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Sustained T-cell reactivity to *Mycobacterium tuberculosis* specific antigens in 'split-anergic' leprosy

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Summary Split anergy represented by delayed-type hypersensitivity skin reaction to tuberculin, but not to leprosin, is known to occur in a distinct proportion of leprosy patients. The mechanism was originally attributed to *Mycobacterium leprae*-specific suppression of T cells toward common mycobacterial antigens. This study ascertained an alternative explanation, attributing the phenomenon to selective responsiveness to *M. tuberculosis*-specific epitopes. Indeed, the results of blood T-cell proliferative responses in 11 split-anergic patients showed normal responsiveness to the *M. tuberculosis*-specific 38 kDa lipoprotein and peptide 71–91 of the 16 kDa antigen but diminished responsiveness to 2 common mycobacterial antigens, represented by the 65 kDa heat shock protein and the fibronectin-binding Ag85 complex, as compared with leprosin responsive patients and healthy contacts. These findings support the hypothesis that split anergy is due to selective recognition of *M. tuberculosis*-specific epitopes and deletion of T cells reacting to shared mycobacterial antigens.

Introduction

The majority of patients with multibacillary leprosy manifest T-cell anergy represented by the lack of delayed-type hypersensitivity (DTH) skin reactions to soluble extracts from both *Mycobacterium leprae* (leprosin) and *M. tuberculosis* (tuberculin). This anergy was attributed originally to a deletion of T cells[1] but various suppressor mechanisms were also proposed.[2] A distinct proportion of lepromatous patients show 'split anergy', manifested by DTH response to tuberculin whilst being anergic to leprosin.[3] It has been

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postulated that the mechanism of split anergy is due to suppression of T-cell responses to common mycobacterial antigens present in both leprosin and tuberculin by T cells to *M. leprae*-specific, either phenolic glycolipid[4] or protein[5] antigens contained only in leprosin. An alternative explanation, which suggested the role of macrophage mediated suppression,[6] did not explain, however, the basis for the split specificity of anergy. A selective defect of IL-2 secretion was suggested following the restoration of T-cell proliferation by exogenous IL-2,[7] but this could be interpreted in support of either reversal of suppression or as amplification of responder T cells.[8] Analysis of the specificity of the T-cell repertoire of leprosy patients using fractions separated by polyacrylamide gel electrophoresis has been inconclusive.[9] Taken together, there is at present no satisfactory explanation of mechanisms underlying the 'split anergy' phenomenon in leprosy.

Our experimental approach hinged on the analysis of specificity of proliferative T-cell responses in leprosy. We considered as our working hypothesis, that 'split anergy' could be explained on the basis of a sustained T-cell responsiveness to *M. tuberculosis*-specific epitopes, coinciding with the deletion of T cells which respond to common antigens. This assumption has been supported by the results presented in this study in which the T-cell proliferative responses toward 2 *M. tuberculosis*-specific antigens or peptides (38 and 16 kDa) and 2 largely cross-reactive antigens (hsp65 and Ag85) have been compared.

Materials and methods

SUBJECTS

We examined 40 leprosy patients of both sexes, 15–50 years old (29 multibacillary; 11 paucibacillary) and 7 professional contacts, selected from the Shashemene Leprosy Control Unit, or the All Africa Leprosy Rehabilitation and Training Hospital (ALERT) or from the Armauer Hansen Research Institute (AHRI). Patients had received multidrug therapy, according to the WHO regimen, for between 1 and 48 months—4 of the paucibacillary and 8 of the multibacillary patients suffered from reversal reactions and were under steroid therapy, and 2 of these patients were in the 'split anergy' group. None of the patients had active tuberculosis. Blood samples were obtained with informed consent of patients and with ethical research committee approval.

SKIN TESTING

Patients were tested for reactivity to intradermal inoculation with 50 μl of leprosin ('Rees antigen', WHO Bank, National Institute for Medical Research, London, UK) and 10 units of tuberculin (Purified protein derivative, PPD, Evans Med. Ltd, Langhurst, UK). Skin indurations of more than 10 cm diameter at 48 hrs after administration were classified as positive reactions.

ANTIGENS

Tuberculin (PPD) was purchased from Evans Medical Ltd (Liverpool, UK). *M. leprae* soluble extract (MLSE), the recombinant 65 kDa heatshock protein (hsp65) from

M. bovis and the recombinant 38 kDa protein (P38kDa) from *M. tuberculosis*[10] were obtained from the WHO Banks in London or Bilthoven. The recombinant purified Ag85 complex from *M. leprae*[11] was a gift from Dr Jelle Thole (The Academic Hospital of Leiden, The Netherlands). The synthetic 20mer peptide 71–91 (RDGQLTIKAER-TEQKDFDGRS), derived from the sequence of the 16 kDa protein of *M. tuberculosis* (p71–91), was prepared as described earlier.[12]

LYMPHOCYTE PROLIFERATION

Peripheral blood mononuclear cells (PBMC) were isolated from fresh defibrinated whole blood by Ficoll gradient centrifugation (Ficoll-Isopack, Pharmacia, Uppsala, Sweden) and resuspended in culture medium RPMI 1640, containing 5% human serum and 1% penicillin & streptomycin and 1% glutamine). PBMCs at $1{\cdot}56 \times 10^6$ in a volume of 100 μl were cultured using routine conditions[9] in round bottomed 96-well microtitre plates in the presence of purified or complex antigens at 5 μg/ml or 40 μg/ml of peptide p71–91. Following incubation in a humidified 5% CO_2 incubator at 37°C for 6 days, 1 μci/well of [^{3}H]TdR (Amersham International, Amersham, UK) was added and radioactive counts were quantified after an overnight incubation using liquid scintillation fluid in a betaplate counter. The results have been expressed as cpm in the antigen stimulated culture following subtraction of background counts (Δcpm). Student's *t*-test was used for statistical evaluation of differences between group mean values.

Results

From the total of 29 patients with lepromatous leprosy, initially included in the study, 18 were found by skin testing to be anergic to both leprosin and tuberculin, and also failed to respond with significant *in vitro* proliferative response to both antigenic extracts. DTH skin responsiveness with split responsiveness to tuberculin, but not to leprosin, was observed in only 11 patients, of whom 7 were clinically classified as borderline lepromatous (BL) and 2 were borderline tuberculous (BT). Despite the lack of skin DTH, the state of T-cell anergy to leprosin was merely partial, since a diminished degree of proliferative responsiveness was sustained. The proliferative responses to individual antigens were analysed also in 7 borderline leprosy patients, including both lepromatous and tuberculoid cases, with positive DTH skin responses to both tuberculin and leprosin and 7 healthy hospital contact responders.

The results of *in vitro* stimulation with: (i) hsp65 and Ag85, representative of common mycobacterial antigens; (ii) the 38 kDa protein and the p71–91 synthetic peptide, representative of *M. tuberculosis* specific antigens; and (iii) tuberculin and leprosin as complex antigenic extracts are shown as individual Δcpm values from split anergic leprosy patients and from responder patients and healthy controls (Figure 1). Responsiveness of PBMCs from the majority of leprosin anergic patients (represented by empty squares) has been characterized by significantly diminished Δcpm values following incubation in the presence of either hsp65 (mean cpm 240, $p < 001$) or Ag85 (mean cpm 766; $p < 001$), when compared to skin-test responders, either on leprosy patients or on healthy contact controls. In contrast, responses to the 38 kDa protein and peptide p71–91 comparing split-anergic patients with DTH-responder

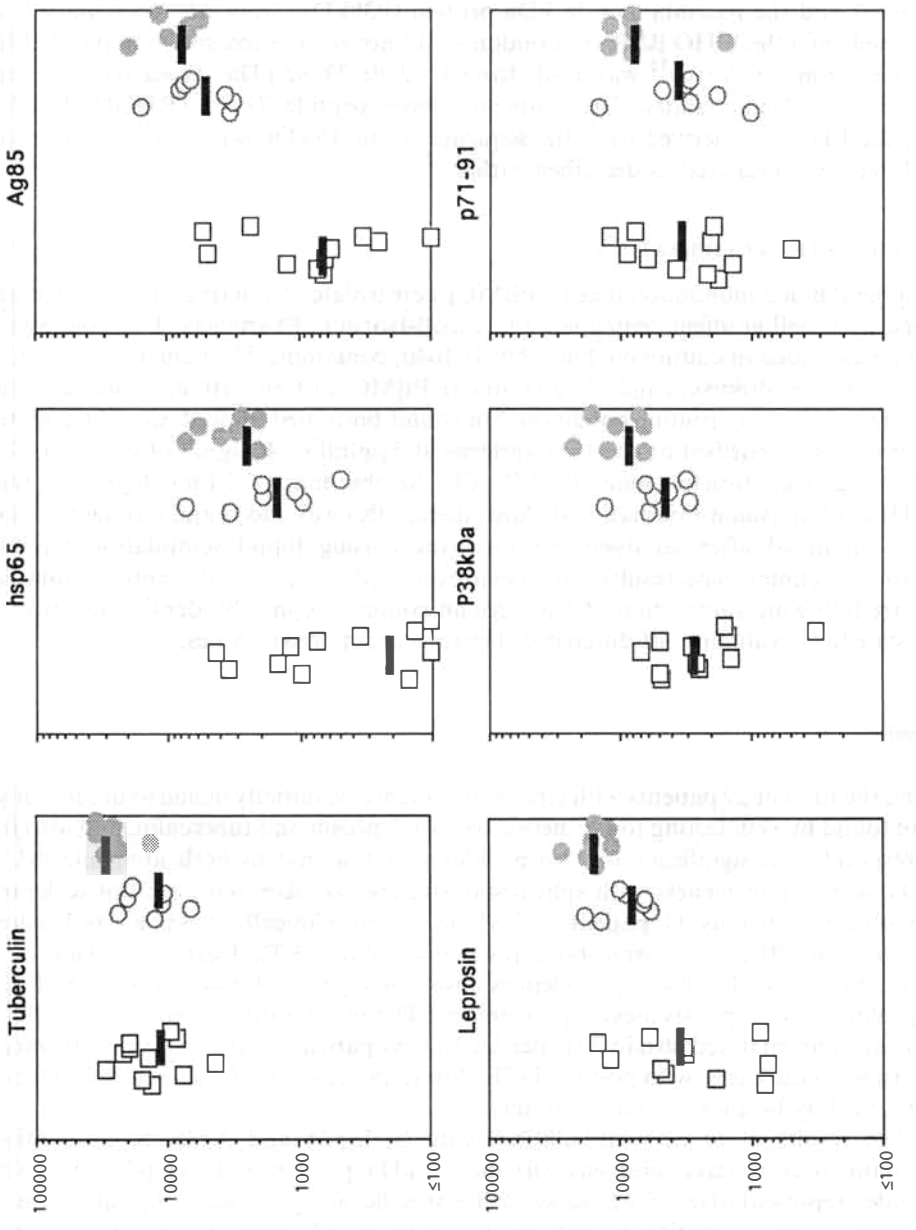

Figure 1. Proliferative responses to common mycobacterial and *M. tuberculosis* specific antigens. Individual Δcpm values obtained from 11 leprosy patients with DTH skin responses negative to leprosin but positive to tuberculin ('split anergy') (□), 7 leprosy patients with skin test responses to both reagents (○) and 7 healthy DTH responder healthy hospital contacts (◎). Vertical scale, thymidine incorporation, Δcpm; horizontal bars, geometric mean Δcpm values.

Table 1. Number of responder individuals based on proliferation to single antigens relative to individual responsiveness to PPD

Group tested	Skin test leprosin	Total tested	Number (%) of *in vitro* Responders*			
			P38kDa	p71–91	Ag85	hsp65
Leprosy	negative	11	10 (90)	9 (82)	4 (36)	2 (18)
Leprosy	positive	7	7 (100)	6 (86)	7 (100)	5 (71)
Contacts	positive	7	7 (100)	6 (86)	7 (100)	6 (86)

* > 75% of the relative response represented by: [Δcpm to Ag/Δ cpm to PPD] × 100, from individual values shown in Figure 1.

leprosy patients or healthy contacts were not significantly different. Despite the skin DTH anergy, significant proliferation in response to leprosin was probably due to the higher sensitivity of the latter assay. Therefore, it is appropriate to qualify the degree of T-cell anergy merely as partial.

In view of the observed pronounced variations in the magnitude of proliferative responses to PPD, it seemed desirable to express the Δcpm count following antigenic stimulation as a relative value in relation to the individual's response to PPD (100%). The relative values were calculated following the formula: [Δcpm with antigen/Δcpm with PPD] × 100 for each antigen. Using these relative values, tested individuals were classified as positive responders when their relative values exceeded the arbitrarily chosen 70% cut-off point. On the basis of such evaluation only 2 hsp65 responders and 4 Ag85 responders were found in the group of 11 split-anergic leprosy patients. However, responses to the 38 kDa protein and the p71–91 peptide were positive in the majority of split-anergic patients. Most patients and healthy contacts with positive skin DTH reactions to leprosin were found to be responders to all 4 antigens.

Discussion

The results obtained in 11 leprosy patients identified by DTH skin testing as 'split anergic', i.e. responders to tuberculin whilst anergic to leprosin, showed significantly diminished lymphocyte proliferation to the hsp65 and Ag85 antigens which are highly cross-reactive between *M. tuberculosis* and *M. leprae*.[13] In contrast, the same patients showed unimpaired responses to 38 kDa protein and the p71–91 peptide derived from the 16 kDa protein antigen which have previously been found specific for the *M. tuberculosis* complex.[12,13] This latter finding corroborates with the previous demonstration of elevated antibody levels to both the 16 kDa and 38 kDA protein antigens in patients with lepromatous leprosy.[14]

The argument in favour of suppressive mechanisms has previously been based on the assumption that partial T-cell responsiveness in leprosy is biased toward common mycobacterial epitopes.[4] However, this view is not supported by recent studies which demonstrated more profound impairment of responses to Ag85 than to whole *M. bovis* BCG.[15] The results of this study confirmed the impaired responsiveness to Ag85 and demonstrated a similar decline of response to hsp65, both antigens being representative

of cross-reactive common mycobacterial constituents. Furthermore, our finding of unimpaired responses in respect of the 2 tested *M. tuberculosis* specific antigens is alone sufficient to reconcile the occurrence of split specificity without a need to invoke *M. leprae*-specific suppression of responses to common epitopes.

The mechanisms of anergy in lepromatous leprosy are of fundamental interest, because they influence the rationale for immunotherapeutic intervention using the BCG vaccine or suitable adjuvants and cytokines.[7] Although the conclusions of this study are limited by the relatively small number of tested patients and by the lack of data on the T-cell cytokine profile, the obtained results clearly suggest a distinct, previously not identified, specificity pattern of the T repertoire in lepromatous leprosy.

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