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Erythema nodosum leprosum is associated with up-regulation of polyclonal IgG1 antibody synthesis

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SUMMARY

Erythema nodosum leprosum (ENL) is a serious complication of lepromatous (L) disease in leprosy. We have previously shown that of the four IgG subclasses, IgG1 and IgG3 *Mycobacterium leprae*-specific antibodies are significantly lower in leprosy patients during ENL reaction compared with untreated L patients. To see if this decrease results from a down-regulation of antibody synthesis during ENL, the frequency of antibody-secreting B cells (ABSC) in the blood compartment was determined by ELISPOT and related to serum immunoglobulin concentrations (μg/ABSC). Control groups consisted of 16 patients with stable L disease and 32 healthy endemic controls (EC). Paired samples were analysed during acute ENLS ($n = 13$) and after the reaction had subsided to identify changes associated with ENL. Polyclonal (PC) IgG1 was elevated in L patients compared with EC (325 μg *versus* 180 μg). Interestingly, patients during acute ENL showed concentrations higher than L patients (419 μg), which decreased after the reaction had subsided (260 μg), indicating the transient nature of the antibody response. IgG2 antibodies showed the reverse trend and were lower during ENL and increased after the reaction had subsided. The mean concentrations for PC IgG3 and IgG4 antibodies were similar during ENL and after the reaction had subsided. Thus, decrease in *M. leprae*-specific IgG1 and IgG3 antibodies is not related to down-regulation of B cell responses. Identification of factors which regulate PC IgG1 antibody synthesis may provide additional insights into determinants of ENL reactions.

Keywords leprosy erythema nodosum leprosum immunoglobulin subclasses antibody-secreting cells

INTRODUCTION

In multibacillary leprosy patients, erythema nodosum leprosum (ENL) is a serious complication of the disease, which may develop any time before or after starting chemotherapy for leprosy. Clinically ENL presents as recurrent crops of painful, red, indurated subcutaneous nodules, which may become necrotic, pustular, and haemorrhagic. Patients with severe ENL can develop fever, lymphadenopathy, albuminurea, iritis as well as iridocyclitis, orchitis and neuritis [1] indicative of Arthus-type phenomenon [2].

Polyclonal (PC) activation of all isotypes (IgM, IgG and IgA) is observed in patients with stable multibacillary disease [3–5]. Human IgG is composed of four subclasses which differ in structure as well as biological activity [6]. Qualitatively, IgG1, IgG3 and IgG4 antibodies recognize predominantly protein antigen requiring $CD4^+$ T cell help, while IgG2 subclass antibody is directed predominantly towards carbohydrate antigens which recognize antigens in a $CD4^+$ T cell-independent fashion [7]. Among the four IgG subclasses only IgG1 and IgG3 fix complement efficiently [8]. In addition, monocytes/macrophages have high-affinity Fc receptors for complement, IgG1 and IgG3 [9]. These properties of IgG1 and IgG3 may have important implications in uptake of *Mycobacterium leprae* (which resides and multiplies within the macrophages) and activation and triggering of macrophages for cytokine release. Our studies have therefore focused on IgG subclass antibodies in leprosy [10,11]. The most significant finding was the dominant antigen-specific response in IgG1 followed by IgG3 antibody in patients with multibacillary or lepromatous form of the disease [10]. Moreover *M. leprae*-specific IgG1 antibody showed a highly significant correlation with bacterial load across the disease spectrum. However, in lepromatous patients undergoing ENL reactions, significantly lower concentrations of *M. leprae*-specific IgG1 and IgG3 antibodies were detected compared with lepromatous patients with stable disease [11]. Although it has been hypothesized that during ENL antibody deposition occurs at the site of disease, lower concentrations of antibodies can also be due to decreased production/synthesis of antibodies at the B cell. To address this issue we have analysed the frequency of B cells secreting each of the four IgG subclass antibodies in the blood compartment of leprosy patients with and

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without ENL and related them to serum concentration. Our results clearly demonstrate that ENL episodes are associated with up-regulation of PC IgG1 antibody synthesis within the tissue compartments. Lower concentrations of *M. leprae*-specific IgG1 and IgG3 antibodies are therefore probably due to antibody deposition in the tissues.

PATIENTS AND METHODS

Patients and controls

Leprosy patients were recruited at The Marie Adelaide Leprosy Centre (Karachi, Pakistan). The criteria used for the diagnosis of leprosy were standard clinical signs [12]. For histological confirmation, a 4 mm punch biopsy was taken from a representative skin lesion and fixed by conventional formol-mercuric chloride-acetic acid fixative (FMA), processed to paraffin and stained with haematoxylin and eosin and Wade–Fite stains for acid-fast bacilli. Sixteen non-reactional patients with stable lepromatous disease (L) were newly diagnosed (<2 weeks treatment). These patients were confirmed histologically as either polar lepromatous (LL; $n = 12$) or borderline lepromatous (BL; $n = 4$). Thirteen lepromatous patients undergoing ENL reactions were studied both during acute reaction (A.ENL) and post-remission of reaction (P.ENL). The clinical diagnosis of ENL reactions has been described in detail previously [13]. Ten of 13 patients were also histologically confirmed as undergoing ENL reactions. The time lag between A.ENL and P.ENL ranged from 10 to 110 days, with a mean of 54 days. The control group consisted of 32 healthy endemic controls with no known contact with leprosy, employed at the Aga Khan University Hospital.

Serum

Peripheral blood (5 ml) was obtained from each patient and control subject. The blood was allowed to clot overnight at 4°C. Serum was removed and centrifuged at 400 ***g*** for 15 min; the clear supernate was distributed in 100-μl aliquots and kept at −70°C until use.

Reagents

Polyclonal rabbit anti-IgG (Fc-specific) was obtained from Boehringer-Mannheim (Lewes, UK). MoAbs specific for human IgG subclasses were HP6069 (anti-IgG1) kindly provided by Dr R. G. Hamilton (John Hopkins University, Baltimore, MD); HP 6002 (anti-IgG2), HP 6047 (anti-IgG3), HP 6023 (anti-IgG4), prepared at the Centers for Disease Control (Atlanta, GA), were a gift from Dr C. B. Reimer. Goat anti-mouse IgG and goat anti-human IgG conjugated to alkaline phosphatase were purchased from Jackson Immuno Research Labs (West Grove, PA) and were used according to the manufacturer's recommendations. RPMI 1640 medium, bovine serum albumin (BSA), fetal calf serum (FCS), 5-bromo 4-chloro 3-indoyl phosphate (BCIP), *p*-nitro-phenyl phosphate, were obtained from Sigma Chemical Co. (St Louis, MO). Nitrocellulose paper (NCP) discs (13 mm diameter) were purchased from Millipore (Bedford, MA).

Quantitative determination of polyclonal IgG subclass antibodies

Polyclonal IgG subclasses in serum were determined as described in detail previously [15]. Briefly, Immulon 4 microtitre plates were coated with optimal dilutions of MoAbs specific for each of the IgG subclasses and incubated at 4°C overnight. The free sites were blocked with 5% BSA in PBS at 37°C for 2 h and then the plates were further incubated with test sera for 2 h at 37°C and subsequently overnight at 4°C. A WHO reference (67/97) with known amounts of the IgG subclass antibodies was used to establish a calibration curve in each assay. The plates were probed with goat anti-human IgG conjugated to alkaline phosphatase and later developed with alkaline phosphatase substrate. Between each incubation, the plates were washed with PBS–Tween 20 (PBS–T) to remove any unbound protein. The reaction was stopped with 3 N NaOH, and the optical density (OD) was read at 410 nm in a microtitre plate reader (Dynatech, MR 600).

Frequency of IgG subclass-producing B cells by ELISPOT assay

Preparation of cells for ELISPOT assay. Heparinized blood (25 ml) was collected in a sterile syringe and diluted with equal volumes of RPM1 in a 50-ml sterile Falcon centrifuge tube. The blood was separated on Ficoll (Histopaque; Sigma) and the buffy coat containing mononuclear cells at the interface was removed and washed three times with RPMI, and resuspended in RPMI containing 10% FCS.

Detection and enumeration of antibody-secreting B cells by ELISPOT assays

ELISPOT assay as developed by Sedgwick *et al*. [14] was modified to assess the frequency of IgG subclass antibody-secreting B cells (ABSC), as follows. Precut NCP discs were placed in 24-well tissue culture plates (Flow Labs, UK). MoAbs to the four human IgG subclasses were diluted in carbonate buffer 0·05 M pH 9·6 and 500 μl were placed in each well. Coating of capture antibodies was carried out at 37°C for 4 h. All capture reagents were titrated with respect to optimal concentrations for coating. Polycolonal rabbit anti-IgG (Fc-specific) from Boehringer-Mannheim performed best at 1 : 100 dilution for determining B cell frequency of IgG-secreting cells. MoAbs to IgG subclasses (HP series 6069 for IgG1, 6002 for IgG2, 6047 for IgG3 and 6023 for IgG4) performed optimally at 1 : 200 dilution. The NCP was washed four times with 1·5 ml PBS containing 0·025% Tween 20. The wash solution was removed from the well by aspiration with a Pasteur pipette by applying vacuum. The remaining sites were blocked with 1% BSA in coating buffer by incubation at room temperature for 1 h. The plates were washed three times as described above. The cell suspension was added in a volume of 500 μl and incubated at 37°C in a CO_2 incubator overnight without disturbing the plates. The NCP discs were again washed four times as above and then goat anti-human IgG conjugated to alkaline phosphatase was added at a concentration of 1 : 1000 in PBT–T containing 0·1% BSA. The bound conjugate was developed with 1 mg/ml of BCIP in amino-methyl-propanol (AMP) buffer. The AMP buffer was prepared in water (warmed to 30°C) containing $MgCl_2$, Triton X-100 (0·005%) and sodium azide (0·05%). The pH was adjusted to 10·25 with concentrated HCl. Colour was allowed to develop for 15 min and the reaction was stopped by washing three to four times with PBS. The ABSC were observed as blue circular foci or spots on the NCP. These spots were counted on each NCP disc, under a dissecting microscope, and frequency per 10^6 cells was estimated.

Statistical analysis

Descriptive analysis including geometric means and s.d. were carried out on a Macintosh Plus microcomputer using Statview software packages and Microsoft Excel packages. Non-parametric analysis (Mann–Whitney *U*-test) was carried out to assess the significance of difference between antibody levels in the different patient groups. The Spearman rank test was used to assess the

relationship between IgG ABSC as detected by anti-IgG or derived from the sum of IgG subclass ABSC.

RESULTS

Optimization and validation of IgG subclass-secreting B cell ELISPOT assay

Figure 1 shows the number of spots detected for IgG and IgG1–4 ABSC. The characteristic spots have homogeneously stained centres and slightly diffuse periphery. The ABSC titered out in a linear fashion and the frequency of B cells was calculated per 10^6 peripheral blood mononuclear cells (PBMC). To confirm that the frequency of IgG subclass ABSC being observed was reliable, the frequency of total IgG ABSC was also determined. The frequency of IgG-secreting cells as obtained by summation of the four IgG subclass ABSC or determined using polyclonal anti-IgG antibodies in 21 control individuals is given in Table 1. A highly significant Spearman rank correlation (rho = 0·751; $P < 0{\cdot}0008$) was observed for the two methods of determining the frequency of IgG ABSC. These results indicate that the proportion of different IgG subclass ABSC being detected was indeed reliable.

Absence of correlation in serum concentrations and frequency of ABSC

Table 2a shows the distribution of PC IgG subclasses in healthy donors (EC) and non-reactional L patients. A significant increase was observed for PC IgG1 ($P < 0{\cdot}0001$), PC IgG3 ($P = 0{\cdot}0001$) and PC IgG4 ($P = 0{\cdot}015$) in L patients compared with EC using Mann–Whitney *U*-test. The mean elevation was two-fold for IgG1 and IgG3 and 1·78 for IgG4 antibodies. PC IgG2 was also higher in L patients, but did not reach statistical significance.

When the frequency of ABSC were determined in the circulation of the same patients at the same time point a different picture emerged (Table 2b). When L patients were compared with EC, IgG1 antibody which was two-fold elevated in patient sera showed no significant difference in the frequency of ABSC between patients (L) and controls (EC). On the other hand, IgG2 antibody which did not show a significant elevation in patients compared with control group showed significant elevation ($P = 0{\cdot}0003$) of IgG2 ABSC.

This discrepancy in serum concentrations and B cell frequency was even more evident when the proportional distribution of IgG subclasses in serum and ABSC cells in the blood compartment was compared in patient and control groups (Fig. 2). While IgG1 and IgG2 constituted >80% of the total serum pool, IgG1 and IgG2 ABSC were a minor proportion of ABSC (<40%) in the blood compartment. On the other hand, IgG3 and IgG4 which constitute <20% of serum immunoglobulin formed a high proportion of ABSC (>60%) in the blood compartment. We therefore felt that both parameters should be considered while analysing the activation of B cell in the tissue compartment.

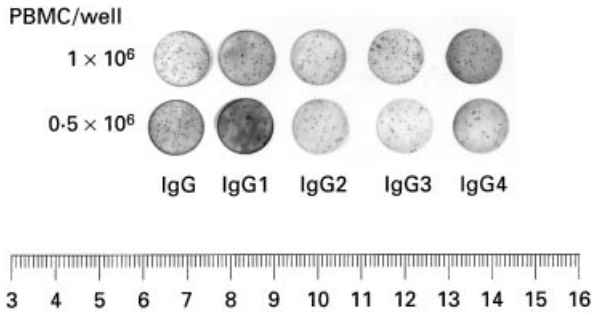

Fig. 1. ELISPOT assay for IgG and IgG subclasses. Antibody-secreting B cells were determined at two concentrations. Each spot indicates a single antibody-secreting cell. The frequency of ABSC ($\times 10^6$) for IgG = 260, IgG1 = 44, IgG2 = 42, IgG3 = 88 and IgG4 = 60.

Table 1. Concordance between B cell frequency for IgG antibody-secreting B cells (ABSC) using polyclonal antibody and IgG subclass monoclonal antibodies

ABSC/10^6	Mean ± s.e.m. ($n = 21$)
Polyclonal IgG	163 ± 21·60
Σ IgG subclasses	146 ± 17·93

rho = 0·751.
$P < 0{\cdot}0008$.

Figure 3 shows the results as a ratio of both parameters (μg of immunoglobulin synthesized/ABSC). The highest amount of IgG subclass antibody synthesized per ABSC was IgG1 in L patients compared with EC (325 μg/ABSC *versus* 180 μg/ABSC). There was no marked difference in the other three IgG subclasses. IgG2 antibodies actually showed a slight decrease in L patients compared with EC. These results strongly suggest activation of B cells in the tissues is restricted to IgG1-secreting B cells in patients with lepromatous disease.

Polyclonal IgG1 subclass antibody synthesis is selectively increased in patients during ENL reaction

While comparisons of patients undergoing ENL reactions with untreated lepromatous patients may show trends in immune responses, it may not be completely appropriate as ENL reactions can occur at variable times during the course of chemotherapy. We therefore carried out paired analysis on a cohort of 13 ENL patients during reactions and after remission of reaction. Figure 4 shows the distribution of IgG subclass antibody responses. The mean concentrations of PC IgG1/ABSC was higher during reaction compared with concentrations after the reaction had subsided. Interestingly, PC IgG2 showed the reverse trend, with lower concentrations during ENL. PC IgG3 and PC IgG4 antibodies did not show any change during ENL reaction. After remission of reaction the pattern of IgG subclass response was comparable to that observed in stable lepromatous disease (L patients), confirming that the changes in PC IgG1 and PC IgG2 antibody subclasses are indeed associated with ENL reactions and are transient in nature.

DISCUSSION

There is increasing evidence from different studies that leprosy patients with lepromatous disease have abnormal B cell functions which may be related to altered T cell function. ENL reactions exclusively occur in patients with lepromatous disease, but little is known about the mechanisms which precipitate ENL reactions. The clinical symptoms of ENL resemble serum sickness and are believed to be due to immune complex deposition in the tissues.

Table 2. Serum concentrations and frequency of IgG subclass antibody-secreting B cells (ABSC) in leprosy patients and endemic controls

a.		Serum concentration, mg/ml			
Group ID		IgG1	IgG2	IgG3	IgG4
	Range	5·12–21·76	2·2–7·2	1·15–3·52	0·3–3·3
L	Mean	12·38	4·16	1·96	1·39
$n = 16$	s.d.	±3·98	±1·55	±0·71	±0·87
	Median	12·55	4·15	1·79	1·24
	Range	3·04–12·4	1·24–5·52	0·26–2·17	0·03–2·6
EC	Mean	6·12	3·29	0·87	0·78
$n = 32$	s.d.	±3·98	±1·18	±0·44	±0·60
	Median	5·8	3·2	0·76	0·62
P L *versus* EC		<0·001	0·086	<0·0001	0·015
b.		ABSC/10^6 PBMC			
Group ID		IgG1	IgG2	IgG3	IgG4
	Range	14–90	17–80	18–140	19–300
L	Mean	38	44	70	77
$n = 16$	s.d.	±19·79	±21·25	±41·09	±73·07
	Median	31	42	61	57
	Range	3–100	3–70	7–160	4–130
EC	Mean	33	22	44	43
$n = 32$	s.d.	±26·29	±16·42	±33·93	±28·66
	Median	25	19	34	41
P L *versus* EC		0·174	0·0003	0·020	0·100

This is based on the observation that during ENL there is a decrease in antibody in circulation. Most of these studies were cross-sectional, single time point studies, except for one study on a small group of ENL patients ($n = 4$) who were followed sequentially [15]. Antibody concentrations show considerable variation among individual patients. Second, ENL reactions can precipitate any time before or during chemotherapy which is given over a period of 2 years for multidrug treatment (MDT) and longer for other regimens. Since chemotherapy is known to result in a decrease in antibodies, the variable duration of chemotherapy that these ENL patients have received further compounds an already difficult issue. To overcome these limitations we have analysed paired samples in a cohort of ENL patients during and after remission of reaction. Tumour necrosis factor-alpha (TNF-α) levels which have been shown to be raised during ENL reactions [16–18] were also increased in our A.ENL group and decreased in P.ENL to the levels observed in L patients, indicating that in the P.ENL group the reaction had indeed subsided ([19].

To determine antibody secretion at the cellular level, ELISPOT assays were optimized with respect to coating surface and optimal coating reagent. Precut nitrocellulose membrane with a larger surface area (13 mm) resulted in better reproducibility and sensitivity than 96-well microtitre plates which are more frequently used in ELISPOT analysis. The frequency of ABSC in ELISPOT assay can also be influenced by overlapping of ABSC cells in the monolayer, staining artefacts, as well as differences in capture ability of various subclass-specific IgG MoAbs. The frequency of IgG-secreting B cells as determined by IgG-specific polyclonal antibodies or IgG subclass-specific MoAbs as capture antibodies showed a highly significant Spearman rank correlation (rho $= 0{\cdot}751$; $P < 0{\cdot}0008$). Also, there was no trend in the limited variability observed in the two methods, indicating that the proportion of IgG subclass ABSC being determined was valid in our system.

Lepromatous patients showed significant polyclonal activation for IgG1, IgG3 and IgG4 subclasses compared with healthy controls, while IgG2 responses were not statistically significant. These results suggest that only T cell-dependent IgG subclass responses are dysregulated during lepromatous disease. Increase in serum concentration of a particular immunoglobulin isotype can be due to an increase in the frequency of ABSC or an increase in the overall rate of synthesis of antibodies by ABSC, which would be reflected in the serum concentrations. The proportion of IgG subclass ABSC showed no relationship with the proportional distribution of IgG subclasses in blood compartment. IgG1 antibody, which showed the most significant elevation in serum concentrations, did not show any difference in frequency of IgG1 ABSC in patients with lepromatous disease compared with healthy endemic controls, indicating an overall increase in the rate of immunoglobulin synthesis rather than expansion of ABSC. Interestingly, IgG2, which did not show significant differences in serum concentrations compared with controls, showed increased

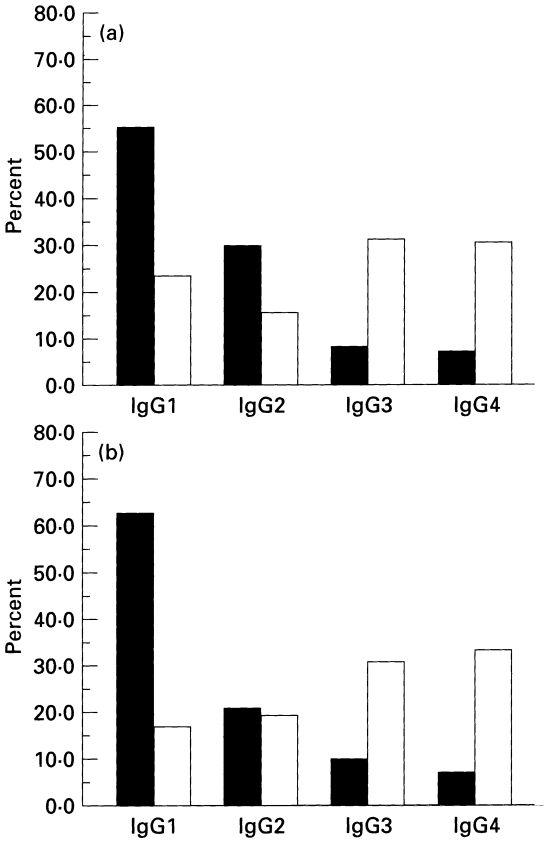

Fig. 2. Proportional distribution of polyclonal IgG subclass antibody in serum and IgG subclass antibody-secreting cells in the blood compartment. (a) Endemic controls ($n = 32$). (b) Leprosy patients with lepromatous type of disease ($n = 16$). ■, Serum; □, antibody-secreting B cells.

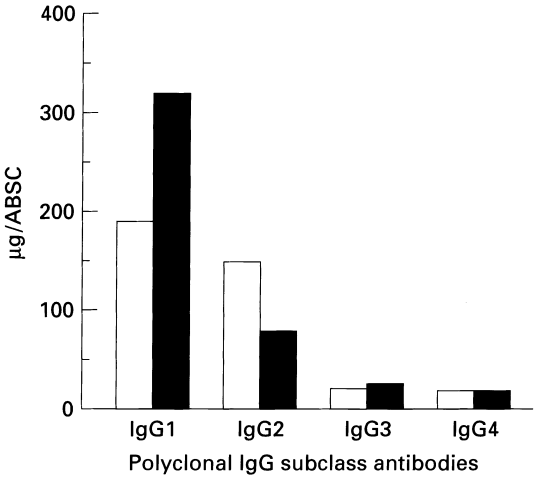

Fig. 3. IgG subclass antibody concentration per antibody-secreting cell in the blood compartment. Healthy endemic controls (□) ($n = 32$); non-reactional leprosy patients with either lepromatous or borderline lepromatous disease (■) ($n = 16$); ABSC, antibody-secreting B cells.

frequency of ABSC in the blood compartment, suggesting decreased overall immunoglobulin synthesis. In paired samples of ENL patients an increase in the overall synthesis of IgG1 antibodies was observed during A.ENL which decreased after remission of ENL, indicating that this was a transient event. This was further supported by our observation that the P.ENL group showed distribution very similar to that observed in stable L patients. However, it needs to be determined if the B cells were being activated at the local disease site or in the lymphoid compartment, and subsequently immune complexes were depositing in the skin. There is some precedence for local activation of B cells at the reaction site in diseases such as arthritis [20] and meningitis [21] where local production of antibody is shown to occur during the inflammatory episodes. In the last 5 years, evidence by different workers suggests the involvement of T cells in the triggering of ENL reactions. For example, there is an increase in $CD4^+$ T cells and interferon-gamma (IFN-γ) expression (Th1 activation) in ENL lesions [22]. Yamamura *et al*. [24] have shown a selective increase in the expression of mRNA for IL-6, IL-8 and IL-10, whereas IL-4 and IL-5 (indication of the Th2 cytokine response) present in L patients persisted during ENL. Naafs [25] suggests the sequence of ENL events as an unidentified trigger which causes $CD4^+$ cells to invade the lepromatous granuloma. These $CD4^+$ cells upon activation would elaborate cytokine which may induce the locally present B cells to differentiate and to secrete antibody. Furthermore, there is indication from our studies that IgG1 antibody-secreting cells are being selectively activated. This antibody is highly efficient in complement fixation and releasing inflammatory mediators. It would therefore be important to identify cytokines which are acting as differentiating factors for IgG1 subclass antibody and growth factors for post-switched B cells. Although human IgG3 and IgG1 antibody is considered to be homologous to murine IgG2a and IgG2b, respectively, based on their ability to recognize predominantly protein antigens and fix complement [26], the factors which result in switching of IgM responses to IgG3 and IgG1 have yet to be identified in the human system. The role of IgG1 in the uptake of antigen by macrophages and subsequent macrophage activation to release proinflammatory cytokine remains to be determined. This will be the future aim of our studies in mycobacterial disease.

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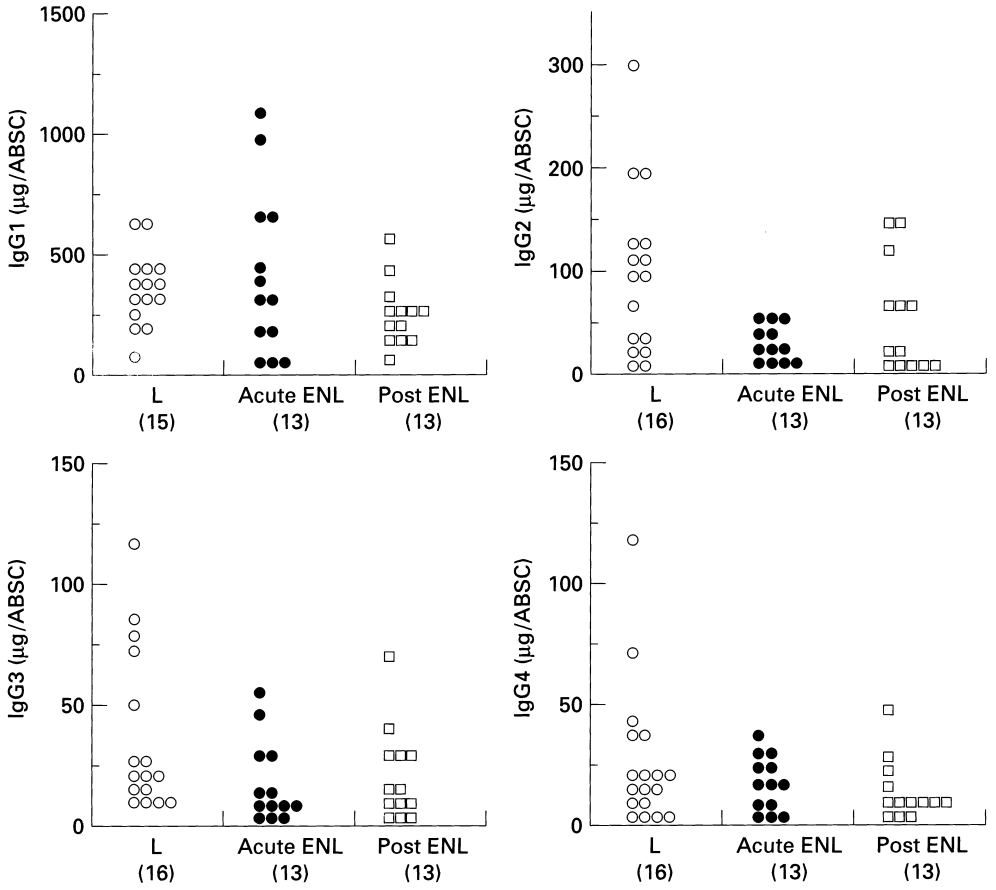

Fig. 4. IgG subclass antibody concentration per antibody-secreting cell in the blood compartment. L, Non-reactional leprosy patients with either lepromatous or borderline lepromatous disease; acute ENL, leprosy patients during acute ENL; Post-ENL, after ENL reaction had subsided. The number of patients in each group is indicated in parentheses. ABSC, Antibody-secreting B cells.

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