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Immunoglobulin G subclass responses to mycobacterial lipoarabinomannan in HIV-infected and non-infected patients with tuberculosis

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SUMMARY

Immunoglobulin G subclass responses to lipoarabinomannan (LAM) of *Mycobacterium tuberculosis* were determined by ELISA in both HIV-1 antibody positive ($n=31$) and negative ($n=43$) patients with tuberculosis (TB). Responses were also studied in a group of healthy controls ($n=16$) and HIV-1 antibody positive ($n=60$) individuals without TB. IgG2 antibodies were the predominant subclass, being present in 25 of 43 non-HIV-infected TB patients (58%) and in 11 of 31 HIV-infected TB patients (35%). However, HIV$^+$ TB patients also showed IgG4 ($n=16$; 52%), and IgG1 ($n=4$, 13%) responses to LAM, whereas these subclasses were absent in sera from HIV$^-$ TB patients. Individuals in both non-tuberculous control groups showed no antibody responses to LAM. The influence of HIV infection on B cell responses to LAM, and possible mechanisms for antibody-mediated regulation of immunity to TB, are explored.

Keywords immunoglobulin G subclasses lipoarabinomannan tuberculosis HIV ELISA

INTRODUCTION

The association between tuberculosis (TB) and HIV infection has been well documented in recent years. TB occurs early on in the course of HIV infection, when cellular immunity is relatively intact [1], and may precede other infections [2]. There is thus a possibility that HIV-1 enhances pathogenic immune responses to *Mycobacterium tuberculosis* through mechanisms that do not require a significant depletion of CD4$^+$ T cells.

Lipoarabinomannan (LAM) is a membrane-associated lipopolysaccharide that is one of the dominant antigens of the mycobacterial cell wall [3]. Due to the presence of repeating D-arabinofuranose residues it has a direct B cell stimulatory capacity, possessing both B cell and T cell epitopes. We have studied antibody responses to T cell-dependent and independent epitopes of LAM in patients with TB and found persistence of IgM responses (attributable to B cell epitopes) in the presence of HIV infection. Total IgG responses were low or absent in this group, but non-HIV infected TB patients showed both types of response (unpublished data). The reduction or absence of total IgG responses was not consistent with the putative relatively 'intact' cellular immune response in the early stages of HIV-TB co-infection.

In order to explore the possibility that this could be accounted for by IgG subclass restriction of T cell-dependent responses, we have developed ELISA techniques to detect subclass-specific responses to LAM. The results reported here show the presence of IgG subclass responses in TB patients regardless of anti-HIV antibody status. HIV-infected TB patients were noted to have a less restricted range of subclass-specific responses, suggesting some influence of the virus on B cell antibody production in response to T cell-dependent epitopes of LAM.

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PATIENTS AND METHODS

Patients

Tuberculosis patients were from a cohort comprising a representative sample of patients attending the TB clinic and medical wards at the University Teaching Hospital, Lusaka, Zambia. All patients had been investigated for clinical, radiological and microbiological evidence of TB and tested anonymously for antibodies to HIV-1 by both Wellcozyme competition ELISA (Wellcome Diagnostics Ltd., Dartford, UK) and DuPont antiglobulin recombinant ELISA (DuPont, de Nemoirs, Belgium). Only patients with confirmed TB and unequivocally positive or negative results in both HIV-1 antibody assays were included in this investigation.

The group of HIV-infected TB patients comprised 31 patients of whom 11 were female (35%) with an age range of 16–42 years (mean 24 years) and 20 were male (65%) ranging from 18 to 66 years (mean 32 years). Twenty-three patients had pulmonary TB, of whom five also had pleural effusions, two had nodal TB, two abdominal TB and one miliary disease. Cavitation was present in only five patients (21%). Seven anti-HIV

antibody-positive patients had pleural effusions only and one nodal disease only, with no evidence of pulmonary involvement.

The non-HIV-infected group of TB patients comprised 43 patients of whom 13 (30%) were female (age range 19–30 years; mean 25 years) and 30 (70%) male (age range 18–84 years; mean 36 years). Thirty-six patients in this group had pulmonary disease, of whom 22 (61%) had cavitation, three had nodal disease, three had pleural effusions, two had miliary disease, and one had bone disease. Five patients had pleural effusions only, with no radiological evidence of parenchymal pulmonary disease, and one had both abdominal TB and a pleural effusion.

Controls

HIV-infected individuals not known to have TB were prospective blood bank donors at the Kenyatta National Hospital, Nairobi, Kenya, who were all positive for HIV-1 antibody by both Wellcozyme and DuPont ELISAs and were therefore excluded as blood donors. There were 60 individuals in this group, 45 (75%) male (age range 20–40 years; mean 32 years) and 15 (25%) female (age range 19–36 years; mean 26 years).

The group of healthy controls consisted of 16 UK laboratory donors (11 male and five female) presumed to be non-HIV-infected and with no history or clinical evidence of TB. Anti-HIV-1 antibody testing was not done for ethical reasons.

Sera from all patients were stored in aliquots frozen in liquid nitrogen until tested. All sera were treated as high risk specimens, and ELISA assays performed in a class I microbiological safety cabinet.

Lipoarabinomannan

LAM, purified from *M. tuberculosis* as described by Hunter *et al.* [3], was kindly provided by Dr P. Brennan, Department of Microbiology, Colorado State University, Fort Collins, CO.

IgG subclass-specific monoclonal antibodies

Mouse MoAbs to human IgG subclasses were derived from clones HP 6091 (IgG1), HP 6014 (IgG2), HP 6050 (IgG3) and HP6023 (IgG4) of the World Health Organisation/International Union of Immunological Societies (WHO/IUIS) panel of Specificity Reagents. These were obtained in the form of ascitic fluid from Dr R. Jefferis (Department of Immunology, University of Birmingham Medical School, Birmingham, UK) and used in assays in dilutions optimized by checkerboard titration.

Anti-LAM IgG subclass ELISA

Immulon I 96-well microtitre plates (Dynatech Laboratories Ltd., Billingshurst, UK) were coated with 1 μg/ml LAM in 0·1 M carbonate-bicarbonate buffer (pH 9·6). Identical plates were coated with 1μg/ml bovine serum albumin (BSA; Sigma Chemical Co., Poole, UK). All plates were incubated at 4°C for 18–20 h and then washed four times with 0·15 M PBS, pH 7·2, containing 0·05% v/v Tween 20 (PBS-Tween). They were then incubated at 37°C for 1 h with 1% w/v BSA in PBS-Tween to block non-specific binding. The blocking buffer was discarded and patients' sera, in three-fold dilutions in PBS-Tween with 10% normal sheep serum (Sigma), ranging from 1:100 to 1:8100, incubated in duplicate wells at 37°C for 2 h in both LAM-coated and BSA-coated wells. The normal sheep serum had been shown to be devoid of any anti-LAM specificity during preliminary assay optimization experiments. Plates were washed four times in PBS-Tween, and anti-human IgG subclass-specific MoAbs, diluted 1:5000 (IgG1), 1:6000 (IgG2), 1:5000 (IgG3) and 1:4000 (IgG4) in PBS-Tween with 10% normal sheep serum, were incubated as appropriate in separate assays on the same microtitre plate at 37°C for 1 h. After washing as above four times with PBS-Tween, horseradish peroxidase-conjugated sheep anti-mouse IgG (The Binding Site Ltd., Birmingham, UK) diluted 1:10000 in PBS-Tween, was incubated at 37°C for 1 h. This conjugate had been shown not to react with human IgG during assay optimization, in which control wells containing diluent, but lacking mouse MoAbs, were included. Plates were washed with PBS-Tween as above, and colour was developed with *o*-phenylenediamine dihydrochloride 0·67 mg/ml in distilled water containing 0·025% v/v hydrogen peroxide. Reactions were stopped after 15 min by the addition of 1 N sulphuric acid and absorbances read at 490 nm in a Dynatech MR600 microplate reader. Corrected mean optical densities (OD) were computed by subtracting mean values for BSA-coated wells from those for LAM-coated wells.

Table 1. Anti-lipoarabinomannan (LAM) IgG subclass ELISA: geometric mean OD, s.d. and confidence limits for all individuals

		IgG1	IgG2	IgG3	IgG4
Cut-off OD†		0·193	0·408	0·198	0·185
$HIV\text{-}TB^+$	Geometric mean	0·064	0·536	0·026	0·013
($n=43$)	s.d.‡	0·066	0·391	0·047	0·028
	CL§	0·085	0·703	0·041	0·022
		0·043	0·386	0·021	0·005
HIV^+TB^+	Geometric mean	0·059	0·361	0·008	0·282
($n=31$)	s.d.	0·089	0·203	0·011	0·296
	CL	0·093	0·458	0·012	0·412
		0·026	0·271	0·004	0·164
HIV^+TB^-	Geometric mean	0·078	0·116	0·025	0·092
($n=60$)	s.d.	0·044	0·070	0·028	0·048
	CL	0·091	0·136	0·032	0·106
		0·066	0·097	0·017	0·079
HIV^-TB^-*	Geometric mean	0·088	0·153	0·093	0·089
($n=16$)	s.d.	0·035	0·085	0·035	0·032
	CL	0·109	0·204	0·113	0·071
		0·068	0·104	0·072	0·107

* Presumed non-HIV-infected.

† Geometric mean plus three times s.d. of logarithmically transformed values for healthy control (HIV^-TB^-) group.

‡ Standard deviation.

§ Five per cent and 95% geometric confidence limits.

Statistical analysis

Geometric mean ODs, s.d., 5% and 95% confidence intervals were calculated for each study group after logarithmic transformation of data. The cut-off OD value for each subclass-specific assay was taken as the geometric mean plus three s.d. of values for the 16 healthy controls (Table 1). Significance testing was done by Yates-corrected χ^2-tests and Fisher exact tests where appropriate. Two-sample *t*-testing of log transformed data was used for comparison of group means based on sex and HIV/TB status, and the relationship between IgG2 and IgG4 responses (see below) was assessed by correlation and linear regression.

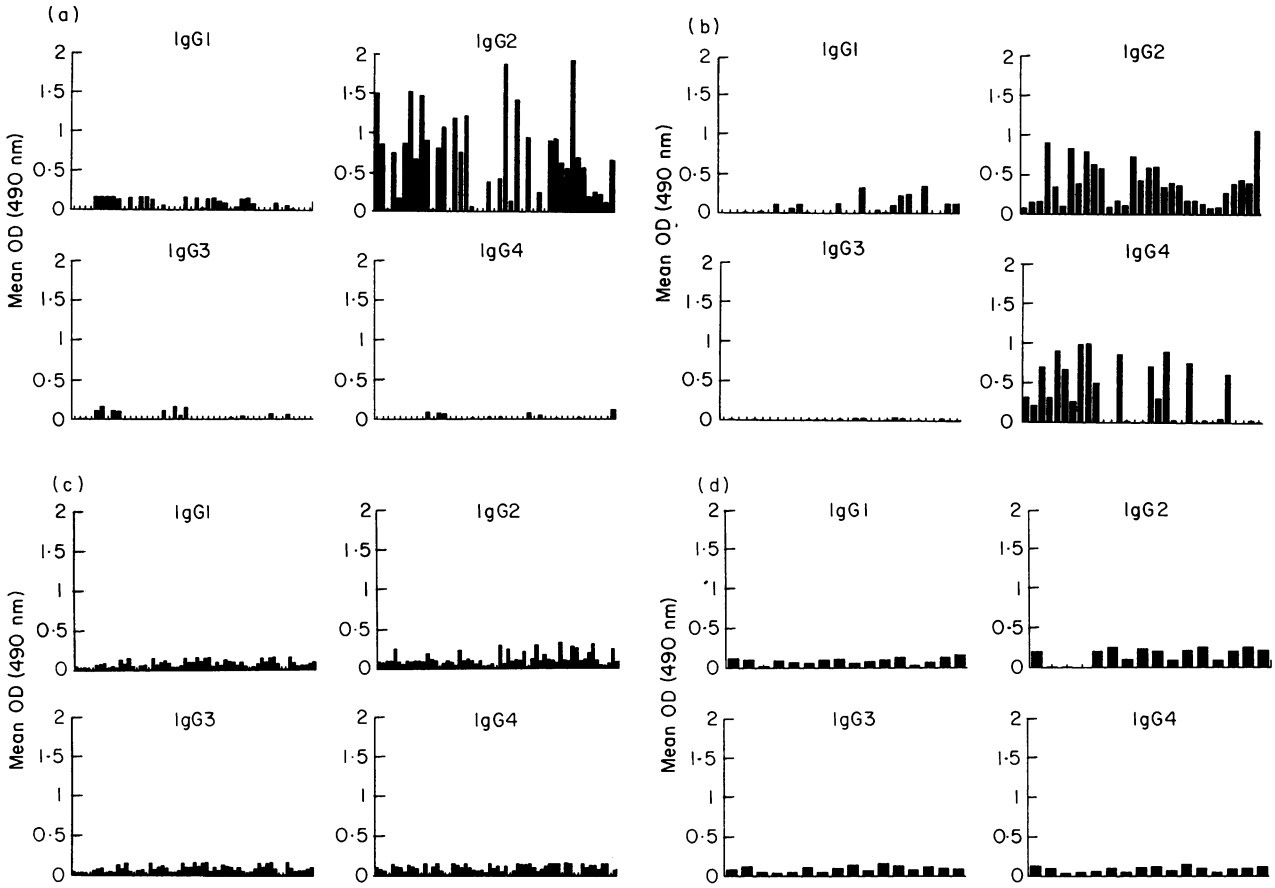

Fig. 1. (a) Anti-lipoarabinomannan (LAM) IgG subclass profile of 43 non-HIV-infected TB patients. (b) Anti-LAM IgG subclass profile of 31 HIV-infected TB patients. (c) Anti-LAM IgG subclass profile of 60 HIV-infected blood donors without TB. (d) Anti-LAM subclass profile of 16 healthy controls.

RESULTS

Figures 1a–d show anti-LAM IgG subclass profiles for individuals in all four groups at a serum dilution of 1:100, representing the plateau of the dilution curve. As LAM is a polysaccharide antigen with multiple repeating epitopes, the effect of antibody affinity on antibody responses as determined by optical density was deemed to be minimal, due to the likelihood of multivalent binding of antibody to surface-bound antigen.

IgG2 was the predominant antibody subclass produced to LAM, being present in 11 of the 31 HIV-infected TB patients and 25 of the 43 non-HIV-infected TB patients. The difference in IgG2 anti-LAM antibody distribution between HIV-infected and non-HIV-infected TB patient groups was not statistically significant ($\chi^2 = 3{\cdot}74$; $P > 0{\cdot}05$).

Geometric mean OD values for IgG2 for each group are listed in Table 1. Non-HIV-infected TB patients had a significantly higher value (0·536) than healthy controls (0·153) ($t = 29{\cdot}99$; $P < 0{\cdot}001$). This was also significantly higher than that of HIV-infected TB patients (0·361) ($t = 2{\cdot}28$; $P < 0{\cdot}05$). Comparison of geometric mean OD values in patients showing positive results for IgG2 in the non-HIV-infected TB group (0·962) with those positive for IgG2 in the HIV-infected TB group (0·677) showed an even greater difference ($t = 4{\cdot}05$; $P < 0{\cdot}001$; Table 2). For HIV-infected non-tuberculous controls the value fell well below the cut-off OD of 0·408.

An IgG4 response was seen in 16 of the 31 HIV-infected TB patients, the only study group with a geometric mean OD above the cut-off value of 0·185 for antibodies of this subclass. The geometric mean OD value for the 16 responders (0·601) was significantly higher than that of the healthy control group (0·089) ($t = 25{\cdot}99$; $P < 0{\cdot}001$). Comparison of the group as a whole (i.e both responders and non-responders; geometric mean 0·282) with healthy controls showed similar trends, although at a lower level of significance ($t = 2{\cdot}59$; $0{\cdot}02 > P > 0{\cdot}01$).

Six patients showing IgG4 responses were also positive for IgG2. However, linear regression analysis of the relationship

Table 2. Anti-lipoarabinomannan (LAM) IgG subclass ELISA: geometric mean optical density and s.d. for individuals showing positive results

		IgG1	IgG2	IgG4
HIV^-TB^+	Positives*	—	25	—
($n = 43$)	Geometric mean	—	0·962	—
	s.d.	—	0·218	—
HIV^+TB^+	Positives	4	11	16
($n = 31$)	Geometric mean	0·281	0·677	0·601
	s.d.	0·048	0·121	0·075

* Number of individuals positive.

between IgG2 and IgG4 levels in all HIV-infected TB patients showed no significant association between them (regression coefficient for IgG4 as dependent variable $= -0{\cdot}011$; $t = 0{\cdot}046$; $P > 0{\cdot}5$; and for IgG2 as dependent variable $= -0{\cdot}006$; $t = 0{\cdot}046$; $P > 0{\cdot}5$). Among patients positive for both IgG2 and IgG4 antibodies, there was a very slight inverse correlation which was not statistically significant (correlation coefficient $= -0{\cdot}156$; $t = 0{\cdot}39$; $P > 0{\cdot}5$). Four HIV-infected TB patients also showed IgG1 responses (geometric mean $= 0{\cdot}281$). This was significantly greater than the negative control value (0·088) ($t = 9{\cdot}21$; $P < 0{\cdot}001$). No IgG3 responses were observed.

Geometric mean OD values for IgG2 for either sex in each group were also computed and analysed statistically. Among healthy controls there was no significant difference in geometric mean values between males (0·157) and females (0·177) ($t = 0{\cdot}41$; $P > 0{\cdot}5$). Female non-HIV-infected TB patients had a higher value (0·582) than males (0·469) but this was not statistically significant ($t = 0{\cdot}62$; $P > 0{\cdot}5$). Among HIV-infected TB patients, however, values were significantly higher in males (0·416) than in females (0·267) ($t = 1{\cdot}98$; $P < 0{\cdot}05$). Values for the HIV-infected non-tuberculous group were not significantly different in either sex (males 0·115; females 0·121) ($t = 0{\cdot}29$; $P > 0{\cdot}5$).

Only HIV-infected TB patients showed IgG4 values above the cut-off. Differences in geometric mean values between males (0·234) and females (0·375) were not statistically significant ($t = 1{\cdot}25$; $P > 0{\cdot}2$).

DISCUSSION

The finding of predominant IgG2 responses to LAM is consistent with the well known association of polysaccharide antigens with antibodies of this subclass that has been previously documented for *Streptococcus pneumoniae* [4,5], Group A Streptococcus [6,7] and Group B Streptococcal type III [8] and type II [9] capsular polysaccharides.

The finding of IgG2 responses in non-HIV-infected TB patients that are greater than those in HIV-infected TB patients suggests the presence of some, albeit small, influence of HIV infection on the integrity of the B cell response to LAM. This is in keeping with studies that suggest the occurrence of impaired IgG2 production in HIV infection associated with increased susceptibility to pyogenic infection [10] and impaired antibody responses to pneumococcal vaccine [11]. We did not quantify total IgG subclass levels in this study, but focused on antigen-specific responses.

HIV-infected non-tuberculous individuals from Kenya showed no response to LAM. Despite previous exposure to *M. tuberculosis* in a large proportion of the endemic African population, it would appear that in the absence of clinical tuberculosis the immune system is not exposed to LAM to a degree sufficient to result in detectable antibody production. The UK control group also did not show detectable responses to LAM. Endemic controls from Africa would need to be studied to confirm these findings.

Although the role of IgG2 in immunity to infections due to encapsulated bacteria is well established, it is unlikely to play a major part in the immune response to mycobacteria. However, a supplementary but crucial role of antibody in boosting protective immune mechanisms above a critical threshold level could partly explain the hitherto unanswered question relating to breakdown of healed foci leading to disease. Due to its relatively poor capacity for complement activation [12] and its low binding affinity for monocyte receptors [13], IgG2 is an unlikely candidate for such a role, but the significance of antibody responses to LAM requires further evaluation. Antibodies to mycobacterial protein antigens, of the appropriate subclass capable of triggering effector functions, are more likely to be involved in such an immunoregulatory function. Indeed we have found predominant IgG1 and IgG3 responses to the 38-kD protein of *M. tuberculosis* in sera of TB patients (unpublished observations).

The production of specific IgG4 in response to LAM is exclusive to the HIV-infected group of TB patients. Its occurrence bears no apparent relationship to IgG2 production, suggesting the possibility of the existence of a different subpopulation of B cells committed to IgG4 production. Further studies are needed to test the validity of this concept. IgG4 has been associated with chronic antigenic stimulation [14]. The presence of increased quantities of mycobacterial LAM, secreted by viable mycobacteria in reactivated tuberculous foci early in HIV infection, and resulting in antigen-driven clonal expansion of IgG4-committed B cell subpopulations, seems a possible explanation for the occurrence of IgG4 responses in this group of individuals. An alternative explanation is HIV-related induction of subclass switching, causing preferential IgG4 production. HIV has been shown to be capable of infecting B cells *in vitro*, and it is tempting to surmise that the virus could induce switching from IgG2 to IgG4 secretion. Although quantitative studies have shown an increase in total IgG1 and IgG3 levels and decreased total IgG2 and IgG4 levels in HIV infection, with a predominance of IgG1 in the specific response to HIV [15], HIV-specific IgG4 has been found to occur with significantly greater frequency in AIDS patients than in those with AIDS-related complex or persistent generalized lymphadenopathy (PGL) [16]. This has also been observed with IgG subclass responses to cytomegalovirus in AIDS and PGL [17,18].

Cytokines are the major regulatory factors in the production of subclass-specific immunoglobulins. It is well known that IL-4 interferes with the activation of B lymphocytes by polyclonal activators such as lipopolysaccharide (LPS), often negating the effects of IL-2 [19]. This is reversible by interferon-gamma (IFN-γ) and various B cell growth factors [20]. IL-4 also induces preferential IgG4 secretion by B cells [21], an action that has been shown to be antagonized by IL-2 [22]. A deficiency of IL-2 and IFN-γ in HIV infection would be consistent with both the observed increase in production of IgG4 as well as the lower incidence of cavitation seen in HIV-infected TB patients. Increased levels of circulating LAM would be expected to lead to further tissue damage due to triggering of cytokine release from macrophages [23]. However, the trapping of LAM in immune complexes would negate this effect, as would HIV-induced deficiency of macrophage responsiveness to circulating immunomodulators.

Sex is a recognized factor in determining total levels of IgG subclass antibodies in serum, IgG2 and IgG3 being slightly higher in females than males, and IgG4 being markedly higher in males [24–27]. However, there have been no reported differences in antigen-specific responses. We found slightly higher IgG2 responses in male HIV-infected tuberculous patients than in their female counterparts. Our results are difficult to interpret in the absence of sufficient background information on the effect of

sex on total IgG subclass concentrations, but it would be interesting to determine whether there are any sex-related differences in susceptibility to TB in the presence of HIV infection.

There are many missing links in the chain of events that determine the course of infection with *M. tuberculosis*. The role of humoral immunity in determining the outcome of TB has been largely relegated to one of marginal importance in comparison to cellular immunity. The emergence of HIV infection and its association with TB provides a window of opportunity for closer scrutiny of mechanisms by which cellular responses may be enhanced or down-regulated by humoral immunity.

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