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Immunoglobulin class and IgG subclass distribution of anticardiolipin antibodies in patients with systemic lupus erythematosus and associated disorders

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SUMMARY

The class and subclass distribution of an antibody response may give insight into the stimulating mechanism and likely effector functions. IgA, IgG and IgM anticardiolipin antibodies (aCL) were quantified in a consecutive series of 200 samples sent to an autoimmune serology laboratory to determine the relationships between aCL responses of each of these antibody classes and, in particular, whether there was any utility in the measurement of IgA aCL. Positive results for one of the three aCL isotypes were found in 105 samples (53%), and in 41 samples IgA aCL was detected (21%). However, amongst these unselected samples, little additional information was obtained by measurement of IgA aCL, which was found in conjunction with IgM or IgG aCL in all but five samples, and in these the isolated elevation of IgA aCL was only slight, and showed no disease specificity. The levels of each of the four IgG subclasses of aCL were measured in a subgroup of serum samples from 28 patients with autoimmune disease and from 29 patients with syphilis. Amongst the SLE patients IgG1 and IgG3 aCL were the predominant IgG subclasses, consistent with an antigen-driven, T cell-dependent antibody response. However, a subgroup of eight of the autoimmune subjects had predominant elevation of IgG2 aCL, possibly implying a role for T cell-independent antibody production to cardiolipin. Amongst the syphilis patients IgG1 and IgG3 aCL were also the predominant subclasses of aCL but IgG4 aCL were also detected in the majority of subjects, consistent with prolonged antigenic stimulation.

Keywords anticardiolipin antibodies IgG subclass SLE syphilis

INTRODUCTION

In patients with SLE and associated autoimmune disorders, elevated levels of antiphospholipid antibodies (aPL) and, in particular, of antibodies against cardiolipin (aCL) are commonly detected. It is not certain whether the clinical syndrome associated with the presence of these antibodies is associated with a particular antibody isotype. Raised levels of IgG aCL have been associated with recurrent venous and arterial thromboses, fetal loss, thrombocytopenia and neurological disorders [1–3]. In contrast, the IgM aCL isotype has been reported to be associated with autoimmune haemolytic anaemia and livedo reticularis [4–6]. For IgA aCL, no definite clinical association has been established, and elevation of IgA aCL appears to usually occur in conjunction with IgG and/or IgM aCL [7]. There has been a single report of an association between IgA aCL and severe Guillain–Barré syndrome [8], and IgA aCL has been identified in some patients with acute infections [9].

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Patients with SLE and associated disorders usually have raised levels of other autoantibodies, such as anti-dsDNA antibodies and antibodies against extractable nuclear antigens (ENAs). The isotypes of both of these groups of autoantibodies have been extensively analysed, and their IgG subclass ditribution has been reported to be mainly restricted to the IgG1 and IgG3 subclasses [10–12], compatible with an antigen-driven antibody response. Although the IgG aCL isotype appears to be most strongly associated with clinical complications, the distribution of the IgG aCL subclasses has not been clearly established. Results from the studies so far have been conflicting and almost all permutations of elevations of the different IgG subclasses have been reported. It has been reported that there is an elevation of all four subclasses of IgG aCL [13], selective increase in IgG1 and IgG3 [14], selective increase in IgG1 and IgG2 [15], and selective increase in IgG2 and IgG4 [16].

We have measured levels of the three aCL isotypes, IgG, IgM and IgA in 200 patient and 100 normal sera, and have studied retrospectively fluctuations in the levels of the three isotypes over periods of up to 48 months. Anticardiolipin

antibodies are often present in syphilis patients, where their specificity is similar but not identical to that of the aCL found in SLE [17], and we have compared the distribution of the IgG aCL subclasses in 28 SLE and 29 syphilis patients. Our aims were to assess: (i) whether aCL in SLE and syphilis patients have a similar subclass distribution, (ii) whether a particular subclass predominates in either of the patient groups, and (iii) whether the presence of a particular subclass has any relationship with total IgG aCL, or with another subclass.

MATERIALS AND METHODS

Serum samples

IgG, IgM and IgA aCL estimations were performed on 200 consective patient sera which were sent to the laboratory for routine aCL estimation, and on 100 normal blood donor sera (kindly provided by Miss Barbara Cant, South Thames Blood Transfusion Centre). The patient sera included 84 samples derived from patients with SLE and 35 from patients with one or more features of the primary antiphospholipid syndrome [18]. In five patients positive for IgG and/or IgM aCL, retrospective serial samples over periods from 9 to 48 months were studied for all three aCL isotypes.

For the IgG aCL subclass study, sera from 28 patients with autoimmune diseases (18 SLE, six primary antiphospholipid syndrome, three unclassified connective tissue disease and one myasthenia gravis), selected to cover a range of positive aCL values from weakly to strongly positive, and from 29 patients with syphilis (kindly provided by Ms H. Wong, STD Lab., PHLS, St Georges Hospital, Tooting, London, UK) were studied.

ELISA for estimation of IgG, IgM and IgA aCL levels

One hundred μl of serum diluted 1:100 in phosphate buffered saline containing 10% fetal calf serum (PBS/FCS) were added to each of three microtitre plates for the simultaneous estimation of all three aCL isotypes.

(a) IgG and IgM aCL were measured using a previously described method [19]. The cut-off for both the IgG and IgM aCL isotypes was set at 5 s.d. above the mean level of 135 normal controls, and a sample was considered positive when it reached $>9{\cdot}0$ units for IgG and $>8{\cdot}0$ units for IgM aCL.

(b) IgA aCL was measured by a modification of the IgG and IgM aCL method; the main change being that the goat anti-human IgA first antibody (Product: 4101; Tago Incorporated, Burlingame, CA) was added at a concentration of 1 μg/well, and this was followed by addition of rabbit anti-goat IgG alkaline phosphatase conjugate (Product: A7650; Sigma Chemical Co, Poole, UK), at a dilution of 1:500, instead of 1:1000 which was the dilution used for the IgG and IgM aCL assay. A standard serum with previously determined high IgA aCL levels was used in each assay to construct a standard curve, from which levels in the samples and controls were read. The standard serum was used at doubling dilutions from 1:100 down to 1:3200, which were used to define arbitrary units, set from 100 (1:100 dilution) down to 1·56 (1:3200 dilution) IgA aCL units. For IgA aCL, a positive result was defined as greater than 5 s.d. above the mean value of the 100 normal blood donor sera, and this corresponded to 10·5 units of IgA aCL. For monitoring the reproducibility of the IgA assay, a normal IgA aCL serum pool (8·8 units, 1 s.d. = 0·60 units), and a moderate positive pool (25·1 units, 1 s.d. = 2·1) were also estimated in triplicate on each microtitre plate.

IgG subclass study

(a) ELISA for quantification of aCL IgG subclasses. The mouse MoAb used in all the subclass studies, HP 6069 against IgG1, HP 6014 against IgG2, HP 6050 against IgG3 and HP 6025 against IgG4 were kindly donated by Dr C. B. Reimer, (CDC, Atlanta, GA). The specificity and reactivity of these monoclonals has been described elsewhere [20,21].

The ELISA method used for IgG aCL subclass estimation was a modification of a previously described aCL method [19], combined with the method of Weetman & Cohen [22] for the determination of the IgG subclasses of thyroid autoantibodies. After coating the microtitre plates with the cardiolipin antigen, blocking and addition of serum samples, 1:1000 dilutions of the four separate subclass monoclonals were added; the plates were incubated overnight at 4°C to achieve maximum binding of the anti-IgG subclass antibodies. The remaining steps of the ELISA were the same as for the standard aCL assay, except for the incubation step with anti-mouse IgG conjugate, which was left for 2, instead of 1 h. The absorbances given by each of the four IgG subclass-specific monoclonals at a single time point (120 min), were summed and individual subclasses were expressed as a percentage of this total. A normal serum pool was included as a control in every assay on every plate.

(b) Affinity purification of IgG subclasses. In three of the autoimmune patients (two SLE, one myasthenia gravis), the different IgG subclasses from the sera were fractionated into the individual IgG subclasses by complete depletion of the other IgG subclasses, by exclusion affinity purification on Sepharose 4B columns, according to the method of Weetman *et al.* [23]. The purity of the affinity purified subclass preparations was checked by two-site immunoenzymometric assay, to determine total IgG subclass concentrations as previously described [24].

Total serum IgA. Total serum IgA was measured using a standard Technicon Immunoturbidimetric technique.

Statistics. Non-parametric tests were used throughout. Statististical significance was determined using Spearman rank correlation (RSp) and the χ^2 test.

RESULTS

Validation of IgA aCL ELISA

Intra-assay coefficients of variation were obtained by testing the same serum samples eight times on the same microtitre plate, and were found to be 9·5% for the normal pool, and 4·4% for the moderate-positive IgA pool.

Inter-assay coefficients of variation were calculated from 10 different assays during a period of 2 months, and these were 6·8% for the normal pool and 8·3% for the moderate positive pool.

No appreciable non-specific binding was found in the IgA aCl assay when purified human IgA at concentrations ranging from 3·1 to 200 μg/ml were tested in the IgA aCL ELISA; all patient and normal control sera were tested on microtitre plates coated with ethanol instead of cardiolipin, and non-specific binding generally accounted for $<10\%$ of total binding.

Table 1. Distribution of the IgG, IgM and IgA aCL isotypes in 105 patients positive for aCL antibodies

aCL isotype	No. positive	% positive
IgG only	27	25·7
IgM only	28	26·6
IgA only	5	4·8
IgG + IgM	9	8·6
IgG + IgA	10	9·5
IgM + IgA	3	2·9
IgG + IgM + IgA	23	21·9

Table 2. Characteristics of 41 patients positive for IgA aCL

No. of patients	Diagnosis	+ ve IgA only
29	SLE	2
6	PAS	—
2	Stroke	—
2	CRF	1
1	Recur. thromb.	—
1	Dementia	1

PAS, primary antiphospholipid syndrome; CRF, chronic renal failure.

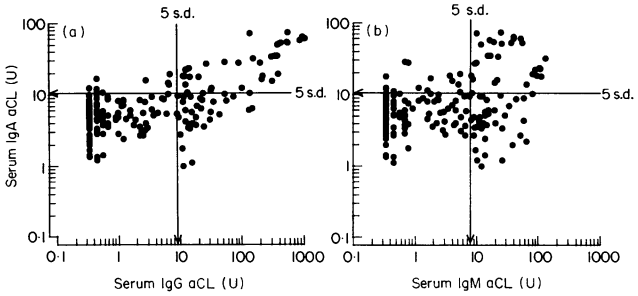

Fig. 1. Correlations between levels of aCL of different isotypes in 200 consecutive patient sera. (a) IgA and IgG; (b) IgA and IgM anticardiolipin antibodies (aCL). Results are expressed in aCL units. The cut-off point between normal and abnormal results for each isotype was set at 5 s.d. above a normal mean, derived from 100 normal blood donors. This is shown by the vertical (IgG or IgM) and horizontal (IgA) arrows.

Prevalence of aCL isotypes

Elevated aCL of one or more isotypes were found in sera from 105 of the 200 patients, 41 of which were positive for IgA aCL. The distribution of the three aCL isotypes in the 105 positive patients is shown in Table 1, and the disease characteristics of the 41 IgA aCL positive patients are shown in Table 2. The IgA isotype was the sole aCL isotype positive in only five (4·8%) of

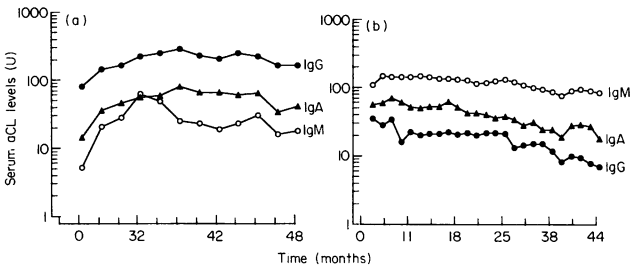

Fig. 2. Serial serum levels of IgG, IgM and IgA aCL levels in two patients during 48 (a) and 44 (b) months of follow-up. IgG aCL (●); IgM aCL (○); IgA aCL (▲).

the 105 aCL-positive patients. In the remaining 36 (34·3%) of the IgA aCL-positive patients, IgA was positive in conjunction with the IgG and/or IgM aCL isotypes (Table 1), and in these patients IgA was positive at a much lower titre than the IgG or IgM isotypes (Fig. 1a, b).

When levels of the three aCL isotypes were compared, a strong positive correlation was found between IgA and IgG aCL (RSp = 0·540, $P < 0·0001$, Fig. 1a), and a positive but weaker correlation was found between IgA and IgM aCL (RSp = 0·287, $P < 0·001$, Fig. 1b).

In the 105 aCL-positive patients, total IgA levels were measured, and were found to be elevated in 13 (12·4%) of these patients. There was no correlation between total IgA immunoglobulin levels and IgG, IgM or IgA aCL levels (data not shown).

Longitudinal aCL studies

Sera from five patients were collected over periods ranging from 9 to 48 months and analysed for IgG, IgA and IgM aCL isotypes. Three had IgG as the highest isotype, and two had IgM as the highest aCL isotype. In each of the five patients, levels of all three aCL isotypes followed parallel patterns, and in the three subjects where IgG was the highest isotype present, the hierarchy for the three aCL isotypes was IgG > IgA > IgM (Fig. 2a), whereas in the two where IgM was the highest isotype this was IgM > IgA > IgG (Fig. 2b).

IgG aCL subclass distribution in autoimmune (SLE) and syphilis patients

Levels of each of the IgG aCL subclasses were estimated in sera from 28 patients with autoimmune disease (18 SLE, six primary antiphospholipid syndrome, three unclassified connective tissue disease and one myasthenia gravis), and compared to those found in 29 syphilis patients. Total IgG aCL in the autoimmune group ranged from 9 to 375 units, and in the syphilis sera from 0·5 to 130 units; total IgM aCL levels were 2–130 units and 3–95 units respectively.

The results for the IgG subclasses expressed as a percentage of the total aCL-specific IgG, are shown for the SLE group in Fig. 3a and for the syphilis group in Fig. 3b. All patients in both groups had detectable levels of IgG1 subclass aCL antibodies; and 21/28 (75·0%) of the SLE group and 25/29 (86·2%) of the syphilis group had aCL IgG1 levels which were within or above the normal range for this subclass in whole IgG (normal range 60–70%). IgG2 subclass antibodies were detected in 19 (67·9%) of patients in the SLE group and in 28 (96·6%) of the syphilis patients; of these 8/28 (28·6%) autoimmune patients and 4/29

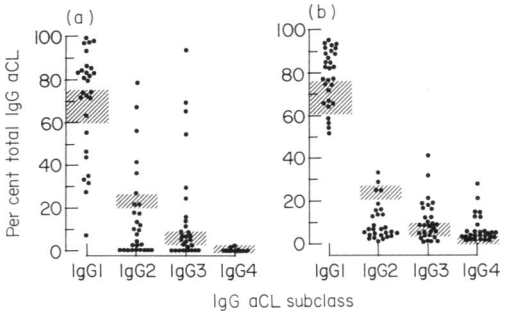

Fig. 3. Distribution of IgG aCL subclasses in 28 autoimmune (a) and 29 syphilis (b) patients. The cross-hatched areas represent normal ranges for each IgG subclass in total IgG from normal sera.

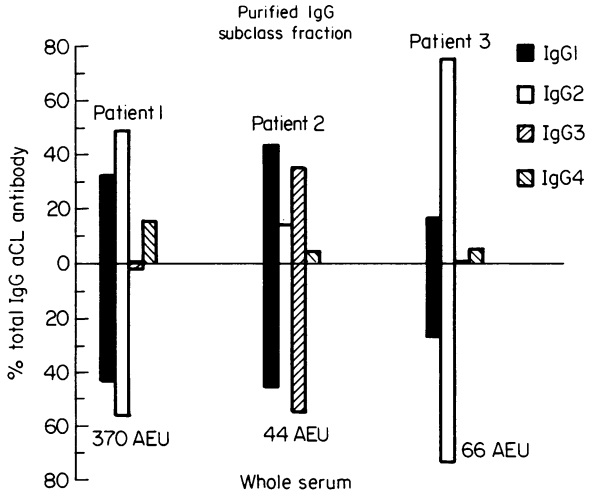

Fig. 4. Measurement of IgG subclass distribution of anticardiolipin antibodies in three patients studied by measurement in whole serum (lower part of panel) and individually purified IgG subclasses (by immunoaffinity exclusion chromatography) (upper part of panel). The percentage of aCL antibody reactivity represented in each subclass is shown for the three patients studied. Total IgG aCL levels for each patient are shown below.

(13·8%) syphilis patients had normal or above normal levels of IgG2 aCL compared to the distribution of this subclass in total IgG (normal range 20–25%). IgG3 aCL antibodies were detected in 25 (89·3%) of the SLE group and in 25 (86·2%) patients with syphilis; antibodies of this subclass were present at levels within or above the expected proportion in 19/28 (67·9%) of the autoimmune patients, and in 23/29 (79·3%) of the syphilis patients (normal range 5–10%); IgG4 on the other hand was undetectable in 26/28 (92·9%) of the autoimmune group patients, whereas it could be detected in all but one (96·6%) of the syphilis sera (normal range $< 5\%$).

As aCL levels were not normally distributed, non-parametric correlations were performed using Spearman rank correlations. Strong correlations were found between total IgG aCL and those of subclasses IgG2 ($RSp = 0{\cdot}70$, $P < 0{\cdot}0001$) and IgG3 ($RSp = -0{\cdot}790$, $P < 0{\cdot}001$) for the SLE patient group, and between total IgG aCL and those of subclasses IgG3 ($RSp = 0{\cdot}444$, $P < 0{\cdot}016$) and IgG4 ($RSp = -0{\cdot}494$, $P < 0{\cdot}006$) for the syphilis group. Correlations were also sought between the different IgG subclasses, and a strong negative correlation was found between IgG1 and IgG2 subclass aCL antibodies ($RSp = -0{\cdot}659$, $P < 0{\cdot}0001$) in the SLE group, and between IgG1 and IgG2 ($RSp = -0{\cdot}607$, $P < 0{\cdot}001$), and IgG1 and IgG4 subclass aCL antibodies ($RSp = -0{\cdot}582$, $P < 0{\cdot}001$) for the syphilis patients.

In order to confirm the results of the assay of the different aCL IgG subclasses, in three of the autoimmune patients, each of the four IgG subclasses were purified by using MoAb on Sepharose B affinity columns, as described above. The resulting IgG fractions were more than 99% pure for each subclass, when tested by two-site immunoenzymometric assay. The starting sera and purified IgG subclasses were then assayed using the IgG subclass-specific aCL ELISA. The results are shown in Fig. 4. From these it may be seen that there was a close correlation between the quantities of aCL of the different IgG subclasses measured in whole serum compared with purified IgG of the different subclasses. The exception was IgG4 aCL, which was only readily measured in the purified IgG4 fraction (Fig. 4).

On close examination of the aCL IgG subclass distribution of the entire autoimmune patient group, it became apparent that all but one of the patients in this group who had IgG2 levels of $> 20\%$ had total IgG aCL levels of > 50 units ($n = 14$), and that all but one of the patients with total IgG aCL levels of < 50 units had IgG3 levels of $> 5\%$ ($n = 14$). For the syphilis patients no marked differences were seen between total IgG aCL levels and IgG aCL subclass distribution.

DISCUSSION

We have assayed aCL of the IgG, IgM and IgA isotypes in a series of 200 consecutive samples sent to the laboratory for aCL estimation. Amongst these samples there was little additional information gained from the assay of IgA aCL, which was found in conjunction with IgG or IgM aCL in all but five samples, in which relatively low levels (10·8–15 units) of IgA aCL were found in isolation. Recent reports on the prevalence and clinical significance of elevated levels of the three aCL isotypes, measured by ELISA, have been discrepant and conflicting [25–28]. These discrepancies are probably due to: (i) methodological differences, such as differences in the sensitivity of the different assays used in the measurement of these autoantibodies; (ii) differences in the definition of positive and negative cut-off points; and (iii) differences in the selection of the patient populations for study.

In the first of these studies [25] in which the IgG, IgM and IgA aCL isotypes were measured, all 40 patients were pre-selected to have aCL-associated clinical complications, and all 40 of them had previously been found to be positive for IgG and/or IgM aCL. Amongst this group, IgA aCL was found to be the sole aCL isotype present in only one of the 40 patients; in the other 20 patients where it was found positive, it was always present in association with IgG and/or IgM aCL. Because of the design of the study and the pre-selection of patients, the authors concluded that measurement of IgA aCL was not really necessary in the routine screening of patients suspected of having raised aCL levels. In a subsequent study where aCL levels were measured in 85 consecutive SLE patients [26], it was found that 42·4% of these patients were positive for aCL of any

isotype; of these 61·1% were positive for IgG, 52·7% for IgM and 63·8% for IgA aCL. These values are broadly similar to the prevalence reported here of 52·5% positivity for any aCL isotype, 65·7% for IgG, 60% for IgM, and 39% for IgA aCL (this latter value is lower than that reported by Kalunian [26]). Weidman *et al.* [12] found a total prevalence of 58% for one or more of the three aCL isotypes in lupus sera. The prevalence of IgA as the sole aCL isotype was not quoted in either of these studies. Our findings that IgA aCL is rarely present as the sole elevated aCL isotype confirm three more recent studies [27,29,30], in which none of the patients was positive for IgA aCL alone.

There are rare clinical circumstances where elevated levels of IgA aCL have been associated with disease, such as an association reported in Guillain–Barré syndrome patients [8], where it was suggested that raised levels of IgA aCL could be the consequence of recent mucosal infection due to an unidentified pathogen. Elevated levels of IgA aCL have also been found in infectious disease patients, and an association has been shown for IgA aCL in mumps patients where this isotype was found to be the only aCL isotype which was significantly raised [9].

Longitudinal studies on fluctuations of aCL levels of all three isotypes over long time periods have seldom been performed, except for the study of Kalunian *et al.* [26] where aCL levels were studied in 10 patients over a period of 20 months, with a maximum of five evaluations over this period. No marked changes were found in aCL levels in half of these patients while very large fluctuations were seen in others, all of whom were treated with anticoagulants, immunosuppression and/or prednisone. As part of the current study we have analysed sequential samples from five patients taken over periods ranging from 9 to 48 months, with a minimum of 12 measurements from each patient on different occasions. aCL antibody levels for all three isotypes in all patients were found to change in parallel; there was a difference between subjects in the hierarchy of aCL levels of different immunoglobulin isotypes, which were found to be IgG > IgA > IgM when IgG was the predominant starting isotype, and IgM > IgA > IgG where IgM was the highest aCL isotype at the start of the study period (Fig. 2a, b).

The study of the IgG subclass distribution of antibodies may give insight into the mechnisms driving antibody production; most foreign proteins induce predominantly T lymphocyte-regulated IgG1 and IgG3 responses, whereas antibodies induced against carbohydrate and carbohydrate-like substances often belong to the IgG2 subclass. The IgG subclass distribution of many commonly occurring autoantibodies has been characterized. In patients with SLE and related diseases it has been found that autoantibodies produced against double- and single-stranded DNA [10,12], ANF [31], Sm, RNP, histone and SS-B [32,33], were mainly restricted to the complement fixing IgG1 and/or IgG3 subclasses, adding to the body of data suggesting that, once initiated, these autoantibody responses are predominantly driven by antigen in a T cell-dependent manner.

The present results show that in patients with SLE and other autoimmune diseases IgG1 and IgG3 were the predominant subclasses of IgG against cardiolipin. However, there was also a subgroup of patients with high levels of aCL amongst whom IgG2 was the prominent subclass of aCL (Fig. 3). IgG4 was barely detectable in whole serum in all but two of the SLE group of patients. Amongst a cohort of patients with syphilis we found that IgG1 and IgG3 were the predominant subclasses present, and that IgG4 was also present in all patients. In four of these patients (13·8%) IgG2 was also detected at normal or above normal levels.

Previous reports of the IgG subclass distribution of aCL have been conflicting. The present results are similar to the IgG aCL subclass distribution reported in SLE patients [14,16,34], lupus anticoagulant-positive SLE and non-SLE patients [15] and syphilis patients [16]. They differ from the study of Versalovic *et al.* [35] in syphilis patients who reported restriction of anti-VDRL IgG activity to the IgG1 subclass, but this conclusion was based on results from only five patients. These results also differ from an earlier study [13], which reported that in 60 patients with SLE and SLE-like diseases, IgG anticardiolipin antibodies were distributed equally in all four IgG subclasses.

Because of the difficulties in the measurement of subclass-specific autoantibody responses and the differences reported in different studies we substantiated our findings by exclusion chromatography purification of the IgG subclassess in three patients (Fig. 4). The IgG aCL subclass concentration in the purified fractions was closely similar to that measured in the original sera for IgG1, IgG2 and IgG3. In the case of IgG4, somewhat higher concentrations of aCL were measured in the purified fractions than in the three starting sera, but despite this it was nonetheless present at very low concentrations in all three patients.

The pattern of subclass restriction of the aCL suggests some hypotheses regarding their production. Two distributions were seen in the autoimmune sera: (i) predominantly IgG1 and IgG3 aCL implying a possible role for a T cell-driven response; and (ii) a subgroup in which aCL levels were high and with a strong predominance of the IgG2 subclass, which may imply a role for T cell-independent antibody production to aCL. In the sera from patients with syphilis, aCL were mainly restricted to IgG1 and IgG3 and most patients also had IgG4 aCL detected in their sera, a subclass whose presence usually indicates prolonged antigenic stimulation.

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