients entering the two studies

| | Immunomotherapy | | Placebo |
|---|---|---|---|
| Age (years) | 40·4±12·3 | | 39·4±16·7 |
| Smoke tobacco | 17/23 (74%) | | 13/17 (76%) |
| Drink alcohol | 13/23 (57%) | n.s. | 12/17 (71%) |
| AFB seen in sputum | 22/23 | | 15/17 |
| Number of bacilli | | | |
| | *0 + ++ +++ | | 0 + ++ +++ |
| | 2 11 6 3 | | 1 8 4 2 |
| Culture positive | 22/23 | | 17/17 |
| Colony count (2nd study only) | | | |
| | 0 <20 <100 100+ | | 0 <20 <100 100+ |
| | 1 4 3 2 | | – 4 5 1 |
| Radiological evidence of disease | | | |
| Bilateral | 14/23 (61%) | n.s. | 13/17 (76%) |
| Unilateral | 7/23 (30%) | n.s. | 2/17 (12%) |
| Miliary | 2/23 | | 1/17 (71%) |
| Pleural | 0 | | 1/17 |
| ESR (mm) | 80·8±34·0 | n.s. | 70·1±32·6 |
| Data available from first study only | | | |
| Haemoglobin ($g\ l^{-1}$) | 11·9±1·6 | n.s. | 11·4±1·3 |
| White cell count | 8577±2101 | n.s. | 8964±3100 |
| Data available from second study only | | | |
| Body weight (kg) | 51·2±5·8 | n.s. | 55·3±8·0 |

*For several patients the numbers of acid fast bacilli seen in positive smears were not recorded.
n.s. not significant.

## Results

The mean age of those receiving immunotherapy was 40·4±12·3 years and of those receiving placebo it was 39·0±16·7 years. In the first study, all subjects were men and in the second study three women received immunotherapy and one received placebo. The 30/40 patients who were smokers and the 25/40 who were alcohol drinkers were evenly distributed between intervention groups, as shown in Table. 1. The results of initial sputum bacteriology are also shown in Table 1. Strains from only two patients were recorded as showing drug resistance, one to streptomycin and the other to ethambutol. By chance, both of these came from the immunotherapy recipient group.

Radiology at entry showed that of those receiving immunotherapy, 14 had bilateral disease, Seven had unilateral disease and two had miliary tuberculosis. Of those receiving placebo, 13 had bilateral disease. Two had unilateral disease, one had miliary and one had pleural tuberculosis.

As shown in Table 2, after 1 month fewer patients receiving immunotherapy had AFB demonstrable in their sputum by smear ($P<0·025$) but by 2 months only a few patients provided samples and results were similar for both groups. Radiological improvement was best amongst immunotherapy recipients at two months, but numbers were too few for statistical significance. The ESR of 80·8 mm fell to 48·6 mm at 2 months in the *M. vaccae* group

TABLE 2. The results of sputum smear examination at entry and after 1 and 2 months of treatment, of x-ray improvement after 2 months and of erythrocyte sedimentation rate (ESR) at entry and after 2 months. Data have been pooled from both studies where this was compatable.

| | Immunotherapy | | Placebo |
|---|---|---|---|
| AFB visible in sputum | | | |
| Entry | 22/23 | | 15/17 |
| 1 month | 3/21 (14%) | $P<0.025$ | 8/16 (50%) |
| 2 months | 1/7 | | 2/8 |
| x-ray improvement by month 2 | | | |
| Very good | 8/19 | ($P=0·11$) | 2/13 |
| Good* | 10/19 | | 9/13 |
| Little of none | 1/19 | | 2/13 |
| ESR (mm) | | | |
| Entry | 80·8±34·0 | | 70·1±32·6 |
| | $P<0·001$ | | n.s. |
| 2 month | 48·6±35·8 | | 62·8±43·4 |

*For the radiological assessment, 'good' indicates that the patient's response is similar to that usually seen in similar patients receiving chemotherapy alone 'Very good' infers that the patient has done better than expected.
n.s. not significant.

($P<0{\cdot}001$), whereas that of the placebo group only fell from 70·1 to 62·8 mm (not significant).

Bodyweights were not recorded longitudinally in the first study, but the second study showed that weight increases (from entry to day 30) were significant on paired *t*-test and Wilcoxon test in *M. vaccae* recipients ($51{\cdot}2\pm5{\cdot}8$ vs. $54{\cdot}8\pm5{\cdot}9$: $P<0{\cdot}05$) but not in the placebo group ($55{\cdot}3\pm8$ vs. $57{\cdot}8\pm10{\cdot}4$).In the first study resolution of pyrexia took $19\pm14$ days for 11 immunotherapy recipients and $22\pm15$ days for six placebo recipients. In the second study two of 10 *M. vaccae* recipients and seven of 10 in the placebo group remained febrile after 2 weeks ($P<0{\cdot}04$; Fisher's exact test). In the first study, when patients were discharged from hospital according to their clinical status those receiving immunotherapy left after $45\pm20$ days. Whereas placebo recipients left after $60\pm15$ days ($P=0{\cdot}1$).

Table 3 shows the serological results. IgG to hsp 65 was marginally raised amongst the tuberculosis patients at the start of treatment and fell to normal in both groups by the end of 1 month. those receiving immunotherapy showing the greatest fall $P<0{\cdot}001$). The IgG to hsp 70 was markedly raised above that of controls at the beginning of treatment and fell almost to normal within 1 month in the *M. vaccae* group and only slightly amongst placebo recipients ($P<0.0001$).

All cytokine levels in the tuberculosis patients were markedly above those of the healthy controls at the start of treatment ($P<0.001$). within 1 month both treatment groups showed reductions in IL-4, IL-10 and TNF-$\alpha$, and an increase in IFN-$\gamma$, but in each case this was most marked amongst immunotherapy recipients ($P<0{\cdot}02$).

## Discussion

Although our studies are of small numbers, they are the first published results of immunotherapy for South American Patients. They include the first study of administration of *M. vaccae* on day 1 of chemotherapy and the first in which serum cytokines have been measured in relation to this.

TABLE 3. Results of ELISA absorption measurements. ± SE of IgG antibodies to heat shock proteins 65 kDa and 70 kDa and of the serum cytokines interleukin-4 (IL-4), interleukin-10 (IL-10), interferon gamma (IFN-$\gamma$) and tumour necosis factor alpha (TNF-$\alpha$) in pg/ml

| | Immunotherapy | | Placebo | Controls |
|---|---|---|---|---|
| hsp 65 k Da | $n=13$ | | $n=11$ | $n=12$ |
| On admission | $0{\cdot}30\pm0{\cdot}03$ | | $0{\cdot}23\pm0{\cdot}04$ | $0{\cdot}20\pm0{\cdot}04$[a] |
| | $P<0{\cdot}001$ | | $P<0{\cdot}05$ | |
| After 1 month | $0{\cdot}19\pm0{\cdot}02$ | | $0{\cdot}2\pm0{\cdot}02$ | |
| % decrease | $32\pm5{\cdot}5$ | $P<0{\cdot}05$ | $15{\cdot}6\pm5{\cdot}4$ | |
| hsp 70 k Da | | | | |
| On admission | $0{\cdot}59\pm0{\cdot}05$ | | $0.62\pm0.06$ | $0{\cdot}25\pm0{\cdot}06$[b] |
| | $P<0{\cdot}001$ | | n.s. | |
| After 1 month | $0{\cdot}31\pm0{\cdot}03$ | $P<0{\cdot}001$ | $0.53\pm0.06$ | |
| % decrease | $48\pm3{\cdot}6$ | $P<0{\cdot}0001$ | $17\pm2{\cdot}6$ | |
| IL-4 | | | | |
| On admission | $685\pm77$ | | $586\pm63$ | $69\pm9$[b] |
| After 1 month | $342\pm36$ | $P<0{\cdot}02$ | $495\pm58$ | |
| % decrease | $47\pm4{\cdot}7$ | $P<0{\cdot}001$ | $15\pm4{\cdot}9$ | |
| IL-10 | | | | |
| On admission | $3800\pm302$ | | $3863\pm270$ | $35\pm6$[b] |
| After 1 month | $2292\pm187$ | $P<0{\cdot}002$ | $3663\pm286$ | |
| % decrease | $38\pm5{\cdot}3$ | $P<0{\cdot}007$ | $16{\cdot}5\pm5{\cdot}8$ | |
| IFN-$\gamma$ | | | | |
| On admission | $524\pm76$ | | $553\pm57$ | $157\pm7$[b] |
| After 1 month | $1172\pm173$ | $P<0{\cdot}05$ | $700\pm99$ | |
| % increase | $124\pm21$ | $P<0{\cdot}005$ | $41\pm20$ | |
| TNF-$\alpha$ | | | | |
| On admission | $86\pm6$ | | $85{\cdot}5\pm3{\cdot}3$ | none |
| After 1 month | $52\pm5$ | $P<0{\cdot}001$ | $74\pm3{\cdot}7$ | detectable[b] |
| % decrease | $38\pm3{\cdot}6$ | $P<0{\cdot}01$ | $14\pm4{\cdot}1$ | |

[a]Different from immunotherapy group. $P<0{\cdot}02$

[b]Different from immunotherapy and placebo groups. $P<0{\cdot}001$

n.s., not significant.

With such small numbers of patients, few clinical differences between treatment groups could be expected to reach statistical significance. Nonetheless, the faster radiological improvement and discharge from hospital associated with immunotherapy in the first study, and the better weight regain and faster resolution of pyrexia in the second, do indicate patient benefit. Improved smear conversion at 1 month suggests a public health benefit and greater fall in ESR indicates faster recovery from disease in those receiving *M. vaccae*. These results concur with those reported from studies of this immunotherapy in Nigeria (3), Romania (4), Uganda (5) and Vietnam but differ from those of a larger study carried out in South Africa (6).

The serological results we describe are striking, with much greater changes occurring within the first month of treatment in the immunotherapy recipients than in those receiving placebo. The initially raised levels of IgG to the 70 kDa stress protein and marginal increase in IgG to the 65 kDa stress protein were particularly interesting, having been recorded in other diseases in which the immune system damages host tissues (7, 8, 9). It has long been known that a part of the pathology of tuberculosis is immunologically mediated. The rapid reduction in antibody to these stress proteins found to follow immunotherapy with *M. vaccae* accords with the better clinical and radiological response of these patients.

Overall, the serum cytokine data on immunotherapy recipients makes a composite picture of regulation of the maturation pathways of T-helper (TH) cells in a way that also occurs though at a slower pace, in the placebo group. In tuberculosis, the toxicity of circulating TNF-$\alpha$ is thought to be responsible for fever, weight loss, raised ESR and death. TNF-$\alpha$ does not have these effects except when raised levels of the type 2 cytokines, IL-4, IL-5, IL-10 etc., are also present (10). Regulation of production of these cytokines could be achieved by reducing the proportion of TH2 cells maturing from stem cells, or by regulating their capacity to produce and release cytokines. The speed with which such changes occur after immunotherapy with *M. vaccae* suggests that it is the latter. In patients with a raised proportion of $CD4^+$ T cells making IL-4, the first change in cytokine production found by flourescence-activated cytometry following a first injection of *M. vaccae*, is a reduction in this proportion within 2 weeks (II). This has been seen in patients with cancers (11) or with infections with opportunist mycobacteria (N. Thapa, personal communication). Down regulation of TH2 would be accompanied by a reduction in T-cell dependant antibody production but the rapid reduction in IgG to the stress proteins found in *M. vaccae* recipients suggests that such antibodies are being used up in the pathological lesions. Regulation of T-helper cell maturation is demonstrable in experimentally infected animals receiving injections of *M. vaccae* (12, 13).

There are doubts about the levels of IL-4 circulating in tuberculosis but this may be due to different severities of disease (14) and it is possible that the technique is more a measure or rheumatoid factor than of IL-4. It is unfortunate that no serum remains for rheumatoid factor to be measured. Whatever the test measures, it represents something changing faster in immunotherapy recipients than in those receiving placebo and is accompanied by a fall in IL-10, also indicating reduced TH2 activity.

The lesser changes in stress protein antibody, TNF-$\alpha$ and IL-4 levels in placebo recipients are an effect of the antituberculosis chemotherapy, most probably due to the rapid killing of bacilli. Live tubercle bacilli have been shown to produce a factor (TEA) increasing the activity of TNF-$\alpha$ which antagonises ACTH (15). This would modify the ratio of cortisone to dehydroepiandrosterone (DHEA) produced in the adrenals and secondarily regulate T-helper cell maturation (16). Additional control mechanisms could be through lesional macrophages changing local production of DHEA sulphatase and/or of enzymes regulating the cortisone-cortisol shuttle (17). There may also be direct effects of drugs such as rifampicin on macrophage function (18).

The enhancement of TH1 in those receiving injections of *M. vaccae* appears to be a separate and slightly later effect, possibly reflecting the time required for cell maturation (11). *M. vaccae* is a potent stimulus for IL-12 production and enhanced TH1 maturation may be the response to this (19). (Tight control of the manufacturing process and various biochemical and immunological tests ensure that SRL 172 is produced consistently.) Injection of *M. vaccae* might also be expected to be a stimulus for the production of $CD8^+$ cytotoxic T cells and this, as well as many other changes following injection of *M. vaccae*, have been shown in experimental animal models (12, 13, 19).

Faster sputum smear convertion in immunotherapy recipients may be associated with increased IFN-$\gamma$ production which, together with the greater reduction in IgG levels to the stress proteins, should enhance granuloma formation without tissue necrosis. Live tubercle bacilli are released into the airways to appear in the sputum as a result of the necrosis and liquefaction of lesions. A change in granuloma formation would change this and should lead to better intracellular killing of bacilli. Reduced numbers of bacilli in the sputum means that fewer are swallowed. Thus, some of the effects observed could be related to changing patterns of immune modulation by the gut-associated lymphoid tissues.

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