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Anti-mitochondrial antibody IgG subclass distribution and affinity in primary biliary cirrhosis

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SUMMARY

We have studied the total IgG subclass and anti-mitochondrial antibody (AMA) specific IgG subclass distribution in primary biliary cirrhosis (PBC) sera. In order to solve the problems caused by the differing affinities of subclass specific monoclonals and the competitive inhibition of antibodies in a whole serum assay, six sera were separated into subclass-specific fractions by affinity-depletion chromatography. AMA subclass distribution of 20 further sera from patients with PBC was assessed using conventional methods and the results were calibrated against one of the fractionated sera. Light chain distribution and AMA functional affinity were also assessed for the fractionated subclasses. Total amounts of IgG3 were significantly increased compared with normal controls. AMA were found in all IgG subclasses and not restricted predominantly to IgG3 as previously described. The functional affinity of IgG3 AMA is generally lower as compared with that of other subclasses. No light chain restriction was found.

Keywords Primary biliary cirrhosis IgG subclasses anti-mitochondrial antibodies pyruvate dehydrogenase complex

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic liver disease of unknown aetiology characterized by destruction of intrahepatic bile ducts, which eventually leads to liver failure. There is evidence of abnormal humoral immune function in PBC patients, for example high immunoglobulin and immune complex levels [1], and, more importantly, the specific presence of anti-mitochondrial antibodies (AMA), which is one of the major diagnostic features of PBC [2]. The role of AMA in the pathogenesis of PBC is unclear. AMA react with enzyme complexes on the inner mitochondrial membrane [3], the dominant antigen being the E_2 component of pyruvate dehydrogenase [4,5], but animals immunized with this antigen do not develop disease [6].

On the basis of differences in heavy chain, human IgG has been divided into four subclasses IgG1, IgG2, IgG3 and IgG4 [7]. There is heterogeneity in the biological properties of the four subclasses; for instance, IgG1 and IgG3 can fix and activate complement efficiently, whereas IgG2 and IgG4 fix complement poorly or not at all [8]. These differences may therefore be important in the pathological behaviour of autoantibodies [9]. IgG1, IgG2, IgG3 and IgG4 concentrations in normal serum constitute approximately 65%, 25%, 6% and 4% of total IgG, respectively [7], but the IgG subclass distribution of autoantibody activity is not necessarily in these proportions [10,11].

Previous studies of AMA isotype distribution showed that AMA are mainly in the IgG3 and IgM fractions [12–14]. These studies used four anti-human IgG subclass-specific MoAbs, and assays were performed on whole serum. This may introduce technical problems due to different binding characteristics of the subclass-specific MoAbs [15] and competitive inhibition between AMA of different IgG subclasses in whole serum, due to their different affinity and limiting amount of antigen.

To avoid the technical problems mentioned above, we have purified six PBC sera into single IgG subclass fractions by depletion of the unwanted IgG subclasses using affinity columns. The isolated IgG subclass fractions were used to test AMA binding and light chain distribution for each subclass, and to assay the relative functional affinity of AMA between different IgG subclasses. The AMA IgG subclass distribution was also investigated in whole sera from 20 further PBC patients, using purified IgG subclass fractions as standard controls.

MATERIALS AND METHODS

Blood samples

Twenty-six serum samples were selected from patients who met the diagnostic criteria of PBC. According to Scheuer's histologi-

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cal classification [16], four were stage I, 10 were stage II/III and 12 were stage IV. Eight samples from healthy individuals were used as controls.

MoAbs

Mouse anti-human IgG1 (HP6069), IgG2 (HP6014 and HP6002), IgG3 (HP6050 and HP6047) and IgG4 (HP 6025) MoAbs were employed in both assays and subclass isolation. Their specificity and reactivity have been described previously [17].

Antigen

Pyruvate dehydrogenase complex was purified as described elsewhere from bovine heart [18]. The complex contained $E1_A$, $E1_B$, E2 and E3 components [19], as judged by SDS–PAGE.

Affinity purification

Mouse anti-human MoAb affinity columns were used for IgG subclass isolation. The preparation of these columns and their absorptive capacity are detailed elsewhere [20]. For IgG1 isolation, the column was coupled with HP6047 (anti-IgG3), HP6025 (anti-IgG4) and two anti-IgG2 MoAbs (HP6014 and HP6002). The column for IgG2 preparation was coupled with HP6069 (anti-IgG1), HP6019 (anti-non-IgG2), HP6047 and HP6025, and IgG4 preparation used a column with HP6069, HP6014, HP6002 and HP6047. IgG3 was prepared by using a protein A column, which binds all IgG subclasses except IgG3. Fractions without IgG were prepared as control samples by passage through a protein G column to remove all IgG subclasses.

IgG subclass fractions were isolated from six PBC sera of different histological stage, one stage I, two stage II/III and three stage IV. To isolate each subclass, 200 μl of serum was loaded onto each column. Fractions (1 ml) were collected with buffer (0·5 M NaCl, 0·01 M Tris–HCl, pH 8·0) running at a flow rate of 1 ml 3 min. The peak fractions, as judged by absorbance at 280 nm, were pooled. Samples were dialysed against PBS, and stored with 0·01% sodium azide at 4°C. The columns were eluted with 2 bed volumes of 3 M NaSCN to remove the bound material.

ELISA

The concentration of IgG subclasses in whole serum and purity of the subclass preparations were estimated using a two-site immunoenzymatic assay [21]. Fractions contaminated with other IgG subclasses were passed again through the MoAb columns. For the ELISA, MoAbs anti-IgG1 (HP6069), anti-IgG2 (HP6014), anti-IgG3 (HP6050 and HP6047) or anti-IgG4 (HP6024), diluted 1:1000 in distilled water, were absorbed to Nunc Immuno plates (Nunc, Glostrup, Denmark). They were air dried at 37°C, and non-specific binding sites were blocked with PBS containing 10% fetal calf serum (FCS) and 0·1% azide. Samples in serial dilution were added and incubated for 2 h at room temperature. The assay was developed by adding 1:1000 sheep anti-human IgG conjugated with alkaline phosphatase (AP; Sigma) followed by *p*-nitrophenyl phosphate (disodium salt) 1 mg/ml (Sigma) dissolved in AP buffer (12 mM Na_2CO_3, 16 mM NaH_2CO_3, and 2 mM $MgCl_2$). The IgG subclass concentration were determined using the WHO International Standard (67/97) as a calibrator [17]. For the detection of AMA distribution in isolated IgG subclasses, an ELISA was developed by modifying procedures previously described [22]. Purified pyruvate dehydrogenase complex (100 μl at 7·5 μg/ml, diluted in 0·05 M sodium carbonate buffer, pH 9·3) was coated onto Nunc Immuno plates, and then blocked with PBS containing 10% FCS and 0·1% azide. By determining the IgG subclass concentrations in whole serum and in the purified fractions, it was possible to dilute the fractions so that each isotype was at dilutions from 1:200 to 1:30 000 with reference to the original serum concentration.

These dilutions were added and incubated at 37°C for 1 h. Bound antibody was detected with a mouse anti-human IgG MoAb HP6043, diluted 1:1000. HP6043 recognizes an epitope common to all four IgG subclasses [17] and has a reactivity identical to that of a polyclonal anti-IgG reagent (unpublished observations). Binding of this MoAb was detected by adding 1:1000 of goat anti-mouse IgG AP conjugate (Sigma). To assess the AMA IgG subclass distribution in 20 more PBC whole sera, one set of subclass fractions from a single serum was selected as a reference standard. Each unknown serum sample was diluted at 1:2500 and 1:5000, and incubated with antigen coated onto the plates. After incubation, HP6069 (anti-IgG1), HP6014 (anti-IgG2), HP6050 plus HP6047 (anti-IgG3) or HP6024 (anti-IgG4) at 1:1000 dilution were added, followed by an anti-mouse IgG AP conjugate, and then substrate.

The plates were washed four times between each incubation. The incubation time was 1 h at 37°C (except where otherwise shown).

Affinity assay

To determine the relative functional affinity of AMA in the IgG subclasses isolated from the six sera, a previously described method was followed [23]. This is based on the effect of the mild chaotropic agent DEA, which inhibits antibody binding to antigen inversely in proportion to the relative functional affinity of the antibody. For each isolated IgG subclass, serial dilutions were made in three panels; 15 mM and 30 mM DEA were used as inhibitors in two of the three panel dilutions, and the remaining panel was an uninhibited control. Binding to pyruvate dehydrogenase complex in the solid phase was then measured as above.

Light chain distribution in AMA

To detect the light chain distribution of AMA within IgG subclasses, the subclass-specific ELISA was modified. The isolated subclasses were diluted at 1:1000, and then incubated on antigen-coated plates. Anti-kappa (BAMO 3) or anti-lamda (BAMO 4) MoAb (Unipath) was added at 1:500 dilution, followed by AP-conjugated anti-mouse IgG and substrate. All of the incubations were performed at 37°C for 1 h.

RESULTS

Total IgG subclass distribution in whole serum

As shown in Table 1, there was an increase in the IgG1 ($P < 0·05$, Student's *t*-test) and IgG3 ($P < 0·01$) subclass concentrations in sera from PBC patients compared with healthy controls, whereas for IgG2 and IgG4 no significant differences were found between patients and controls. After correcting for multiple comparisons by multiplying the *P* values by 4, only the difference in IgG3 concentration remained significant. No significant correlation was found between serum IgG1 or IgG3 and serum total IgG (data not shown).

Table 1. IgG subclasses distribution in primary biliary cirrhosis (PBC)

	IgG1	IgG2	IgG3	IgG4
PBC sera (n = 26)				
Mean ± s.d.	10·23 ± 4·57*	4·40 ± 2·55	2·36 ± 1·34†	0·36 ± 0·29
Range (mg/ml)	2·38–19·14	1·44–12·06	0·79–4·68	0·033–0·89
Total IgG (%)	59	25	14	2
Control sera (n = 8)				
Mean ± s.d.	6·32 ± 1·83	4·37 ± 2·12	0·46 ± 0·10	0·24 ± 0·16
Range (mg/ml)	3·24–8·53	1·18–7·65	0·30–0·56	0·084–0·51
Total IgG (%)	55	39	4	2

* $P < 0·05$ compared with healthy controls.
† $P < 0·01$ compared with healthy controls.

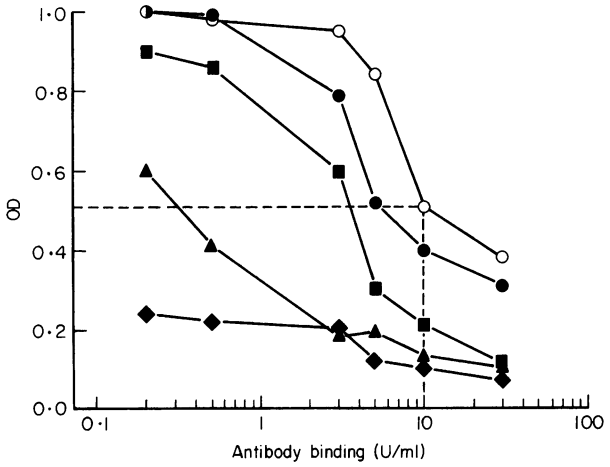

Fig. 1. Anti-mitochondrial antibody (AMA) IgG subclass distribution of a PBC serum (sample 1) determined in an ELISA using a MoAb to human IgG Fc. Dilution curves of AMA activity in the four isolated IgG subclasses and serum were obtained by plotting an optical density (OD) *versus* log dilution. The AMA activity of serum at the point of half-maximal binding (in this case, 0·5 absorbance units, at 405 nm) was designated at 10 U/ml. The AMA activity in IgG subclass fractions could be calculated as follows, 2·9 U/ml for IgG1, 0·2 U/ml for IgG2, 6·9 U/ml for IgG3, and less than 0·1 U/ml for IgG4. ■, IgG1; ▲, IgG2; ●, IgG3; ◆, IgG4; ○, serum.

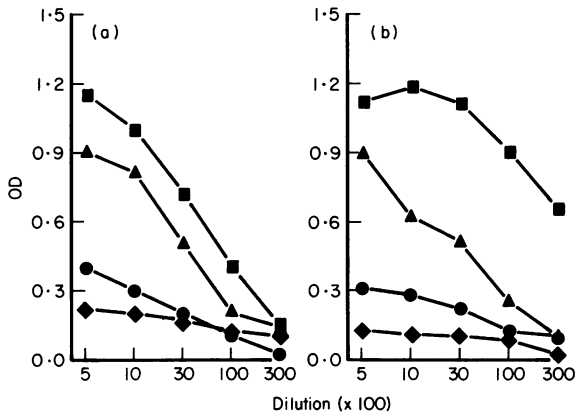

Fig. 2. The difference between anti-mitochondrial antibody (AMA) IgG subclass distribution as the result of using the two detection systems. Dilution curves in (a) and (b) were from the same patient and at the same dilutions: (a) using a single anti-human IgG Fc MoAb, assayed on subclass fractions; (b) using four IgG subclass-specific MoAbs for detecting the binding of AMA, assayed on whole serum. ▲, IgG1; ●, IgG2; ■, IgG3; ◆, IgG4.

Table 2. Anti-mitochondrial antibody activity in IgG subclasses of fractionated samples as a percentage of summed total of all classes

Patient no.	IgG1	IgG2	IgG3	IgG4
1	29	2	69	< 1
2	59	13	10	18
3	54	26	19	< 1
4	46	52	1	< 1
5	68	5	23	4
6	25	38	24	13

AMA IgG subclass distribution

IgG subclasses were isolated from six samples selected from the 26 sera mentioned above. Each isolated subclass was more than 98% pure when tested by two-site immunoenzymatic assay. This purity was achieved by passage for IgG2 and IgG3 once, and IgG1 and IgG4 twice through the respective columns.

AMA activity in the subclass fractions was assessed in the following manner. Each fraction was used to construct a dilution curve such that the relative concentrations of each subclass were in the same proportion as in whole serum (based on the results for total IgG in the immunoenzymatic assay). The half-maximum absorbance of whole serum, assayed in parallel, was used to define the point at which the antibody activity distribution was read in U/ml from the subclass fraction binding curves (Fig. 1). The AMA percentage of each subclass was calculated from the summed units of all four subclasses. The summed AMA units were approximately equal to that contained in serum. Non-IgG fractions were used to control for background binding. As shown in Table 2 AMA were found in all four IgG subclasses; no consistent pattern of AMA IgG subclass distribution was found in the six fractionated samples.

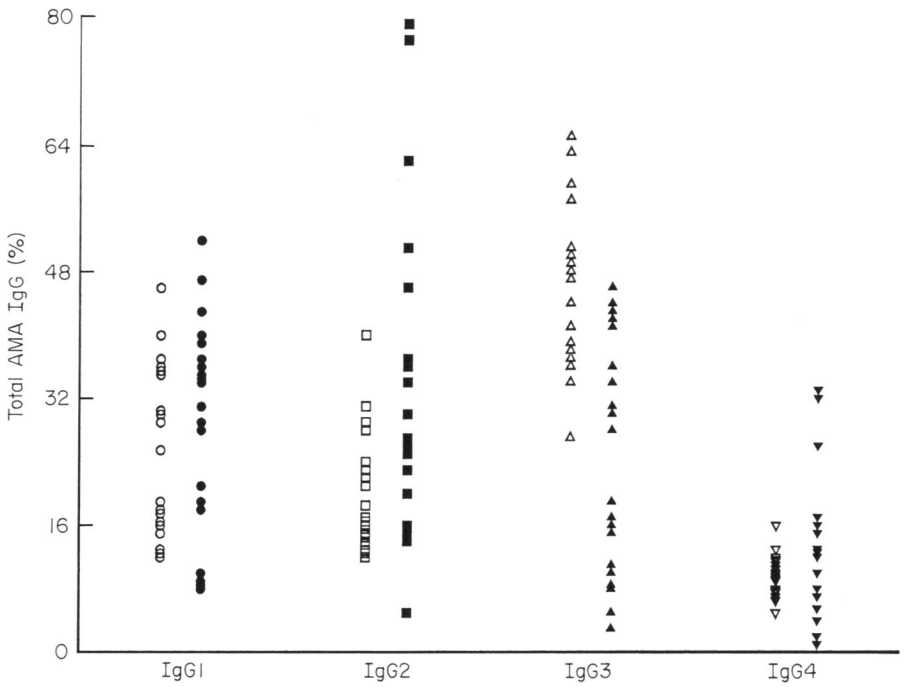

Fig. 3. Comparison of anti-mitochondrial antibody (AMA) IgG subclass distribution from 20 sera before (open symbols) and after (solid symbols) calibration against the standard curves.

Table 3. Relative functional affinity of anti-mitochondrial antibodies in IgG subclass fractions

Patient no.	IgG1	IgG2	IgG3	IgG4
1	0·90	0·30	1·00	*
2	0·48	0·52	1·10	0·38
3	0·44	0·25	0·52	0·56
4	0·59	0·15	0·41	0·29
5	0·11	0·34	0·59	*
6	0·30	0·26	0·32	0·46

Results are the log shift in the dilution curve produced by 30 mM DEA at half-maximal binding.

* Activity too low for affinity determination.

By calculation of the area under the dilution curves, the same results were obtained.

To compare the conventional and our method for detection of AMA subclass distribution, AMA activity in fractionated sample 1, which has relatively parallel AMA IgG subclass dilution curves, was assayed at the same dilution in the two assay systems. The two assay systems differ in detecting the AMA binding by using either a single anti-human Fc MoAb to detect binding from the subclass fractions or using four anti-human subclass-specific MoAbs to detect binding from whole serum. The AMA distribution differed in the two assays, as shown in Fig. 2. IgG3 AMA were much higher in the conventional system using the four subclass-specific MoAb system.

We then assayed subclass-specific AMA activity in 20 unfractionated whole sera (Fig. 3). In order to correct for the difference introduced by the varying affinities of these subclass-specific MoAbs (compare Fig. 2a and 2b), we used the subclass distribution of AMA activity from serum 1, derived from Fig. 1, to recalibrate the curves shown in Fig. 2b. To do this, a 1:100 dilution of the IgG4 fraction was arbitrarily assigned an AMA activity of 1 U/ml; therefore, 1:100 dilutions of IgG1, IgG2 and IgG3 would contain 29, 2 and 69 U/ml, respectively (from Table 2). These values, and the corresponding subclass dilutions, were then used to construct individual abscissa (in U/ml) for each of the subclass curves shown in Fig. 2b. It was then possible to convert the optical density to U/ml for each individual subclass at one particular dilution and derive the corrected percentage distribution of AMA in whole sera (Fig. 3).

AMA relative functional affinity

The functional affinity of AMA was tested in the isolated subclasses and expressed as the log leftward shift. The log shift was measured as the distance between the two points of 50% maximal binding in the log dilution working curves, with and without DEA [23]. The shorter the distance of this shift, the greater the relative functional affinity. The results in Table 3 were obtained using 30 mM DEA dilution curves because 15 mM DEA gave a much smaller shift. The affinity of AMA in IgG4 of samples 1 and 5 could not be measured because activity of the IgG4 AMA in these two samples was too weak to produce meaningful dilution curves.

AMA light chain distribution

The absorbances given by ELISA using kappa and lambda light chain-specific MoAbs were summed. Results were calculated as the percentage contribution of each light chain to the total absorbance in a given IgG subclass. The mean percentage distribution of lambda light chain reactivity of AMA in the fractions was $36{\cdot}17 \pm 3{\cdot}54$ for IgG1, $36{\cdot}5 \pm 4{\cdot}32$ for IgG2, $34{\cdot}5 \pm 4{\cdot}32$ for IgG3 and $33{\cdot}67 \pm 2{\cdot}73$ for IgG4; for kappa

reactivity, the mean percentage distribution was 63·83 ± 3·54 for IgG1, 63·5 ± 4·32 for IgG2, 65·5 ± 4·32 for IgG3 and 66·33 ± 2·73 for IgG4. No individual subclass fractions were found to consist of only a single light chain type.

DISCUSSION

We found that the mean total concentrations of both IgG1 and IgG3 were increased in our patients (1·6-fold and 5·10 fold, respectively), but that after correcting for multiple comparison, only the concentration of IgG3 was significantly increased. This is consistent with previous studies [14,24,25]. Since the contribution of IgG1 to total IgG in our PBC sera was 4·2 times that of IgG3, the raised total IgG concentration must be due to increases of both IgG1 and IgG3. However, for individual sera there was no correlation between levels of IgG1 or IgG3 and total IgG.

The analysis of antigen-specific IgG subclass distribution using whole sera in solid-phase assays is subject to a number of problems: (i) there is competition between subclasses reflecting differing affinities for the target antigen; (ii) the behaviour of anti-human subclass reagents changes in different assay systems; and (iii) the assay does not allow assessment of relative subclass affinity. In order to address these problems, we isolated IgG subclasses by affinity-depletion chromatography and then quantified subclass binding using a common detection system. We found AMA activity in all four IgG subclasses, with the proportion of AMA in each IgG subclass varying greatly in different individuals.

As shown in Fig. 2, the use of MoAbs resulted in a much higher AMA IgG3 activity, compared with the fractionated subclasses assayed separately, using a common anti-human Fc detection system. After correction for this, in the 20 whole sera, IgG3 was no longer the predominant subclass, its proportion being exceeded by those of IgG1 and IgG2 (Fig. 3).

In the six fractionated sera, we used serial dilution curves rather than a single dilution for measuring the AMA distribution. As shown in Fig. 1, the AMA dilution curves are not perfectly parallel, particularly in the case of IgG4. This introduces a further problem, not always addressed in previous studies, in that choosing a different single dilution of sera will change the pattern of subclass distribution. There is no ideal solution to this, and we arbitrarily determined the AMA activity units by choosing the point of half-maximal binding, at which point the dilution curves are relatively parallel. Choosing a different point would not have altered significantly our finding that the contribution of IgG3 is overestimated by using an assay based on subclass-specific MoAb.

Affinity is a major determinant of the biological effect of an antibody [26] and the isolation of IgG subclasses gives additional advantage in the affinity analysis of antibodies, because it avoids competitive inhibition between antibodies of different subclass. IgG3 AMA generally showed lower affinity than the other subclasses, but no other consistent pattern was found in the six tested samples.

AMA are present in more than 95% of PBC patients, but their role (if any) in pathogenesis remains unknown. Other autoantibodies which are known to be pathogenic, such as anti-glomerular basement membrane antibodies and anti-acetylcholine receptor antibodies, are often restricted to the IgG1 and IgG3 subclasses which are particularly efficient at fixing complement [10,27]. The heterogeneity of AMA may suggest that AMA are less important in pathogenesis.

The light chain distribution of AMA showed the same proportion between subclasses, indicating that the AMA were not of oligoclonal or monoclonal origin. This is consistent with a previous study which found no evidence for clonally restricted synthesis of IgG3 by PBC blood lymphocytes [28]. Thus AMA secreting B cells are not the result of clonal expansion of one (or a few) B cells.

We have found that (i) anti-human subclass-specific MoAbs may bias the results of autoantibody subclass distribution assays due to their variable affinity. This can be compensated for by using affinity depletion chromatography and a single anti-human Fc monoclonal detection system; (ii) dilution curves of autoantibodies are important in the analysis of subclass distribution, particularly for antibodies which have significantly different distribution in individual subclasses; (iii) AMA IgG subclass distribution is heterogeneous; (iv) the AMA affinity of IgG3 is generally lower than that of other subclasses and (v) AMA were not light chain restricted.

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REFERENCES

1 Sandilands GP, Reid FM, Galbraith I, *et al.* Immune-complex inhibition of lymphocyte Fc-receptors in primary biliary cirrhosis: a possible immunomodulatory mechanism. Lancet 1980; **ii**:9–13.
2 Mitchison HC, Bassendine MF, Hendrick A, *et al.* Positive antimitochondrial antibody but normal alkaline phosphatase: is this primary biliary cirrhosis? Hepatology 1986; **6**:1279–84.
3 Berg PA, Klein R, Lindenborn-Fotison J, *et al.* ATPase-associated antigen (M2): marker antigen for serological diagnosis of primary biliary cirrhosis. Lancet 1982; **ii**:1423–6.
4 Yeaman SJ, Fussey SPM, Danner DJ, *et al.* Primary biliary cirrhosis: identification of two major M2 mitochondrial autoantigens. Lancet 1988; **i**:1067–70.
5 Bassendine MF, Yeaman SJ. Primary biliary cirrhosis: nature of autoantigens. Springer Semin Immunopathol 1990; **12**:73–83.
6 Krams SM, Surh CD, Coppel RL, *et al.* Immunization of Experimental animals with dihydrolipoamide acetyltransferase, as a purified recombinant polypeptide, generates mitochondrial antibodies but not primary biliary cirrhosis. Hepatology 1989; **9**:411–16.
7 Grey HM, Kunkel HG. 11 chain subgroups of myeloma proteins and normal 7S gamma globulin. J Exp Med 1964; **120**:253–66.
8 Burton DR. Immunoglobulin G: functional sites. Mol Immunol 1985; **22**:161–206.
9 Jayne DRW, Weetman AP, Lockwood CM. The IgG subclass distribution of anti-neutrophil cytoplasm autoantibodies in systemic vasculitis. Clin Exp Immunol 1991; **84**:476–81.
10 Bowman C, Ambrus K, Lockwood CM. Restriction of human IgG subclass expression in the population of auto-antibodies to glomerular basement membrane. Clin Exp Immunol 1987; **69**:341–9.
11 Davies TF, Weber CM, Wallack P, *et al.* Restricted heterogeneity and T Cell dependence of human thyroid autoantibody immunoglobulin G subclasses. J Clin Endocrinol Metab 1986; **62**:945–9.
12 Feltkamp TE. HLA and uveitis. Int Ophthalmol 1990; **14**:327–33.

13 Nouri-Aria KT, Hegerty JE, Neuberger J, *et al. In vitro* studies on the mechanism of increased serum IgM levels in primary biliary cirrhosis. Clin Exp Immunol 1985; **61**:297–304.
14 Surh CD, Cooper AE, Coppel RL, *et al.* The predominance of IgG3 and IgM isotype antimitochondrial antibodies against recombinant fused mitochondrila peptide in patients with primary biliary cirrhosis. Heptatology 1988; **8**:290–5.
15 Weetman AP, Cohen SB. The IgG subclass distribution of thyroid autoantibodies. Immunol Lett 1986; **13**:335–41.
16 Hostikka SL, Eddy RL, Byers MG, *et al.* Identification of a distinct type IV collagen α chain with restricted kidney distribution and assignment of its gene to the locus of X chromosome-linked Alport syndrome. Proc Natl Acad Sci USA 1990; **87**:1606–10.
17 Jefferis R, Reimer CB, Skavaril F, *et al.* Evaluation of monoclonal antibodies having specificity for human IgG subclasses: results of an IUIS/WHO collaboration study. Immunol Lett 1985; **10**:223–52.
18 Stanley CJ, Perham RN. Purification of 2-oxo dehydrogenase multienzyme complexes from ox heart by a new method. Biochem J 1980; **191**:147–54.
19 Yeaman SJ. The 2-oxo acid dehydrogenase complexes: recent advances. Biochem J 1989; **257**:625–32.
20 Weetman AP, Black CM, Cohen SB, *et al.* Affinity purification of IgG subclasses and the distribution of thyroid autoantibody reactivity in Hashimoto's thyroiditis. Scand J Immunol 1989; **30**:73–82.
21 Black CM, Plikaytis BD, Wells TW, *et al.* Two-site immunoenzymatic assays for serum IgG subclass infant/maternal ratios at full-term. J Immunol Methods 1988; **106**:71–81.
22 Schardt CW, McLachlan SM, Matheson J, *et al.* An enzyme-linked immunoassay for thyroid microsomal antibodies. J Immunol Methods 1982; **55**:155–60.
23 Gorevic PD, Kassab HJ, Levo Y, *et al.* Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. Am J Med 1980; **69**:287–308.
24 Riggione O, Stokes RP, Thompson RA. Predominance of IgG3 subclass in primary biliary cirrhosis. Br Med J 1983; **285**:1015–16.
25 Floreani A, Bird P, Mitchison H, *et al.* Abnormal subclass distribution of immunoglobulin in primary biliary cirrhosis. Ital J Gastroenterol 1987; **19**:325–8.
26 Devey ME, Lee SR, Le Page S, *et al.* Serial studies of the IgG subclass and functional affinity of DNA antibodies in systemic lupus erythematosus. J Autoimmunity 1988; **1**:483–94.
27 Nielsen FC, Rodgaard A, Djurup R, *et al.* A triple antibody assay for the quantitation of plasma IgG subclass antibodies to acetylcholine receptors in patients with myasthenia gravis. J Immunol Methods 1985; **83**:249–58.
28 Bird P, Calvert JE, Mitchison H, *et al.* Lymphocytes from patients with primary biliary cirrhosis spontaneously secrete high levels of IgG3 in culture. Clin Exp Immunol 1988; **71**:474–80.