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Tumour necrosis factor-alpha (TNF-α) synthesis is associated with the skin and peripheral nerve pathology of leprosy reversal reactions

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SUMMARY

Leprosy may be complicated by episodes of increased cell-mediated immunity towards *Mycobacterium leprae* (reversal reactions) which result in severe local immunopathology in skin lesions and peripheral nerves. Using *in situ* hybridization and MoAb techniques we have demonstrated TNF-α mRNA and TNF-α protein in macrophages infiltrating leprosy skin and peripheral nerve. Levels of TNF-α mRNA are significantly increased in reactional skin and nerve, particularly in borderline tuberculoid patients. TNF-α mRNA and TNF-α protein levels are higher in reactional nerves then reactional skin. In both reactional skin and nerve TNF-α mRNA is more abundant than TNF-α protein; this may reflect the rapid turnover of TNF-α protein in an immunologically dynamic situation, such as is seen in reversal reaction. Our findings emphasize the importance of documenting both mRNA and protein production when assessing the role of cytokines in pathology. The leprosy reversal reaction may be regarded as a useful model of tissue immunopathology in which TNF-α is generated as part of the host response to infection, but also produces local tissue damage.

Keywords leprosy reversal reaction TNF *in situ* hybridization nerve damage

INTRODUCTION

Leprosy is one of the commonest causes of peripheral nerve damage world-wide, yet little is known about cellular events that are associated with acute nerve damage. The disease is caused by *Mycobacterium leprae*, which has a predilection for skin macrophages and Schwann cells. Localization of *Myco. leprae* organisms at these sites leads to a striking chronic granulomatous reaction, and the resultant tissue immunopathology manifests clinically in the form of skin lesions and peripheral neuropathy, but the cellular mechanisms of the nerve lesion have not been examined previously.

The clinical and histological presentation of leprosy is determined by the host response to *Myco. leprae* and the spectrum of responses was described by Ridley & Jopling [1]. At the lepromatous (LL) pole there is no effective cell-mediated immunity to *Myco. leprae* and bacilli proliferate, producing large bacillary loads. At the tuberculoid (TT) pole there is cell-mediated immunity towards *Myco. leprae* and elimination of mycobacteria with the formation of granulomata [2]. Between the two poles are borderline patients who are immunologically unstable. At least 15% of borderline patients experience episodes of immunological hypersensitivity, called reversal (Type 1) reactions [3] which are the consequence of increased cell-mediated immunity towards *Myco. leprae* resulting in the elimination of bacilli [4] and an upgrading of the patient towards the tuberculoid end of the spectrum. This upgrading and destruction of leprosy bacilli is only achieved at the expense of local tissue damage [5], which is expressed as acute inflammation of pre-existing skin lesions and acute neuritis. The neuritis is often rapid and severe, resulting in the permanent nerve damage which leaves the patient at risk of developing deformities.

The relationship between immunopathology and antibacterial immunity is poorly understood, but is fundamental to our understanding of how immune responses to pathogens are regulated and for devising intervention strategies for immunologically mediated tissue damage resulting from increased antibacterial immunity. Leprosy reversal reactions provide an ideal opportunity for investigating this relationship. TNF-α in particular is implicated in the pathogenesis and natural history of mycobacterial disease [6]. TNF-α is regarded as crucial for

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granuloma formation [7], thus contributing to protective immunity. However, TNF-α has also been proposed as an important mediator in local tissue damage [8].

In this study we have investigated the role of TNF-α in mediating tissue damage in nerve and skin of leprosy patients undergoing such reversal reactions. Our results support the notion that TNF-α may play a key role in the nerve lesion as well as in the skin pathology, and suggest that detection of local TNF-α mRNA synthesis as well as TNF-α protein may be a useful indicator of tissue immunopathology.

PATIENTS AND METHODS

Tissue samples

Skin and nerve biopsies were taken from patients in reversal reaction attending Dhoolpet Research Centre. A reversal reaction was diagnosed when a patient presented with reactional skin changes (erythema and/or oedema of existing lesions, new skin lesions that were not relapsing leprosy or Erythema Nodosum Leprosum) or acute neuritis (peripheral nerve tenderness, new sensory symptoms or signs or new motor symptoms or signs). Patients were graded clinically and histologically on the leprosy spectrum according to the Ridley–Jopling classification [1] as TT, borderline tuberculoid (BT), borderline lepramotous (BL) and LL. All reversal reactions were confirmed histologically. Partial thickness nerve biopsies were taken from the radial cutaneous nerve at the wrist. Skin biopsies were taken from the active edge of lesions. Additional skin biopsies included in this study were of new, non-reactional, untreated leprosy patients as well as normal skin. Normal control nerves were obtained from fresh post-mortems (> 12 h) in the UK. Nerves from non-reactional patients were not available since there was no clinical indication for a nerve biopsy in these patients. Cryostat sections (6 μm) were stained with haematoxylin and eosin (H&E) for general morphological examination and a modified Fite–Faraco procedure for staining *Myco. leprae*. The bacterial load in each site was counted and expressed on a logarithmic scale as the Bacillary Index (BI) [9].

Immunocytochemistry

Cryostat sections (6 μm) were fixed in acetone and immunostained with antibodies against macrophages (CD68) (EBM11, MoaHu, 1/50) (Dakopatts, Glostrup, Denmark), S100 protein (RbaCw, 1/500) (Dako) and TNF-α protein (MON 5006, 1/20, equivalent to 12 μg/ml) (Bradsure Biological Ltd, Leicester, UK) [10]. EBM11 was detected with a horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum (BioRad, Richmond, CA) at a dilution of 1:200. RbaCw and MON 5006 were detected using a biotinylated rabbit anti-mouse antiserum (Dako) at a dilution of 1:300 followed by streptavidin–biotin complex/HRP (Dako) for 20 min. Positive staining was visualized for both antibodies using 3,3 diaminobenzidine (Sigma, Poole, UK)/H_2O_2. Sections were counterstained with haematoxylin and mounted in a resinous mountant. Controls for the specificity of staining included using normal serum, omitting the primary antibody, and using a similar isotype antibody with different specificity (anti-*Schistosoma mansoni*). Cells from a clonal carcinoma known to produce TNF-α protein were used as a control for positive staining. Blocking of TNF-α protein staining was not attempted.

Preparation of probe

A 738-bp EcoRI fragment of human TNF-α cDNA clone 142-4, corresponding to nucleotides 337–1070 of TNF-α mRNA, was subcloned into the EcoRI site of pBluescript SK+ (Stratagene, San Diego, CA). An antisense strand RNA probe for TNF-α transcripts was synthesized from Not I-linearized plasmid, using T7 RNA polymerase. A control sense strand RNA probe was synthesized from Xho I-linearized plasmid, using T3 RNA polymerase. Both probes were labelled with digoxigenin (Boehringer Mannheim, Lewis, UK). Probes were reduced in length to approximately 200 bases by limited alkaline hydrolysis in 50 μl hydrolysis buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3 pH 10·2, 10 mM DTT) at 60°C for 20 min. After precipitation the probes were resuspended in 50 μl of diethylpyrocarbonate-treated water containing 1 μl RNAse inhibitor (500 μg/ml) and stored at −20°C.

In situ *hybridization*

Cryostat sections (6 μm) were cut on to Vectabond-coated slides (Vector Labs, Peterborough, UK), air-dried and fixed in fresh 4% (w/v) paraformaldehyde in PBS. Sections were washed in PBS, digested with proteinase K (Boehringer Mannheim) (6 μg/ml in 100 mM Tris/HCl pH 8·0, 1 mM $CaCl_2$) for 15 min at 37°C, washed in PBS and post-fixed in 4% (w/v) paraformaldehyde. Before hybridization, sections were rewashed in PBS, dehydrated through an ascending ethanol series and air dried. Sections were prehybridized for 30 min with 150 μl of hybridization solution (4 × SSC, 50% (w/v) formamide, 1 × Denhardt's solution, 5% (w/v) dextran sulphate, 0·5 mg/ml salmon sperm DNA and 0·25 mg/ml yeast tRNA). This was drained and replaced with an equal volume of hybridization solution containing 35 ng digoxigenin-labelled probe. Sections were covered with siliconized coverslips (Sigmacote) and incubated at 55°C overnight in a humid chamber lined with paper soaked in 2 × SSC/50% (w/v) formamide. After hybridization, coverslips were removed by immersion in 2 × SSC at 45°C. Slides were then washed twice in 2 × SSC, once in 0·2 × SSC and twice in 0·1 × SSC, each for 15 min at 42°C. Control sections were included in each batch of hybridizations. Sections were washed for 5 min in buffer A (100 mM Tris/HCl pH 7·5, 150 mM NaCl) and placed in blocking solution (buffer A containing 0·3% (w/v) Triton X-100, 2% (w/v) normal sheep serum) for 30 min. The sections were then incubated for 2 h at room temperature with alkaline phosphatase-conjugated antibody (1:500) in buffer A containing 0·3% (w/v) Triton X-100, 1% (w/v) normal sheep serum, washed twice for 15 min in buffer A and once in buffer B (100 mM Tris–HCl pH 9·5, 100 mM NaCl, 50 mM $MgCl_2$) at room temperature, and placed in freshly prepared development solution (220 μl of 75 mg/ml nitroblue tetrazoleum chloride (NBT), 165 μl of 50 mg/ml 5-bromo-4-chloro-3-indoylphosphate (X-phosphate), 1 mM levamisole in 50 ml buffer B). Slides were left in this solution, in the dark, and checked for the development of a blue colour reaction by light microscopy. After 5 h the reaction was terminated by immersing the slides in 100 mM Tris–HCl (pH 8·0)/2 mM EDTA. Sections were counterstained with 2% (w/v) nuclear fast red and mounted in a resinous mountant (Eukitt).

Statistical analysis

Slides were evaluated by two independent observers who counted the number of positive cells in each granuloma. The

percentage of positive cells (TNF-α mRNA, TNF-α protein-producing cells or macrophages) were counted by microscopic evaluation of several fields corresponding to > 1000 cells. Statistical analysis was done using the Mann–Whitney test on the Minitab statistical package.

RESULTS

TNF-α mRNA expression in lesions

Skin and nerve biopsies were obtained from 21 patients. Fourteen of these (seven BT, one borderline (BB) and six BL) were in reversal reaction and seven were skin biopsies from untreated, non-reactional patients (four BT, three BL). Concurrent skin and nerve pairs were available for 11 of these patients. The cellular localization of TNF-α mRNA was determined by *in situ* hybridization using a digoxigenin-labelled antisense probe (Figs 1, 2 and 3). TNF-α mRNA production was visible as darkly staining areas within the cytoplasm of positive cells. In both BT skin (Fig. 1b) and nerve (Fig. 2a,b) intense staining was observed and co-localized with areas of granuloma formation. Staining of BL skin and nerve (Fig. 3a,b) was less intense, but again TNF-α mRNA was seen primarily in areas of granulomata. No staining was seen in any cases when probed with the control sense construct (Fig. 1a). The number of cells containing detectable TNF-α mRNA and protein were increased in both BT and BL leprosy reactional skin compared with non-reactional skin ($P = 0{\cdot}03$). Reactional BT patients had a significantly higher number of cells containing TNF-α mRNA in their skin lesions than their non-reactional counterparts ($P = 0{\cdot}01$). Reactional BL patients also had more cells containing TNF-α mRNA in their skin lesions than non-reactional BL patients, but this difference did not reach statistical significance. Significantly more TNF-α mRNA-positive cells ($P = 0{\cdot}03$) were present in BT than BL reactional patients.

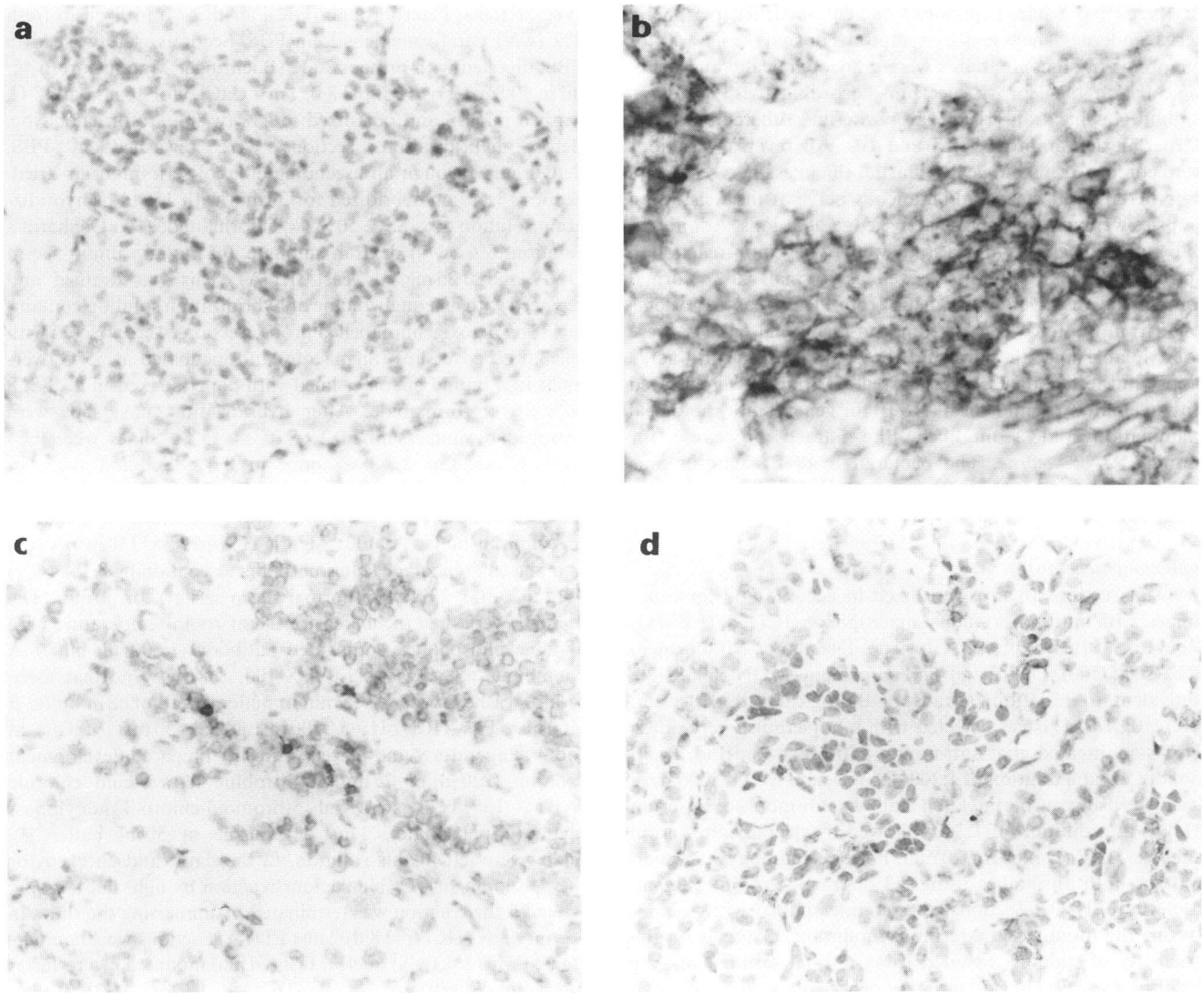

Fig. 1. Detection of TNF-α mRNA and protein staining in leprosy skin lesions (BT in reversal reaction). (a) Negative control using TNF-α sense probe showing absence of signal. (b) TNF-α mRNA-positive cells within the tuberculoid granuloma of skin. (c) Immunocytochemical staining of skin lesion showing fewer cells positive for TNF-α protein. (d) A section of the same lesion stained with the monoclonal antibody EBM11 (macrophage marker) indicating large number of positive cells (× 345).

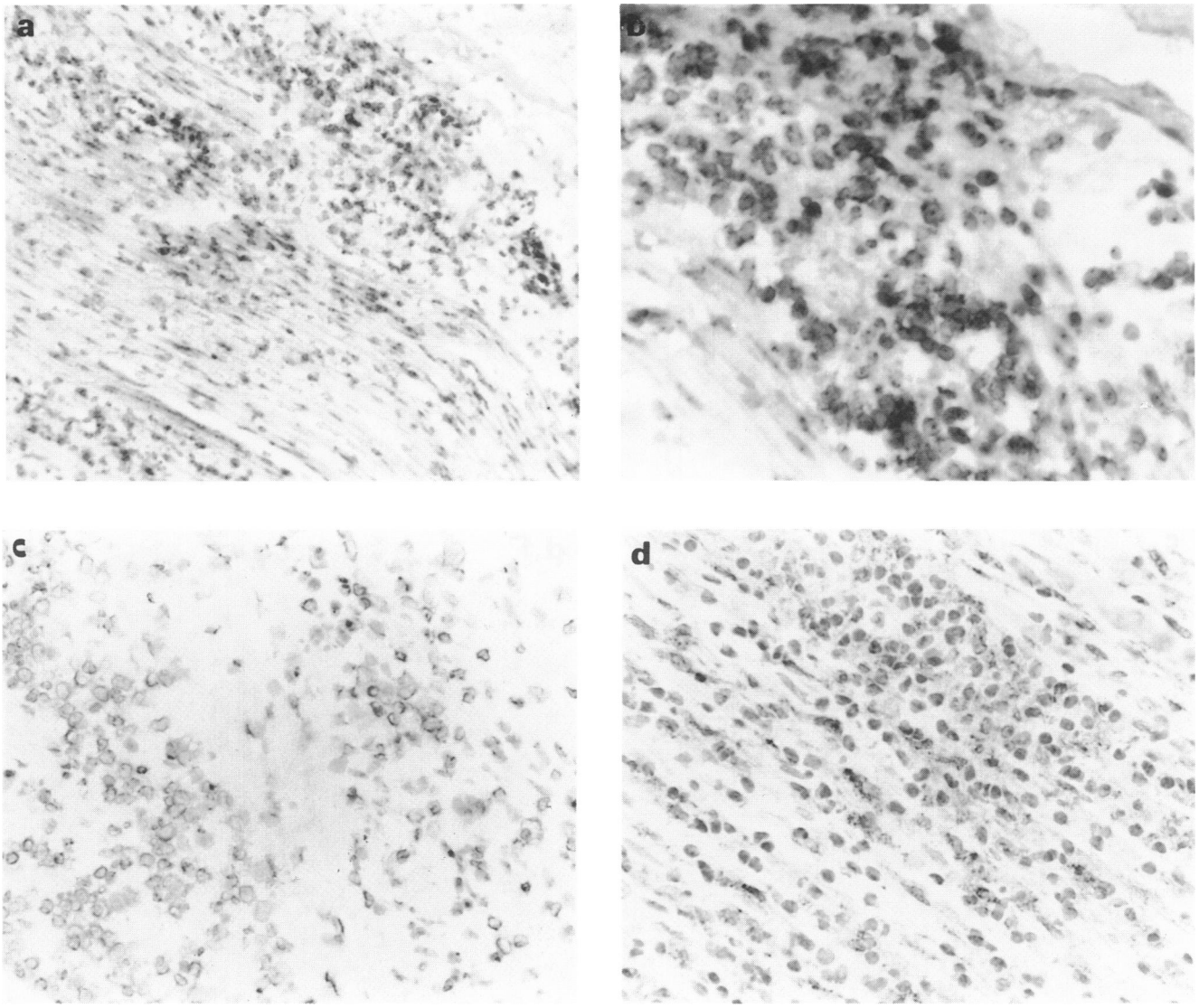

Fig. 2. *In situ* hybridization was performed on cryostat sections of reactional borderline tuberculoid nerve using TNF-α riboprobe. (a) Positive cells expressing TNF-α mRNA seen within nerve lesion (×250). (b) High power field of (a) (×400). Serial cryostat sections immunostained for TNF-α protein (c) and macrophages (d) (×345).

Immunocytochemical analysis

In order to investigate the relationship between TNF-α mRNA expression and protein expression in lesions, we compared the number of cells with mRNA detected by *in situ* hybridization and the number of cells with protein detected by immunocytochemistry. Localization of TNF-α protein (Figs 1c, 2c and 3c) showed a similar distribution to TNF-α mRNA, but fewer cells were positive for protein than were positive for mRNA. The numbers of cells in skin expressing TNF-α protein were higher in reactional patients than non-reactional patients, and highest in reactional BT patients (Table 1), but in contrast with the numbers of cells containing TNF-α mRNA the differences were not statistically significant. There were proportionately more cells with TNF-α mRNA and protein in reactional nerve lesions than in reactional skin lesions (Fig. 4). Furthermore, BT patients had higher numbers of cells containing both TNF-α mRNA and protein in their reactional nerves than BL patients, the percentages of positive cells for mRNA and protein for BT and BL patients respectively being 3·39% compared with 1·36% (mRNA) and 0·61% compared with 0·33% (protein). Staining for CD68 as a marker of macrophages identified a large number of positive cells in both skin and nerve biopsies (Fig. 1d, 2d and 3d), although not $CD68^+$ cells stained for TNF-α mRNA or protein. Demonstration of Schwann cells by S100 protein staining did not correlate with cells responsible for TNF-α mRNA and protein production.

DISCUSSION

In this study we demonstrated that TNF-α mRNA and TNF-α protein are significantly increased in leprosy skin and nerve during reversal reactions. Reactional BT patients have higher amounts of both TNF-α message and protein in their skin and nerve than their reactional BL counterparts. This is probably in part due to the greater degree of macrophage activation seen at

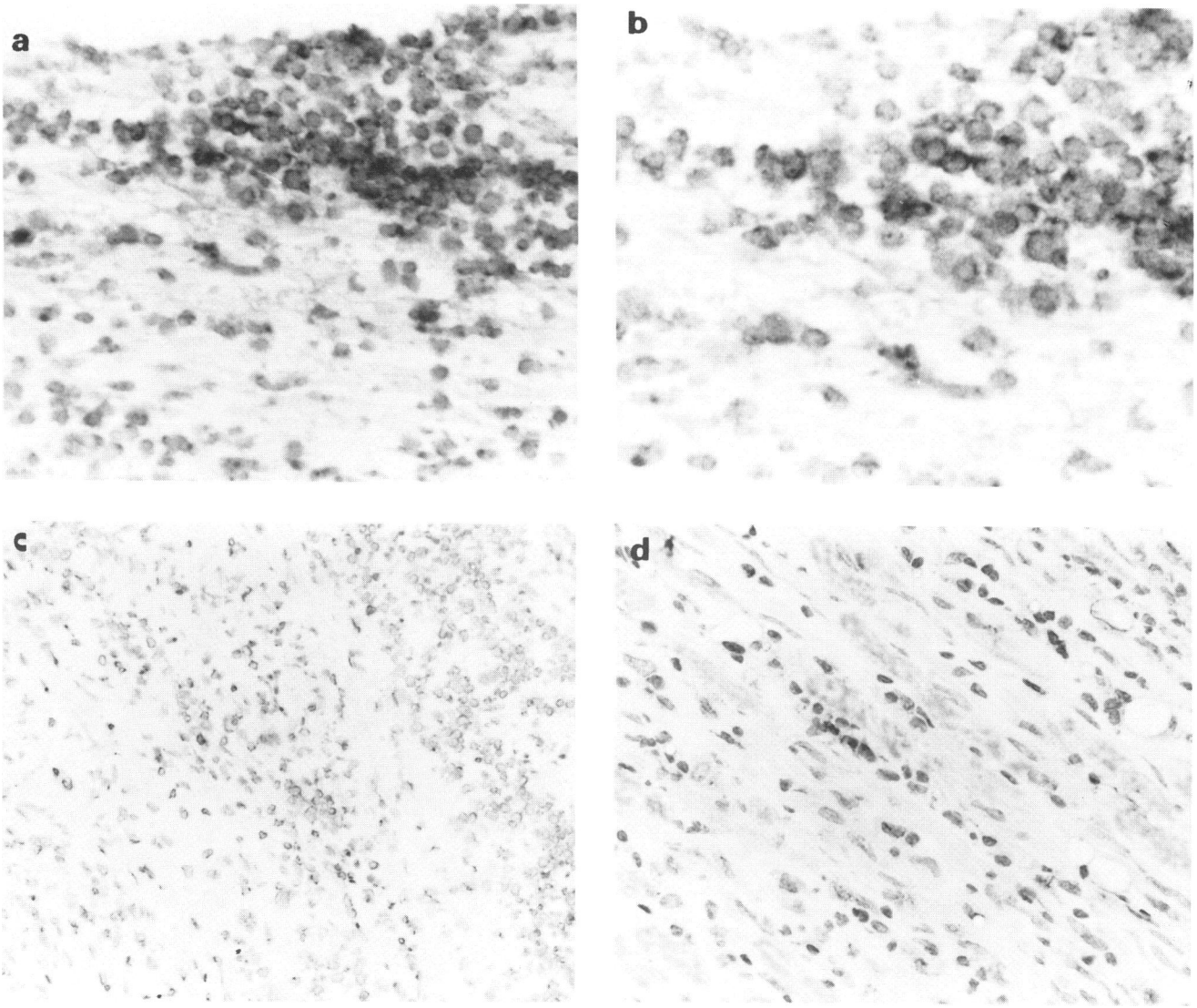

Fig. 3. Representative example of reactional lepromatous nerve lesions showing differential *in situ* expression of TNF-α mRNA and protein. (a) *In situ* hybridization with antisense TNF-α riboprobe demonstrating few positive cells within the granuloma ($\times$ 345). (b) High power image of (a) ($\times$ 550). Immunocytochemical detection of TNF-α protein (c) and macrophage marker (d) ($\times$ 345).

the tuberculoid end of the leprosy spectrum. BL patients, with low basal level production of cytokines, are probably slower to initiate cytokine production than BT patients when they go into reaction. Other workers have shown that there are both qualitative and quantitative differences in cytokine production between tuberculoid and lepromatous patients. Arnoldi *et al.* [11] using antibodies in an immunocytochemical technique showed that IL-1, TNF-α and interferon-gamma (IFN-γ) were present in tuberculoid lesions but absent in lepromatous lesions. A similar tuberculoid/lepromatous dichotomy was found for TNF gene expression in skin biopsies by Yamamura *et al.* [12]. Yamamura *et al.* [13] using a polymerase chain reaction (PCR) based method showed that mRNAs for cytokines associated with a Th1 type response such as IL-2 and IFN-γ were abundant in tuberculoid lesions, whereas mRNA for Th2-associated cytokines such as IL-4, IL-6 and IL-10 predominated in lepromatous lesions. During reversal reactions patients are moving towards the tuberculoid end of the spectrum, so one would predict a dynamic switch to be occurring, with reactional patients changing to produce Th1 type and macrophage cytokines in their lesions. Beissert *et al.* [14] demonstrated an increase in mRNA for IL-1β, TNF-α, IL-2 and IFN-γ during reversal reaction.

It was striking that in all cases the number of cells positive for TNF-α mRNA was higher than the number positive for TNF-α protein. Such low levels of staining are consistent with similar findings reported in renal and ovarian carcinomas [15,16]. These low levels of TNF-α protein detectable only with relatively concentrated antibody, support the notion that our findings are unlikely to be due to decreased detection by the anti-TNF-α antibody compared with *in situ* hybridization. It is possible that macrophages in these pathological sites are only partially responsive to the signals required for TNF-α mRNA translation, or do not permit TNF-α cleavage from its membrane-bound form. Since similar numbers of macrophages are present in the reactional and non-reactional samples, it is clear

Table 1. *In situ* production of TNF-α in leprosy lesions

Diagnosis	Type of tissue	No. of patients	Bacillary index	Percentage of positive cells (± s.d.)*		
				TNF mRNA	TNF protein	Macrophages (CD68)
BT	Skin	4	0–1+	0·55 (±0·36)	0·17 (±0·12)	60·1 (±6·4)
BL	Skin	3	3+	0·43 (±0·40)	0·10 (±0·17)	57·3 (±6·0)
BT in RR	Skin	7	0	2·60 (±1·80)	0·38 (±0·27)	55·1 (±15·0)
	Nerve	7	0–1+	3·39 (±2·77)	0·61 (±0·37)	59·5 (±12·37)
BL in RR	Skin	6	1–3+	0·78 (±0·59)	0·22 (±0·29)	64·0 (±12·21)
	Nerve	3	2–4+	1·36 (±1·40)	0·33 (±0·35)	52·6 (±6·8)

* Percentage of positive cells was estimated by microscopic evaluation of several fields corresponding to > 1000 cells.
BT, Borderline tuberculoid leprosy; BL, borderline lepromatous leprosy; RR, reversal reaction.

that macrophage activation has a role in the development of the local lesions, but it is possible that the precise nature of the activation signal may determine different cytokine outcomes. The number of cells synthesizing TNF-α (and hence, presumably active TNF-α levels) may represent an important index of local tissue pathology. Alternatively, TNF-α protein may be rapidly degraded in the very active milieu of reactional pathology. Irrespective of which of these explanations is correct, it is clear that the difference in detection rates between TNF-α mRNA and protein highlights the importance of using both *in situ* and antibody detection methods if one is to gain insight into the patterns of cytokine expression in this dynamic pathology.

Local tissue damage is one of the hallmarks of reversal reactions. This is probably caused in part by TNF-α, which has tissue-damaging effects such as stimulating the production of reactive oxygen intermediate metabolites, stimulating collagenase production, activating fibroblasts and promoting production of adhesion molecules [17]. More pertinently in this context of peripheral nerve damage are the observations that TNF produces demyelination of cultured spinal cord tissue [18] and is a potent inducer of inflammation when injected into rat spinal cord. In the latter experiments TNF and IFN-γ had additive effects on the induction of inflammation when injected together [19]. Local TNF-α synthesis has not been demonstrated previously in any form of peripheral neuropathy. The known actions of TNF-α in other sites suggest that this cytokine could well be important in any neuropathy where there is focal, localized nerve damage. Local TNF synthesis may produce damage not only through its proinflammatory and demyelinating effect but also by its local actions, since the tight structure of the nerve fascicle does not permit the expansion that accompanies the secretion of biologically active molecules, and nerve compression rapidly ensues. There was good correlation of maximal TNF-α mRNA synthesis in skin and nerve with the clinical findings of acute inflammation and neuritis during reversal reactions.

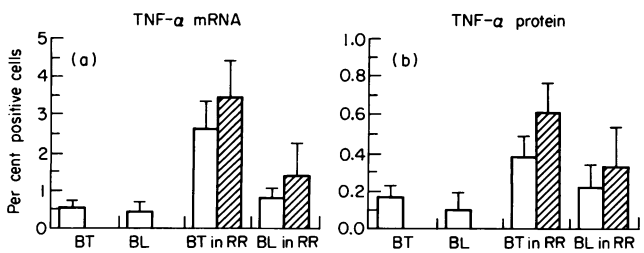

Fig. 4. Cytokine TNF-α mRNA in leprosy lesions as determined by *in situ* hybridization (a) and immunocytochemistry (b). The results show the percentage of positive cells in skin (□) and nerve lesions (▨) of reactional and non-reactional leprosy biopsies. Bars represent mean value ± s.e.m.

Kindler *et al.* [7], using antibodies to TNF, has demonstrated the critical role of TNF-α in the development, maintenance and mycobactericidal functions of granuloma formation. Previous work, using the *in situ* hybridization approach, has demonstrated a significant elevation of IFN-γ mRNA in reactional skin lesions [20]. Knowing that IFN-γ is produced locally and that it is a known inducer of macrophage TNF-α [21,22], one may postulate that in reactional lesions the infiltrating $CD4^+$ lymphocytes secrete IFN-γ which then induces macrophage activation and subsequent TNF-α production [20]; and that this TNF-α may in turn have an autocrine and auto-amplification role favouring further macrophage accumulation and differentiation. This type of positive feedback loop which increases local tissue pathology is consistent with the clinical experience of reversal reactions in which devastating tissue damage may develop in the presence of only a small amount of antigen and require large amounts of steroids in order to dampen down the effect. This local TNF-α synthesis may be a critical component in confining mycobacterial disease to a small focal area, rather than allowing for dissemination [23]. Other macrophage produced cytokines probably play a role in local tissue damage, and are currently being investigated.

In this study we have shown a close association between the presence of TNF-α and local tissue immunopathology. Thus, while TNF-α production may have beneficial effects, the dynamics of the cellular events in reversal reactions mean that disease confinement and bacterial elimination are only achieved at the expense of considerable local tissue immunopathology. If this is correct, then this work provides a useful basis for future rational immunological intervention, aimed at shifting the balance away from tissue damage and towards protective immunity.

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