Clin Exp Immunol 1994; 95:35–41

Low antibody affinity restricted to the IgA isotype in IgA nephropathy

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(Accepted for publication 22 September 1993)

SUMMARY

Antibody affinity affects the handling and behaviour of immune complexes, and experimental studies have shown that animals which produce predominantly low-affinity antibody are prone to immune complex deposition resulting in glomerulonephritis. In order to investigate the potential role of antibody affinity in the pathogenesis of IgA nephropathy, affinity of both IgA and IgG antibody isotypes during secondary response to systemic immunization with tetanus toxoid was studied in 20 patients with IgA nephropathy. Patients with IgA nephropathy produced IgA antibodies of significantly lower affinity than controls ($P < 0{\cdot}001$), whereas IgG antibody affinities were similar. Contrasting with controls, patients' IgA antibody affinity was inversely related to antibody concentration, with higher responders producing large amounts of low-affinity antibody. IgG antibody affinity increased with time, and maturation of IgG antibody affinity was similar in both controls and patients. IgA affinity in controls decreased with time, and this lack of IgA affinity maturation may explain the relative unimportance of IgA in normal systemic immunity. This temporal decrease in IgA affinity was not observed in patients with IgA nephropathy. The production of low-affinity IgA in IgA nephropathy may provide an explanation for the predominant deposition of IgA in this disease.

Keywords IgA antibody affinity IgA nephropathy IgG tetanus toxoid

INTRODUCTION

IgA nephropathy (IgAN) is the commonest glomerulonephritis in the Western world, characterized by the predominant or co-dominant deposition of IgA in the glomerular mesangium [1]. The origin of this IgA and the reason(s) for its deposition are not known. Despite extensive investigation, no consistent antigenic target has been satisfactorily identified for this IgA, although cytomegalovirus (CMV), food antigens, and hepatitis B have been amongst the candidates [2–4]. Nonetheless, IgAN is widely regarded as an immune complex-mediated disease [1]. While the role of IgA in the progressive renal damage in IgAN remains controversial [5], numerous abnormalities in the IgA immune system, including raised serum IgA and IgA immune complexes, and increased *in vitro* IgA synthesis, strongly suggest a pathogenic role for IgA in IgAN [6].

One factor that significantly influences the handling and behaviour of immune complexes is antibody affinity [7]. Antibody affinity has been shown to be biologically important in providing effective immunity, affecting functions such as neutralization of virus and toxins, agglutination, complement fixation, and elimination of antigen [7]. Excessive production of low-affinity antibodies has been considered an expression of immunodeficiency [8].

Antibody affinity is genetically controlled and, at least in mice, the genes controlling affinity are not linked to the major histocompatibility locus [9]. Affinity is not dependent on antibody levels or the type of antigen administered [7]. In many secondary antibody responses, particularly those following immunization with adjuvants, IgG antibody affinity increases with time. This antibody maturation is generated by a combination of somatic hypermutation of V region germ-line genes [10,11], and selection by antigen of B cells bearing the highest affinity surface antibody [12]. Affinity maturation is fundamental to IgG secondary responses, but IgA affinity maturation has yet to be studied.

There is considerable evidence from experimental animal studies to suggest that susceptibility to chronic immune complex disease is associated with the production of low-affinity IgG antibodies and failure to eliminate antigen [13–18]. Strains of mice producing low-affinity IgG antibody deposit immune complexes in their glomeruli and eventually die from renal failure [15,17,19]. In addition, those low-affinity mice who further failed to produce IgG affinity maturation to adjuvantized antigen were susceptible to a more rapid and severe glomerulonephritis [20].

The present study was undertaken in order to investigate IgA antibody affinity and IgA affinity maturation following

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systemic immunization with tetanus toxoid (TT) in IgAN, to determine whether there are qualitative abnormalities of IgA production in IgAN.

PATIENTS AND METHODS

Subjects

Twenty patients with biopsy-proven IgAN (mean age 36 years, range 16–60 years; 16 males, four females) and 20 age- and sex-matched controls from hospital staff (mean age 36 years, range 17–64 years; six females, 14 males) were studied. Renal function in the patients was preserved (serum creatinine within the reference range in all but one patient (350 μmol/l)). None of the patients had excess alcohol consumption, parenchymal liver disease, or systemic lupus erythematosus (SLE). Seventeen out of 20 of the patients had abnormal dipstick urinalysis at the time of testing: 16/20 had microscopic haematuria and 13/20 had proteinuria, but none had macroscopic haematuria or heavy proteinuria at the time of the study. No patient was receiving immunosuppressive therapy. Prior approval for the studies was obtained from the Leicestershire Health Authority Ethical Committee.

All individuals had been previously immunized with TT at least 12 months before the study. Subjects were given 40 U TT adsorbed onto aluminium hydroxide (Merieux) intramuscularly.

Blood samples were taken preimmunization and 2 and 10 weeks post-immunization, and sera were aliquoted and stored at −75°C.

ELISA

Ninety-six-well plates (Nunc Immunoplate, Life Technologies, Paisley, UK) were coated with TT in simple solution (Wellcome, Beckenham, UK) diluted 1:20 in 0·05 M bicarbonate buffer pH 9·6 at 4°C. After washing with washing buffer (PBS/0·1% Tween 20/0·3 M NaCl), non-specific binding sites were blocked with 2% bovine serum albumin (BSA) for 1 h at room temperature. After further washing, 50 μl test sample (diluted in washing buffer 1:200–1:20 000 for antibody levels; starting 1:5–1:200 for affinity measurement) were added to each well in duplicate and incubated overnight at 4°C. Bound antibody was detected with 50 μl horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG or IgA (P214, P216, Dako, High Wycombe, UK) (diluted in washing buffer 1:500–1:2000) incubated for 1 h at room temperature. IgG anti-TT standards were obtained from Blood Products Laboratories (Elstree, UK); IgA anti-TT antibody standards were not available and therefore were made internally from a pool of high responders and assigned arbitrary units (AU).

After further washing, 50 μl substrate (8 mg of 1,2-phenylenediamine dihydrochloride (OPD) (Dako) in 12 ml 0·1 M citric acid/phosphate buffer pH 5 plus 5 μl H_2O_2) were added and the reaction stopped after 20 min with 75 μl 2 M H_2SO_4. Colour development was read at 492 nm on a Titertek Multiskan (ICN, High Wycombe, UK).

The ELISA method has been shown to be a quantitative assay for antibodies which is not influenced by the affinity (except for very low affinities) of the antibody [21].

Antibody affinity

The relative functional affinity of IgG and IgA anti-TT was assessed by ELISA in the presence and absence of diethylamine (DEA) (Fig. 1). The use of mild chaotropic agents in ELISA has been shown to have a differential effect on the dose-response curve, producing a marked shift to the left with low-affinity antibodies, but having less effect on high-affinity antibodies [22,23].

Serial dilutions of serum (starting at 1:5 for IgA and 1:100 for IgG) were made in duplicate in the presence or absence of 15 mM DEA for IgG assays, or 20 mM DEA for IgA, on plates coated with TT (having previously been blocked with 2% BSA). The optimum concentration of DEA for each assay was previously determined as that which gave the maximum shift of the dose-response curve without affecting the maximum optical density (OD). After overnight incubation at 4°C, the amount of IgG or IgA bound to antigen was detected using HRP-conjugated rabbit anti-IgA (diluted 1:500) and anti-IgG (diluted 1:2000) and developed as described earlier. OD was plotted against serum dilution and the leftward shift in dose-response curve in the presence of DEA was measured at 50% of the maximum OD (Fig. 1). Results were expressed as the $\log_{10}$ of this shift.

Statistical analysis

Statistical analysis was by Mann–Whitney U-test, and data are presented as mean ± s.e.m.

RESULTS

IgA and IgG antibodies to tetanus toxoid

IgA and IgG antibodies to TT were measured by ELISA in the sera of controls and patients with IgAN before and 2 and 10 weeks after immunization. Comparable amounts of serum IgG and IgA anti-TT were produced by patients with IgAN and

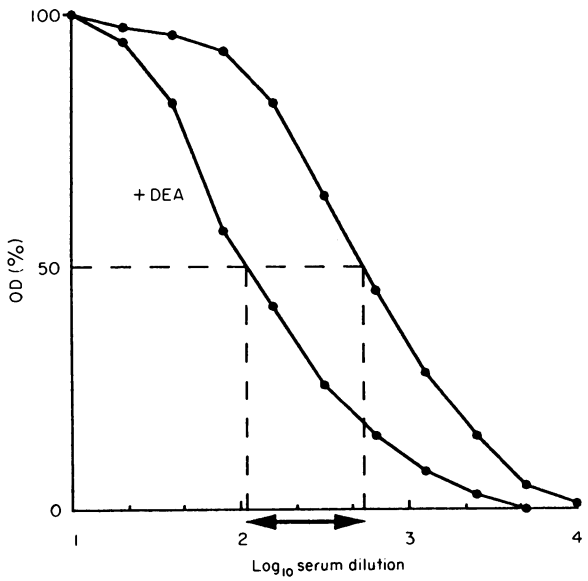

Fig. 1. The curve obtained by serial dilutions of serum in the tetanus toxoid (TT)-specific ELISA is shifted to the left in the presence of the chaotropic agent, diethylamine (DEA). Relative functional affinity of sera was assessed by measuring the $\log_{10}$ leftward shift of the curve. Low-affinity antibodies are more affected by DEA than high-affinity antibodies, resulting in a greater curve shift.

Table 1. Serum IgA and IgA anti-tetanus toxoid (TT) levels

	IgA anti-TT			IgG anti-TT		
Week	0	2	10	0	2	10
Control	5·8±0·8	62·3±23·3	17·0*±3·2	1·68±0·37	20·28±7·24	8·3±1·63
IgAN	5·9±0·8	57·5±15·4	12·0*±6·3	1·2±0·3	17·03±5·29	8·75±2·5
P†	0·82	0·87	0·9	0·44	0·74	0·54

IgA anti-TT (arbitrary units (AU)/ml) and IgG anti-TT (U/ml) (mean±s.e.m.) in the sera of controls and patients with IgA nephropathy (IgAN) taken before, and 2 and 10 weeks after immunization.

*$P<0·01$ compared with week 2.

†*P* values refer to comparison of patients and controls.

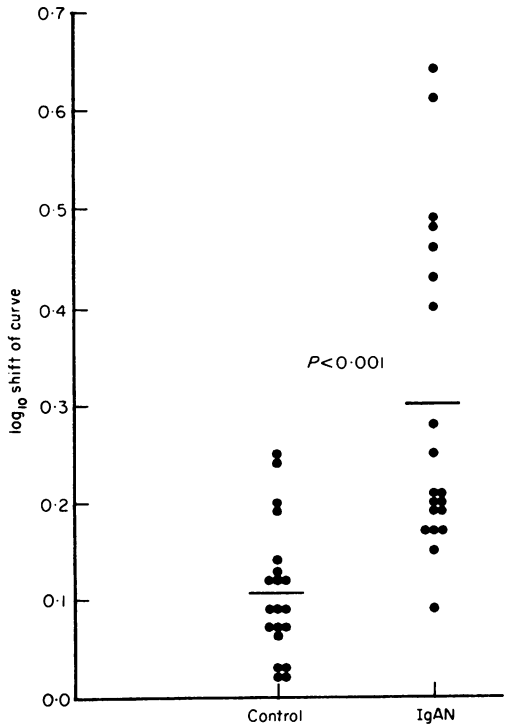

Fig. 2. Serum IgA anti-tetanus toxoid (TT) affinity in patients with IgA nephropathy (IgAN) and controls 2 weeks after immunization expressed as the $\log_{10}$ shift of the curve on the addition of 15 mM diethylamine (DEA).

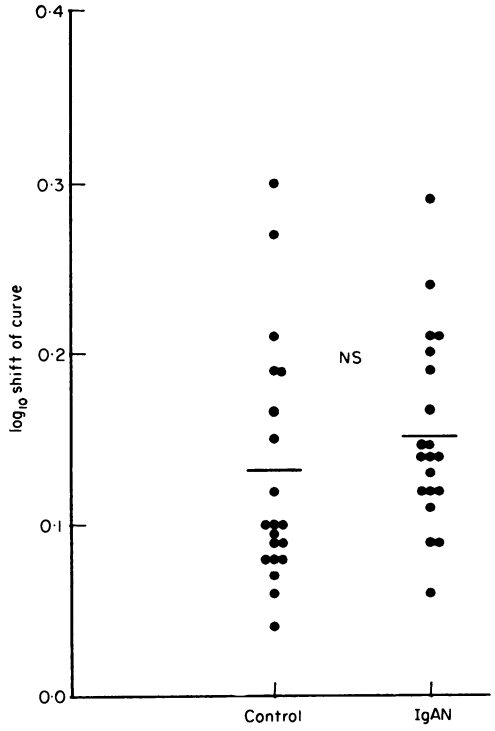

Fig. 3. Serum IgG anti-tetanus toxoid (TT) affinity in patients with IgA nephropathy (IgAN) and controls 2 weeks after immunization expressed as the $\log_{10}$ shift of the curve on the addition of 20 mM diethylamine (DEA).

controls at each time point (Table 1). The levels of IgA anti-TT in both patients and controls fell significantly between 2 and 10 weeks after immunization ($P<0·01$). While the level of IgG antibodies also fell in both patients and controls, the differences between weeks 2 and 10 were not significant.

Antibody affinity

There was a marked difference in the functional affinity of serum IgA anti-TT antibodies produced by IgAN compared with controls (Fig. 2). The mean $\log_{10}$ shift of the curve in the presence of DEA was significantly greater (denoting lower affinity) in IgAN (mean±s.e.m. $\log_{10}$ shift 0·30±0·036) compared with controls (0·107±0·015; $P<0·001$). By contrast, there was no difference in IgG anti-TT affinities (controls mean±s.e.m. $\log_{10}$ shift 0·124±0·17 *versus* IgAN 0·15±0·14; $P=0·22$) (Fig. 3).

There was no correlation between the quantity of IgA anti-TT antibodies produced and IgA antibody affinity in controls ($r=-0·33$; $P=0·1$). However, there was a correlation between affinity and quantity of IgA anti-TT in IgAN ($r=0·55$, $P=0·011$), with higher responders having a larger curve shift with DEA, i.e. tending to produce lower affinity IgA antibodies. IgG affinity was independent of IgG anti-TT concentration in both controls and IgAN (controls $r=0·05$, $P=0·83$; IgAN $r=0·35$, $P=0·15$).

Serum IgA levels are often raised in IgAN, which is polyclonal [24]. Since there was a negative correlation between IgA antibody levels and IgA antibody affinity in IgAN, we

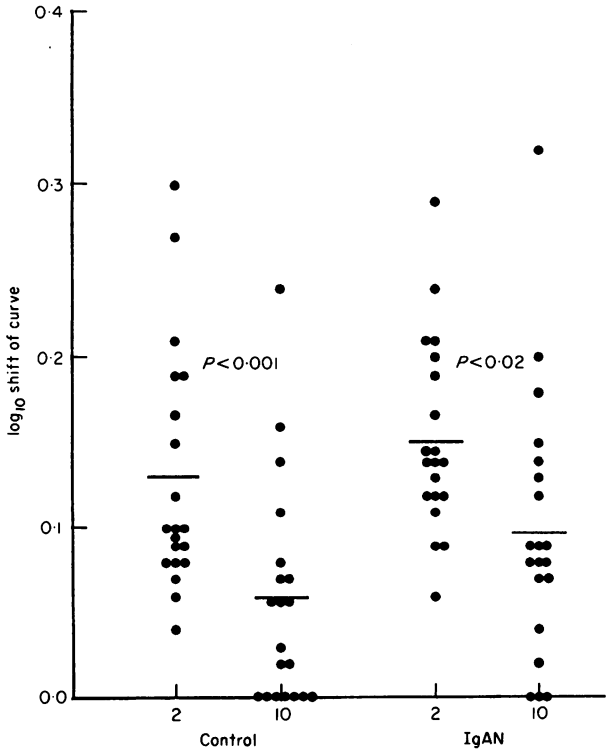

Fig. 4. Serum IgG anti-tetanus toxoid (TT) affinities in patients with IgA nephropathy (IgAN) and controls at 2 and 10 weeks after immunization expressed as the $\log_{10}$ shift of the curve in the presence of 20 mM diethylamine (DEA). The significant reduction in the leftward shift of the curve denotes increasing IgG affinity with time (affinity maturation), in both patients with IgAN and controls.

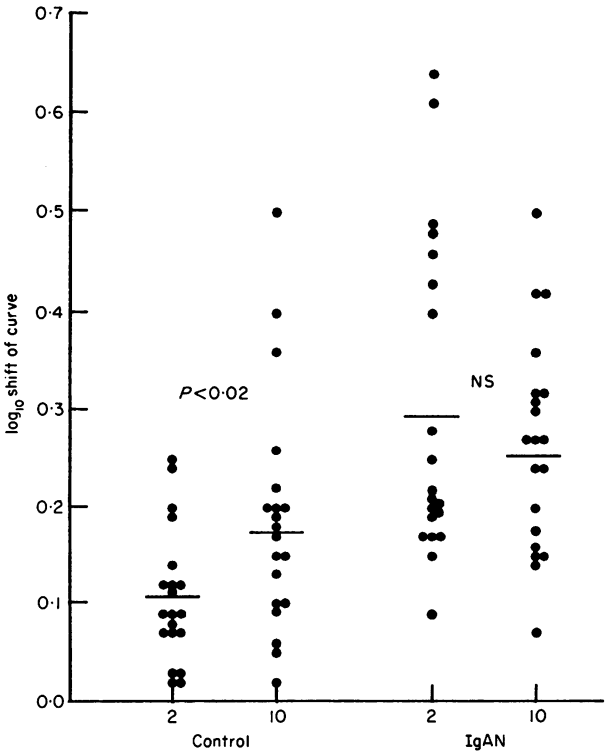

Fig. 5. Serum IgA anti-tetanus toxoid (TT) affinities in patients with IgA nephropathy (IgAN) and controls at 2 and 10 weeks after immunization expressed as the $\log_{10}$ shift of the curve in the presence of 15 mM diethylamine (DEA). The significant increase in the leftward shift of the curve in controls denotes decreasing IgA affinity with time (showing a lack of affinity maturation). There was no significant difference in IgA affinities between 2 and 10 weeks in patients with IgAN.

investigated whether there was a link between levels of total serum IgA and IgA affinity. There was, however, no correlation between total serum IgA levels and IgA affinity in either patients with IgAN or controls (controls $r=0{\cdot}25$, $P=0{\cdot}28$; IgAN $r=0{\cdot}29$, $P=0{\cdot}20$).

Affinity maturation

In order to investigate the maturation of antibody affinity following immunization, serum IgG and IgA anti-TT affinities were measured again, 10 weeks after immunization. As expected, there was a significant increase in IgG affinity with time (a reduction in the leftward shift of the curve) for both controls and IgAN (Fig. 4), demonstrating IgG antibody affinity maturation. Similar to the results 2 weeks post-immunization, there was no significant difference between IgAN and controls in IgG anti-TT affinity (controls mean s.e.m. $\log_{10}$ shift $0{\cdot}06\pm0{\cdot}16$ *versus* IgAN $0{\cdot}10\pm0{\cdot}19$; $P=0{\cdot}1$).

There was, however, no affinity maturation of IgA antibodies. Indeed, a reduction in IgA antibody affinity in controls (indicated by an increase in the leftward shift of the curve) was observed (Fig. 5). Patients with IgAN also did not demonstrate IgA affinity maturation, with no significant difference in IgA affinities detected between 2 and 10 weeks (no decrease in IgA affinity with time was observed in IgAN).

Similar to the results obtained 2 weeks post-immunization, 10 weeks after immunization the IgA anti-TT antibodies produced by patients with IgAN were of significantly lower affinity compared with controls (controls mean $\pm$ s.e.m. $\log_{10}$ shift $0{\cdot}186\pm0{\cdot}026$ *versus* IgAN $0{\cdot}274\pm0{\cdot}024$; $P<0{\cdot}03$).

DISCUSSION

This study investigated the relative functional affinity of antibodies produced in IgAN. Following systemic immunization with TT, patients with IgAN produced lower affinity IgA antibodies than controls. This abnormality was specific to the IgA isotype, since the IgG antibody affinity did not differ from controls. The results confirm that IgG antibody affinity is independent of IgG antibody concentration, and that this also holds true for IgA affinity in controls. However, in IgAN, IgA affinity inversely correlated with IgA antibody levels, with high responding patients tending to produce large amounts of IgA antibodies of low average affinity.

The relative functional affinity of antibodies, as opposed to absolute affinities, was measured in this study. The use of DEA as the chaotropic agent has been shown to rank the affinities of antibodies in the same order as the equilibrium dialysis [25] and ammonium sulphate precipitation assay method of Steward & Petty [26,27]. Such methods require large amounts of antibody, and the use of DEA has proved to be a simple and convenient method of assessing relative functional antibody affinities [27]. The use of chaotropic agents in ELISA has been to measure antibody affinity in man and experimental animals to a wide range of antigens [22,23,25,27–29]. The use of chaotropic agents

to measure antibody affinity, as with all affinity assays, measures the average affinity of polyclonal antibodies which contain a mixture of both high- and low-affinity antibody.

Since IgG antibody affinity is independent of the type of antigen administered [15], it is likely that the same is true for IgA (although there is no direct information for this). Low-affinity IgA antibodies are therefore likely to be produced in response to all antigens in IgAN. Experimental evidence has shown that mice producing low-affinity IgG antibodies are more susceptible to glomerular immune complex deposition and glomerulonephritis [15,17,19]. The production of low-affinity IgA (but not IgG) antibodies demonstrated in this study may therefore result in the production of pathogenic IgA immune complexes, and may thus explain the predominance of IgA in the glomeruli in IgAN.

In experimental models of IgAN, the size of immune complexes has been shown to play a critical role in not only glomerular deposition but also the subsequent appearance of haematuria and proteinuria [30]. This is consistent with the observation that pathogenic complexes are large and insoluble, whereas non-pathogenic complexes tend to be relatively small and soluble [31]. Low-affinity IgA antibodies are thought to result in the formation of large immune complexes which are nephritogenic.

Abnormalities in IgG affinity have been demonstrated in a number of diseases, including low IgG antibody affinity in rheumatoid arthritis in man [32] and experimental animals [33] with large amounts of low-affinity antibody predicting severe arthritis. Low IgG affinity to viral antigens has been demonstrated in multiple sclerosis [29], and low-affinity anti-hepatitis B antibodies detected in the sera of patients with chronic liver disease [34]. Disease associations with abnormal IgA affinity, however, have not been previously reported.

As anticipated, affinity maturation of IgG anti-TT antibodies was observed between 2 and 10 weeks post-immunization in controls. This is an important parameter of secondary immune responses resulting in the long-term production of high-affinity IgG antibody. In contrast, IgA antibody affinity maturation was not observed during the same time period; indeed, there was a significant decrease in the IgA affinity in controls. Should a similar decrease in IgA affinity with time occur for other antigens, such a lack of IgA affinity maturation could explain the short-lived duration of the serum IgA response, and its relative unimportance in the systemic immune response compared with IgG. By 10 weeks post-immunization not only had the amount of IgA anti-TT fallen, but its affinity had also declined.

In IgAN, no abnormality in either IgG affinity or IgG affinity maturation was demonstrated. IgA affinity in IgAN was low, and remained significantly lower than controls at 10 weeks. Similar to controls, patients with IgAN showed no IgA antibody affinity maturation, although the decrease in IgA antibody affinity with time observed in controls was not apparent in IgAN. The lack of a decline in IgA affinity in IgAN may indicate a difference in IgA affinity maturation in this condition, or may simply reflect the low inherent affinity of the IgA antibodies, which is unable to be reduced further. Further experiments would be required to elucidate this point.

Affinity measurements in both experimental animals and man have concentrated mainly on IgG and IgM, with little information on IgA affinity. Primary (IgM) immune response is of low affinity, secondary (IgG) responses are of greater and increasing affinity [22]. IgG subclasses have been reported to have different patterns of affinities and affinity maturation [35,36]. Differences in antibody affinity and affinity maturation of different immunoglobulin isotypes may reflect diverse intrinsic affinity of each isotype, or that affinity maturation proceeds at differing rates, under different mechanisms of control.

The cellular control of antibody affinity includes the selective proliferation of B cells driven by a process of cellular competition for antigen, with B cells bearing high-affinity receptors preferentially stimulated when antigen becomes limiting [12]. Somatic mutation of germ line-derived variable region genes has been shown to be involved in the generation of high-affinity binding sites for subsequent selection by antigen [10]. T cells can control the production of high- and low-affinity responses [37,38], with $CD8^+$ cells playing an important role in the regulation of affinity maturation [39]. Interferon-gamma (IFN-γ) (compared with IL-1 which only increases antibody levels) has been shown to potentiate antibody affinity in mice, indicating a role played by Th1 cells, possibly acting by enhancing MHC class II expression [40]. Other factors such as dietary and hormonal levels, and reticuloendothelial function may also affect affinity [7]. The abnormal IgA affinity observed in IgAN in this study may therefore result from an abnormality intrinsic to IgA B cells, or from abnormalities in T cell activity, lymphokine production, or factors independent of immune function.

Following TT immunization in humans, both IgA1 and IgA2 antibodies are produced, although the vast majority of IgA antibody is of the IgA1 subclass [41]. The affinity measurement of IgA2 antibodies was not possible because of low IgA2 antibody concentration (data not shown), therefore preventing comparison of affinities of the two IgA subclass antibodies in this study. Shortly after systemic immunization, a substantial amount of serum polymeric IgA antibodies are produced [42]. This response is short-lived, and by 4 weeks after immunization serum polymeric IgA antibodies are undetected [42,43]. Both polymeric IgA and IgA1 antibodies have been demonstrated to be high in IgAN [43,44], and their relative contributions to overall antibody affinity need further investigation. While polymeric IgA would be expected to have higher functional affinity than monomeric IgA, at the time of maximal polymeric IgA production (2 weeks after immunization), overall IgA affinity was significantly lower in IgAN.

The route of immunization in experimental animals has been shown to influence the subsequent antibody affinity, with subcutaneous immunization resulting in higher IgG affinity antibody production compared with antigen administered via the peritoneum [39]. It is therefore possible that IgA produced at the mucosa is of different affinity and affinity maturation to systemic IgA, and that similar or more profound abnormalities in IgA affinity may exist at the mucosa in IgAN.

It is not known whether the mesangial IgA in IgAN has low or high affinity for antigen, or indeed whether the deposited IgA is produced in response to antigen challenge. The antibody measured in the circulation may not be the same as that deposited in the tissues [15,45], and therefore the affinity of the mesangial IgA in IgAN requires further investigation.

ACKNOWLEDGMENTS

L. L. is supported by the National Kidney Research Fund, and S.J.H. is a Wellcome Trust Medical Graduate Research Fellow, grant 034937/Z/91/Z/1.4G/DG/SC.

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