NERVE GROWTH FACTOR (NGF) CONCENTRATIONS IN CULTURED HUMAN KERATINOCYTES EXPOSED TO *MYCOBACTERIUM LEPRAE* CELL FREE EXTRACT

Editor,

The classical neutrotrophic factor, nerve growth factor (NGF), is a small protein normally produced by cells in the target organ, such as skin; NGF is taken up by sympathetic and small sensory nerve fibres via a high-affinity receptor (trkA), and retrogradely transported to the cell body. In adults, NGF is necessary for the survival of sympathetic fibres, and for phenotypic properties of small sensory fibres, including expression of neuropeptide substance P, and their response to noxious stimuli.[1] Our recent studies showed decreased NGF staining in keratinocytes in leprosy skin patches, as well as mirror-image clinically unaffected skin in the same patients: we postulated that a sub-clinical decrease on NGF immunostaining may explain lack of pain in this condition.[2] However, the mechanism of the decrease of NGF immunostaining is unknown.

In vitro studies of NGF production may help in understanding the cellular and molecular mechanisms involved in changes observed in the clinical studies. A number of potential agents, including cytokines and growth factors, have been shown to affect NGF expression in different cultured cells,[1,3] but it is not known whether these mechanisms operate in leprosy skin. Although infection of human keratinocytes has not been observed, it may be possible for *Mycobacterium leprae* and substances derived from them to interact with keratinocytes to affect NGF production. Despite multidrug therapy, *M. leprae*-derived antigens persist in skin,[4] and keratinocytes may be therefore exposed to them for long periods. In the present study, we have challenged normal human keratinocytes in culture with *M. leprae* cell free extracts, and measured keratinocyte NGF concentrations using a specific NGF immunoassay, and immunocytochemistry.

Keratinocytes were obtained with permission from patients ($n = 3$) undergoing surgical resection of normal skin, and grown at a minimum starting density of $1{\cdot}25 \times 10^4$ per well on a mouse fibroblast 3T3 (gamma irradiated) feeder cell layer in keratinocyte medium for 5 days until established. To eliminate any possible influence of medium-soluble molecules, the cells were transferred to serum and calcium-free medium for a further 3 days. Cells were cultured, in duplicate, for a further 24 or 48 h with or without *M. leprae* cell free extract (kindly provided by Dr M. J. Colston, National Institute for Medical Research, London) at a concentration of 10 μg/ml. Cells from at least two wells per culture were detached with trypsin, washed in medium, centrifuged and re-suspended in 1 ml phosphate-buffered saline (PBS). A 900 μl aliquot was taken for NGF assay and the remainder for protein assay. Both of these samples were centrifuged and supernatant removed. The samples for immunoassay were then re-suspended in NGF extraction buffer (100 mM Tris-HCl, 0·2 M NaCl, 2% BSA, 0·05% sodium azide, 1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride, 4 mM ethylenediaminetetracetic acid, 7 μg/ml bovine aprotinin, pH 7·0) and stored frozen at −80°C. NGF immunoreactivity in extracts of keratinocytes was measured in duplicate by fluorometric, enzyme-linked immunoabsorbant assay (ELISA) using recombinant human NGF as standard and biotinylated anti-NGF and a streptavidin-β galactosidase detection system (Genentech, Inc., USA), as previously described.[3] Protein concentrations were determined colorimetrically with a commercially available kit (Bio-Rad Labs, UK). NGF concentrations in keratinocyte extracts are shown in Figure 1. There was no significant difference (Student's t-test, $p > 0{\cdot}05$) of NGF content for keratinocytes cultured with or without *M. leprae* extracts at either 24 or 48 h of exposure. NGF levels for 3T3 fibroblast control cultures were below the detection limit (5 pg/ml) of the assay at all times, with or without *M. leprae* extracts.

Replicate cultures on glass slide wells were frozen for NGF immunostaining. The slides were

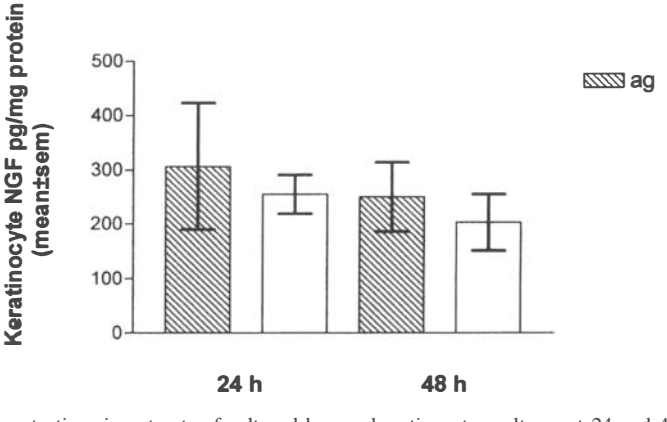

Figure 1. NGF concentrations in extracts of cultured human keratinocytes cultures at 24 and 48 h. The effect of *M. leprae* cell free extract ('ag') at a concentration of 10 μg/ml is shown in shaded columns, while the clear columns show NGF concentrations without addition of *M. leprae* cell-free extract.

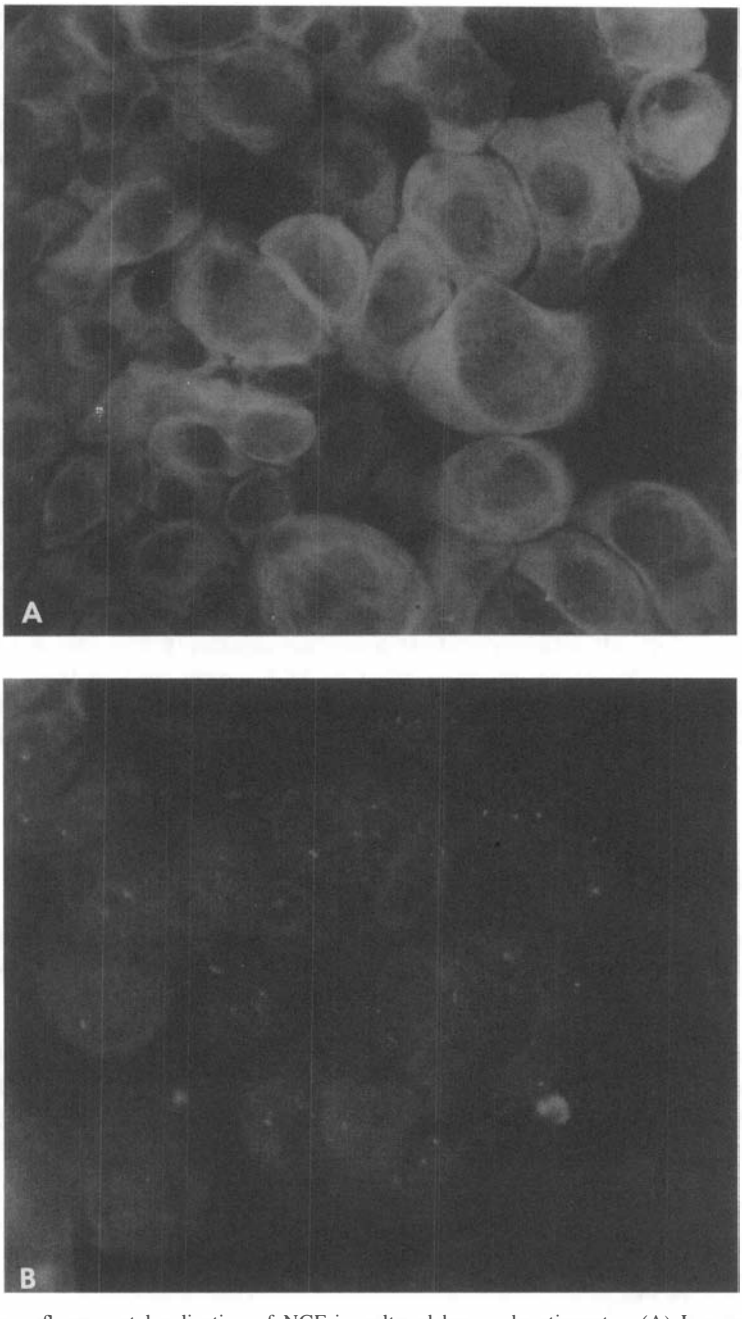

Figure 2. Immunofluorescent localization of NGF in cultured human keratinocytes. (A) Immunopositive cells incubated with anti-NGF; (B) negative control cells incubated with non-immune serum. ×300 magnification.

immersed in 0·4% w/v paraformaldehyde in PBS for 30 min at room temperature, rinsed in PBS then de-ionized water, and air dried for 2–3 h. Cells were then soaked in 0·2% v/v Triton X-100/PBS for 30 min. After washing in PBS, cells were dehydrated and re-hydrated through graded (70–100%) ethanol and returned to PBS. Cells were then incubated overnight with either normal non-immune rabbit serum (1:30 in PBS; negative control) or rabbit antibodies to rhNGF at 1·6 μg/ml. To detect antibody-binding sites, the slides were washed in PBS and incubated with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) for 60 min. After a further wash, slides were mounted in glycerine-PBS including anti-fade reagent (Vector Labs, UK), assessed by an independent observer without knowledge of the identity of each slide, and photographed using epi-fluorescence. Specificity of immunoreaction was determined by pre-incubation of rhNGF antibodies with rhNHF antigen. There was substantial NGF-like immunoreactivity seen throughout the cytoplasm in the majority of keratinocytes (Figure 2A). However, there was no detectable difference of NGF immunofluorescence intensity at 24 or 48 h of incubation with or without *M. leprae* extracts. Control cultures, in which primary antibodies were replaced with non-immune serum or pre-incubated with homologous rhNGF, showed little or no immunofluorescence, and only a few scattered granular deposits which were non-specific (Figure 2B). Fibroblast feeder cell (3T3) cultures showed no immunostaining with anti-rhNGF antibody.

The present study is the first to demonstrate NGF immunoreactivity cytochemically in cultured human keratinocytes, and is in accord with earlier work which described keratinocytes as a major source of NGF in skin.[1,2] The intensity of NGF immunofluorescence appeared not to change with exposure to *M. leprae* cell-free extract. The measurement of NGF in keratinocyte extracts by immunoassay showed only a slight decrease of NGF content with time, and no significant effect of exposure to *M. leprae* cell free extract. The decreasing NGF concenrations with duration of culture was an expected trend, reflecting the positive relationship of NGF production with rate of proliferation of keratinocytes.

The dose of *M. leprae* cell free extract we used has been shown to be critical for effects on the proliferation of lymphocytes in a recent study.[5] Further studies with a range of antigen concentrations, and with *M. leprae* cell wall antigen or whole *M. leprae*, are necessary. It is possible that *M. leprae* cell free extract, although having no detectable effect on NGF protein concentrations, may influence turnover or secretion of NGF. It is also possible that conditions *in vitro* do not reflect the conditions *in vivo*. In addition, it would be of interest to study the effect of sera from leprosy patients, and cytokines known to be involved in leprosy skin, on NGF concentrations in cultured keratinocytes.

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Academic Department of Neurology,
St Bartholomew's and
The Royal London School of Medicine and Dentistry,
Whitechapel,
London E1 1BB, UK
**Blond McIndoe Research Centre,*
Queen Victoria Hospital,
East Grinstead, Sussex, UK
Fax: +44 171 377 7286
e-mail: P.Anand@mds.qmw.ac.uk

P. FACER
D. MANN*
G. TERENGHI*
P. ANAND

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