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IgG subclass antibody to *Mycobacterium leprae* 18 000 MW antigen is restricted to IgG1 and IgG3 in leprosy

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SUMMARY

IgG subclass responses to *Mycobacterium leprae* 18 000 MW recombinant antigen (18K) were determined in sera from untreated leprosy patients using an ELISA-based assay with specific monoclonal antibodies. Antibodies to *M. leprae* 18K were restricted to IgG1 and IgG3 antibodies with higher seropositivity in lepromatous patients (25·5% for IgG1 and 12·8% for IgG3) compared to patients with tuberculoid disease (11·5% for IgG1 and 5% for IgG3). No significant antibody response was detectable in IgG2 and IgG4 in patients with either lepromatous or tuberculoid leprosy. The selective production of antibodies in IgG1 and IgG3 subclasses could not be related to polyclonal activation in these subclasses as all IgG subclasses showed similar elevated levels at the polyclonal level. The major difference noted between lepromatous and tuberculoid leprosy patients with the IgG subclass antibody response was a strong linear correlation between IgG1 and IgG3 responses to *M. leprae* 18K in lepromatous patients ($r = 0{\cdot}703$, $P < 0{\cdot}001$) but not in tuberculoid leprosy patients ($r = 0{\cdot}007$, $P > 0{\cdot}10$) which may be related to immunoglobulin class switching of IgG3 to IgG1 rather than selective shifts in T-helper subsets. Our results therefore, do not support the hypothesis that activation of Th2 cells occurs in lepromatous leprosy; this issue needs further examination.

INTRODUCTION

Switching of IgM antibody responses to IgG requires factors from activated T cells. It is, therefore, paradoxical that in leprosy strong IgG antibody responses occur at the lepromatous pole in the absence of detectable T-cell-mediated responses while in tuberculoid leprosy where strong T-cell-mediated responses are detected, much lower IgG antibody responses are observed.[1,2] This dichotomy between IgG antibody and T-cell-mediated responses was first described by Parish *et al.* in the murine model.[3] An explanation was provided when Mosmann *et al.*[4] demonstrated two subsets of T-helper cells (Th1 and Th2) in the murine system that differentially augment cell-mediated or antibody responses by virtue of the cytokines they elaborate. In addition, cytokines of each subset were also shown to down-regulate the function of the other subset resulting in the dichotomy observed between cell-mediated and antibody responses. When the IgG antibody responses were studied in greater detail, it was further demonstrated that the four subclasses of murine IgG are also differentially regulated by cytokines secreted by different T-helper cell subsets.[5]

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In humans although counterparts of murine Th1/Th2 cells have been identified based on their cytokine secretion patterns,[6] detailed analysis of the regulation of different isotypes and subclasses is still lacking. Leprosy provides an ideal model to investigate whether differential T-cell activation is reflected in qualitative differences in IgG subclasses. Results with IgG subclass antibodies in leprosy are scarce and inconsistent. Wright *et al.*[7] have reported polyclonal activation for all subclasses of IgG as well as IgE in Vietnamese leprosy patients compared to endemic controls. For *Mycobacterium leprae*-specific antibody, Dhandhayuthapani *et al.*[8] have reported a predominant antibody response in the IgG2 subclass with lower but similar distributions of the IgG1 and IgG3 subclasses. We have also observed antibody responses in IgG1, IgG2 and IgG3 subclasses to *M. leprae* sonicate (R. Hussain, H. M. Dockrell & T. J. Chiang, manuscript submitted). However, the relative difference between tuberculoid and lepromatous patients was most marked for IgG1 followed by IgG3. *Mycobacterium leprae* sonicate is a mixture of carbohydrate, protein and lipid antigens containing both T-dependent and T-independent antigens. Several proteins of *M. leprae* have now been cloned, purified and sequenced of which *M. leprae* 18 000 MW recombinant antigen (18K)[9] has been extensively tested for both T- and B-cell reactivity in mice.[10–12] In leprosy patients we have previously reported that T-cell proliferation[13] and IgG responses to *M. leprae* 18K[14]

show an inverse relationship across the leprosy spectrum, with T-cell responses highest towards the tuberculoid pole and IgG antibody responses highest towards the lepromatous pole. We have now extended these studies to IgG subclass responses to see if qualitative differences exist across the leprosy spectrum which may reflect differential activation of T-cell subsets.

MATERIALS AND METHODS

Patients and controls

Untreated leprosy patients presenting at the Marie Adelaide Leprosy Center were recruited to our studies and have been described in detail elsewhere.[15,16] Patients were diagnosed clinically as well as histologically on a 4 mm punch biopsy taken from the edge of an active lesion.[17] Eighty-one patients from across the leprosy spectrum who had not been treated for leprosy previously were included in the study.

Antisera

Five millilitres of blood collected from leprosy patients was allowed to separate overnight at 4°. Serum was removed and centrifuged at 400*g* for 15 min; the clear supernatant was distributed in small aliquots and frozen at −70° before use.

Antigens

The recombinant *M. leprae* 18K preparation used in the study was the full length 18K[9] and was kindly provided by Drs J. D. Watson, R. Booth and R. Prestidge (University of Auckland, New Zealand). The 18K antigen was *Escherichia coli* derived, purified by ammonium sulphate precipitation and further subjected to high-performance liquid chromatography (HPLC) and the protein was >95% pure as assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (R. Prestidge, personal communication).

Reagents

Monoclonal antibodies specific for human IgG subclasses used in the study were HP 6069 (anti-IgG1), kindly provided by Dr R. Hamilton (John Hopkins University, Baltimore, MD) and HP 6002 (anti-IgG2), HP 6047 (anti-IgG3) and HP 6023 (anti-IgG4) which were a gift from Dr C. Reimer prepared at the Centers for Disease Control (Atlanta, GA). The specificity evaluation and performance characteristics of these antibodies in ELISA systems have been described in detail elsewhere.[18,19] Mouse anti-human IgG conjugated to alkaline phosphatase was commercially obtained (Jackson Laboratories, NJ) and used according to the manufacturer's recommendation.

IgG antibody to 18K

Detection of IgG anti-18K has been described previously.[14] Plates were coated with 100 μl of antigen at 1 μg/ml in carbonate buffer pH 9·6 for 4 hr at 37° and the overnight at 4°. Phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA) was added for 2 hr at 37° to block free sites. One hundred microlitres of sera diluted in PBS containing 0·5% Tween-20 and 1·0% BSA were added and incubated for a further 2 hr at 37°. Alkaline phosphatase-labelled goat anti-human IgG (Fc specific; Jackson Laboratories) was used as the revealing probe. Each incubation was followed by three washes with PBS containing Tween-20 (0·05%) to remove unbound protein. All test sera were run at 1:20 dilution for IgG antibody determination. A high titre reference serum pool and a control negative serum pool with no activity to 18K were repeatedly run to monitor the interassay variability and the background binding.

IgG subclasses to 18K

All incubation and wash steps were similar to those described for IgG antibodies except for one additional step introduced after the addition of patients' sera. After washing, monoclonal antibodies specific for each of the subclasses were added at a dilution of 1:1000 and incubated overnight at 4°. These plates were developed with goat anti-mouse IgG conjugated to alkaline phosphatase. The reaction was again monitored with a positive reference serum pool and a negative control serum pool as described above. Initially all test sera were run at a single dilution of 1:20. Positive sera were further titrated using twofold serial dilutions.

Total IgG subclasses

The concentration of total IgG subclasses in serum was assessed as described in detail previously.[20] Briefly Immulon 4 microtitre plates (Dynatech, Arlington, VA) were coated with the appropriate monoclonal antibodies with the relevant specificity and incubated at 4° overnight. A WHO reference serum (67/97) with known amounts of the four subclasses was run as a standard each time. At least four serial dilutions of the test serum were run in each assay. The sera were incubated for 2 hr at 37° and subsequently overnight at 4°. The plates were developed with anti-IgG alkaline phosphate conjugate. Between each incubation step the plates were washed three times to remove unbound proteins. The plates were developed with the alkaline phosphatase substrate, and the reaction was stopped with 3 N NaOH. Optical density was read at 410 nm in a microtitre plate reader (Dynatech).

Statistical analysis

Non-parametric analysis (Mann–Whitney) was carried to out to assess the significance of differences in groups with negative and positive antibody to *M. leprae* 18K. Linear correlation as assessed by Kendall's method was used to determine the relationship between IgG1 and IgG3 antibodies to 18K.

RESULTS

IgG and IgG subclass antibody responses to *M. leprae* 18K

Table 1 shows the percentage of leprosy sera giving low, intermediate, or high optical density readings for IgG and IgG subclass antibodies to *M. leprae* 18K. Detectable antibody responses were observed only with the IgG1 and IgG3 subclasses. The frequency of IgG1 response was twice that of IgG3 antibodies. Very little binding was observed with either IgG2 or IgG4 antibodies with the exception of one patient where IgG2 antibodies showed detectable binding.

We next addressed the question of whether IgG1 and IgG3 responses were restricted to a certain type of leprosy. Antibody responses to 18K in leprosy patients across the disease spectrum are shown in Fig. 1. Quantitative differences among the different IgG subclasses are difficult to assess on the basis of optical density as different monoclonal antibodies may have different sensitivities of detection. However,

Table 1. Frequency of 18K antibody response in leprosy

	% responders (n = 81)				
Optical density	IgG	IgG1	IgG2	IgG3	IgG4
⩽0·3	72	67	99	86	100
0·3–0·6	13	16	1	5	0
>0·6	15	17	0	9	0

All sera were tested at 1:20 dilution.

comparison of a single IgG subclass in different groups of leprosy patients is valid. We have therefore determined a cut-off which identifies only high responders (OD > 0·6) in both groups. As observed with other *M. leprae* antigens, lepromatous leprosy patients showed a higher rate of seropositivity for both IgG1 and IgG3 subclasses than tuberculoid leprosy patients (25·6% versus 11·5% for IgG1 and 12·8% versus 5% for IgG3 respectively).

Seropositivity to 18K was further confirmed by titrating both the tuberculoid and lepromatous sera. A representative group of sera is shown in Fig. 2. Although fewer patients with the tuberculoid type of disease showed positive antibody responses to the 18K antigen, the signal when present was as high as that seen with lepromatous patients.

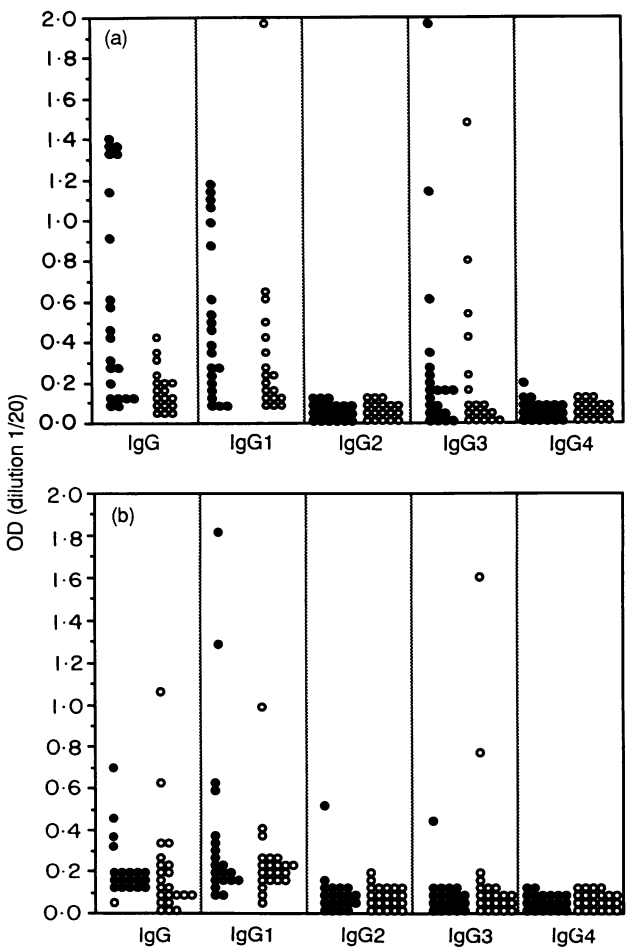

Figure 1. Scatter plot of IgG and IgG subclass responses to *M. leprae* 18K. Sera were tested at a dilution of 1:20. Each dot represents a single patient. (a) Antibody responses in lepromatous (●; n = 21) and borderline lepromatous (○; n = 18) leprosy patients; (b) responses in borderline tuberculoid (●; n = 20) and tuberculoid (○; n = 22) leprosy patients.

Total IgG subclass responses in leprosy patients

To investigate if the IgG1 and IgG3 response in leprosy patients was owing to polyclonal activation in these two subclasses we assessed total levels of IgG1 and IgG3 subclasses in patients with and without detectable IgG1 or IgG3 antibodies to *M. leprae* 18K (Table 2). IgG1 was higher in patients with lepromatous disease than patients with the tuberculoid disease. However, no significant differences were seen between 18K-negative and 18K-positive sera from patients in either form of the disease confirming that polyclonal activation was not responsible for the 18K antibody response. Similar results were obtained with IgG3 subclasses (data not shown)

Correlation of IgG1 and IgG3 antibody responses in leprosy patients

To investigate if IgG1 and IgG3 responses are linked in leprosy as is shown in other disease conditions we analysed the linear correlation between IgG1 and IgG3 responses in patients with either lepromatous (LL/BL) or tuberculoid (BT/LL) leprosy. Figure 3 shows the scatter plot, regression and significance as assessed by Kendall's correlation between IgG1 and IgG3 anti-18K antibodies. A significant correlation ($r = -0{\cdot}703$, $P < 0{\cdot}001$) between IgG1 and IgG3 was observed with lepromatous leprosy patients but no such correlation was seen in the group with the tuberculoid type of leprosy ($r = 0{\cdot}007$, $P > 0{\cdot}1$).

DISCUSSION

In patients with active leprosy IgG subclass response to *M. leprae* 18K was detected in IgG1 and IgG3 subclasses across the leprosy spectrum. Among the four subclasses of IgG, IgG1 and IgG3 are biologically the most active and may play an important role in pathogenesis and progression of disease.

In both the murine model and in humans, progression of IgM antibody responses to IgG subclasses *in vitro* is driven by cytokines from activated T cells. The intriguing aspect in this study is the presence of IgG1 and IgG3 antibodies in patients with lepromatous leprosy where T-cell activation is not detectable. The murine counterparts of human IgG1 and IgG3 are considered to be IgG2a and IgG2b owing to similarities in biological activity such as complement fixation and binding to Fc receptors. Murine IgG2a is selectively up-regulated by interferon-γ (IFN-γ) produced by activated T cells and IgG2b by transforming growth factor-β (TGF-β).[21] One explanation for the presence of IgG1 and IgG3 in patients with lepromatous leprosy may be that some switch factors such as IFN-γ may also be derived from alternative cell sources. Interferon-γ is also secreted by natural killer (NK) cells[22] and $\gamma\delta$ T cells[23] and activated macrophages secrete TGF-β.

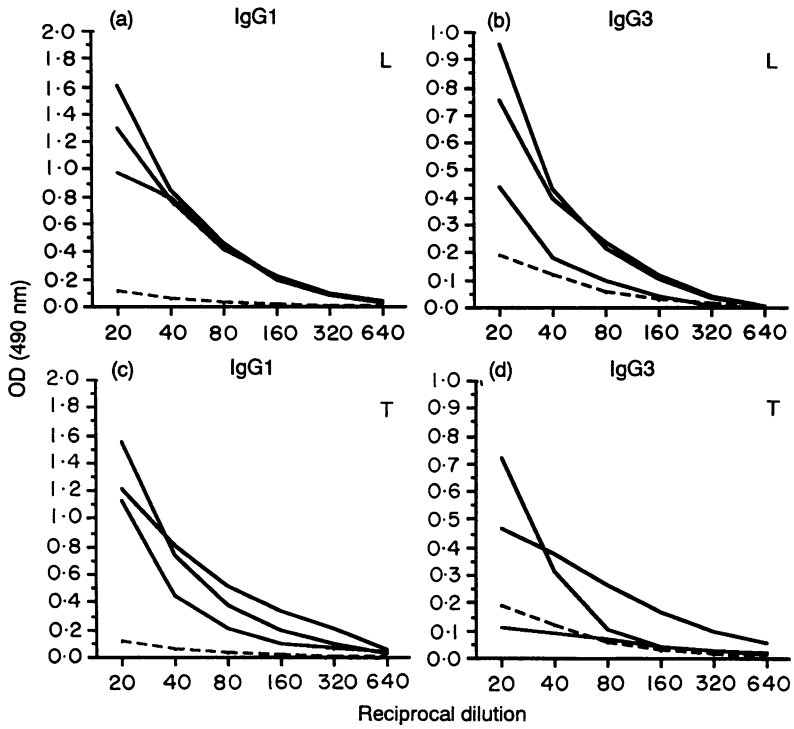

Figure 2. Representative dilution curves with sera from patients with lepromatous or tuberculoid leprosy. Solid lines are dilution curves obtained with individual sera from leprosy patients and broken lines are pooled serum from healthy endemic donors. (L) sera from patients with lepromatous disease; (T) sera from patients with the tuberculoid type of leprosy. (a, c) IgG1 antibody responses to *M. leprae* 18K; (b, d) IgG3 antibody responses to *M. leprae* 18K.

Mycobacterial antigens are known to selectively activate $\gamma\delta$ T cells[24] and may well be the alternative source for this cytokine. Alternative sources of switch factors such as the NK or $\gamma\delta$ cells would be able to bypass T-cell help requirement and switching to IgG1 and IgG3 would still occur in the absence of T-cell activation. Interestingly, several viral infections which are potent inducers of interferons also show a restricted antibody response in IgG1 and IgG3 subclasses.[25]

We have observed a highly significant correlation ($P < 0{\cdot}001$) between IgG1 and IgG3 antibodies in patients with lepromatous leprosy. It is believed that intermediate double producer stages of B cells exist where the new isotype is first synthesized from alternative splicing of multi-CH (heavy chain constant region) RNA transcripts and have been demonstrated in various systems.[26,27] This intermediate double producer stage could be the target for additional, as yet unidentified, T-cell factors that promote recombination and further downstream switching from IgG3 to IgG1. In lepromatous leprosy, this event would not occur owing to the absence of relevant T-cell factors resulting in concomitant production of IgG3 and IgG1. On the other hand, the absence of correlation between IgG1 and IgG3 in tuberculoid leprosy ($r = 0{\cdot}007$) may indicate maturational events resulting in downstream gene switching. One way of testing this hypothesis would be to analyse the epitopes of 18K being recognized by IgG1 and IgG3 antibodies in different groups of leprosy patients; if differential splicing is occurring as we propose in lepromatous patients, IgG1 and IgG3 antibodies should show recognition of distinct epitopes within the same individual. However, if switching is occurring, IgG1 and IgG3 would show recognition of similar epitopes. Our results with 15-mer synthetic peptides of 18K are consistent with this hypothesis (R. Hussain *et al.*, manuscript submitted).

Table 2. Total IgG1 concentration in IgG1 anti-18K responders and non-responders

Group ID	*n*	18K IgG1(+) (mg/ml)	*n*	18K IgG1(−) (mg/ml)	*P**
BL/LL	10	11·94 ± 1·67	29	11·10 ± 0·96	NS
BT/TT	5	9·92 ± 1·12	37	9·67 ± 0·52	NS

(+) Responders (OD > 0·6); (−) non-responders (OD ≤0·6).
*Statistical significance analysed using Mann–Whitney test.

The absence of response in the IgG2 subclass was not surprising as IgG2 antibodies are mostly directed to carbohydrate antigens[28] and we were assessing responses to a non-glycosylated purified protein.

In leprosy patients a shift towards Th2 responses has been reported towards the lepromatous pole based on the cytokine message detected in the skin lesions.[29] IgG4 responses similar to IgE responses are up-regulated by the Th2 subset.[30] We detected no IgG4 antibody response to *M. leprae* 18K. The absence of IgG4 antibodies to *M. leprae* 18K across the disease spectrum was also seen with *M. leprae* sonicate (R. Hussain *et al.*, manuscript submitted) where we have reported low to

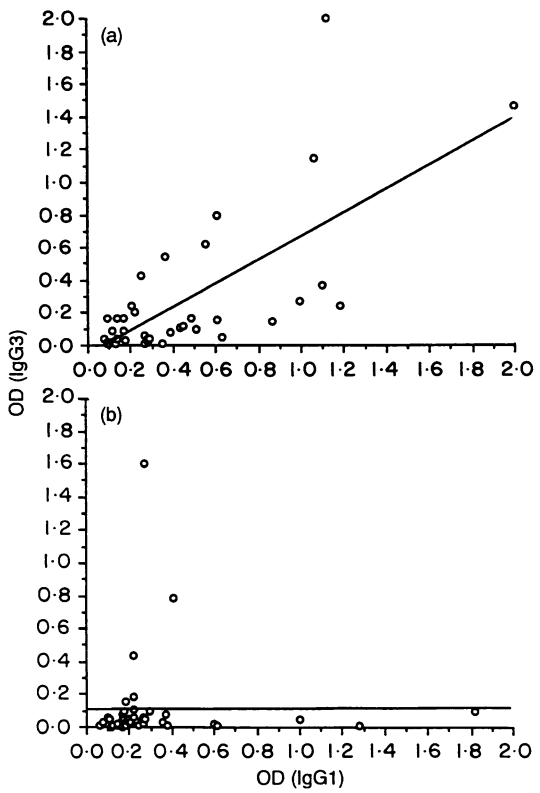

Figure 3. Linear regression analysis between IgG1 and IgG3 antibody responses to *M. leprae* 18K. (a) Correlation of IgG1 and IgG3 responses in sera from patients with lepromatous leprosy ($n = 39$, $r = 0{\cdot}703$; $P < 0{\cdot}001$). (b) Antibody responses in sera from patients with tuberculoid type of leprosy ($n = 42$; $r = 0{\cdot}007$; $P > 0{\cdot}10$).

undetectable levels of both IgG4 and IgE antibodies. The IgG4 response to 18K was therefore, not limited because of poor immunogenicity of 18K. Furthermore, polyclonal activation has been observed for both IgG4 and IgE in patients with leprosy in the Vietnamese population[7] as well as in our population (R. Hussain *et al.*, manuscript submitted) with higher responses towards the lepromatous pole compared to the tuberculoid pole indicating that the permissive signals for proliferation and secretion of these isotypes are adequate. Our results therefore, do not support the hypothesis that a Th2 shift is occurring in leprosy; this issue needs further examination.

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