Correction for Age of Anticardiolipin Antibodies Cut-Off Points

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Immunoglobulin (Ig) isotypes G, M, and A of anticardiolipin antibodies (aCL) are considered markers of antiphospholipid syndrome (APS). They were determined by ELISA in sera of 100 healthy children aged between 6 months and 16 yrs (mean 5.4 yrs $\pm$ 3.4 SD) and 100 healthy elderly subjects aged between 65 and 103 yrs (mean 84.2 yrs $\pm$ 8.1 SD). The frequency with which they were detected was compared to that in sera of 100 healthy adults aged between 21 and 47 yrs (mean 25.8 yrs $\pm$ 5.2 SD) in order to evaluate if adult aCL cut-off levels were fit for pediatric and elderly populations. The cut-off points were calculated adding 2.5 SD to the mean values and, in the adult group, the results were 11.6 GPL, 7.5 MPL, and 23.9 APL for IgG, IgM, and IgA, respectively. In the children, IgG aCL were positive in 26 cases (26%), IgM and IgA aCL in 1 case (1%) respectively. Statistical comparison of these results to those of adults showed a higher significant frequency for children IgG aCL

($P = 0.0001$) with the major contribution by children aged between 6 months and 5 yrs, and a lower significant frequency for children IgA aCL ($P = 0.041$). In elderly subjects IgG aCL were positive in 12 cases (12%), IgM aCL in 4 (4%), and IgA aCL in 37 (37%). In a comparison of these values to those of adults, only elderly IgA aCL frequency was significantly higher ($P = 0.0001$) with the major contribution by the oldest subgroup. In order to avoid false positive results for IgG aCL in children and for IgA aCL in elderly, as well as false negative results for IgA aCL in children, we introduced three new cut-off points: (1) 27.7 GPL for IgG aCL in children; (2) 13.8 APL for IgA aCL in children; and (3) 51.1 APL for IgA aCL in elderly subjects. These data suggest that a correction for age of aCL cut-off levels should be considered to raise specificity and sensitivity for APS in pediatric as well as in elderly populations. *J. Clin. Lab. Anal.* 14:87–90, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Today, the anticardiolipin antibodies (aCL) represent one of the serological markers of antiphospholipid syndrome (APS), an autoimmune disease characterized mainly by venous and/or arterial thrombosis, thrombocytopenia, and recurrent fetal losses. These antibodies have also been described in several other diseases as infections, malignancies, and adverse reactions to drugs (1,2).

In order to increase the diagnostic specificity and sensitivity of aCL for APS, it is necessary to define with accuracy their cut-off levels in detection during the routine analysis. These values could be different among subjects of various ages considering that a child's immune system is not necessarily similar to that of adults. In fact, the concentration levels of immunoglobulins in sera of children are often lower than those of adults, and only during puberty do they reach adult mean values (3). Also the immune system in the elderly

shows some alterations such as abnormalities in immunoglobulin production (4,5) and a raised prevalence of a number of organ and non-organ-specific autoantibodies including aCL (6,7). To the best of our knowledge, a correction for aCL cut-off points in relation to age have not yet been determined. Thus we have determined the aCL in sera of healthy children and healthy elderly subjects to compare their frequencies to those obtained in healthy adults in order to evaluate if the normally used adult aCL cut-off levels were fit for pediatric and elderly populations. The results are described and discussed here. Moreover, two new cut-off points for children and one for the elderly are suggested.

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MATERIALS AND METHODS

Subjects and Serum Samples

The study groups included 100 healthy children—50 females and 50 males, with a mean age of $5.43 \text{ yrs} \pm 3.48$ who underwent routine biochemical assays for functional disorders or who were hospitalized for minor surgical procedures, and 100 healthy elderly—50 females and 50 males, with a mean age of $84.22 \text{ yrs} \pm 8.13$, all of whom lived in a retirement home. They were selected according to the SENIEUR protocol for immunogerontological studies in man (8). Among the children, 14 were 6–12 months old, 43 were 1–5 yrs, 31 were 6–10 yrs and 12 were 11–16 yrs. Of the elderly subjects 13 were 60–74 yrs, 36 were 75–85 yrs, and 51 were >85 yrs. The control group was composed of 100 healthy adults—50 females and 50 males, all blood donors, and with an average age of $25.86 \text{ yrs} \pm 5.24$. In the three groups all the subjects had no history of rheumatic autoimmune disorders including APS. In addition those treated with drugs involved in the induction of autoantibodies (9) were not included in the study.

After venous drawings the specimens were centrifuged at 3000 rpm for 10 min and subsequently sera were stored at -20°C until use.

aCL Detection

aCL of immunoglobulin (Ig) classes G, M, and A were tested by an “in house” enzyme-linked immunosorbent assay (ELISA) method according to E.N. Harris (10). To build the calibration curve we used Harris’s standard sera as well. Microtiter plates (Falcon, Becton Dickinson Labware, CA) were coated with $30 \mu\text{l/well}$ of $50 \mu\text{g/ml}$ cardiolipin (Sigma Chemicals, St. Louis, MO) in absolute ethanol and dried overnight at 4°C . Plates were washed 2 times with $200 \mu\text{l/well}$ of PBS (phosphate buffer solution) $\text{pH} = 7.2$, then they were blocked for 1 hr at room temperature (RT) with $75 \mu\text{l}$ of PBS + 10% FCS (fetal calf serum, Gibco, Paisley, UK). Subsequently, plates were washed once with PBS. The diluted 1:50 in PBS + 10% FCS sera were added to wells ($50 \mu\text{l/well}$ in double) and incubated for 3 hr at RT. After 3 washings of the plates with PBS, $50 \mu\text{l}$ of antihuman alkaline phosphatase labelled (Sigma) IgG, IgM, and IgA, diluted 1:1,000 in PBS/FCS, were added to each well and the plates were incubated for 90 min at RT. Plates were washed 3 times with PBS and $50 \mu\text{l/well}$ of *p*-nitrophenylphosphate (Sigma) in diethanolamina buffer (diethanolamina 97g, $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ 0.1g, sodioazide 0.2g, H_2O until 1 litre, $\text{pH} = 9.8$) at a concentration of 1mg/ml were distributed. The plates were incubated at 37°C for 20–30 min, and were then read at 405 nanometers when concentration of the highest standard was at 0.900 ± 0.100 of optical density (OD).

All cut-off points were calculated adding 2.5 standard deviations to the mean values. IgG, IgM, and IgA cut-off levels of control group were 11.6 GPL, 7.5 MPL, and 23.9 APL,

respectively. Following Harris’s suggestion, we considered sera “low positive” when the aCL values were between 11.6 and 20 GPL, 7.5 and 15 MPL, and between 23.9 and 30 APL; “moderate positive” between 20 and 80 GPL, 15 and 60 MPL, and between 30 and 90 APL; “high positive” >80 GPL, >60 MPL, and >90 APL.

Statistical Analysis

Comparison of the results obtained in the different age groups was carried out using the χ^2 test.

RESULTS

IgG, IgM, and IgA aCL determinations in children, adults, and the elderly are illustrated in Figure 1. The children were positive for IgG aCL in 26 cases (26%): 15 (57.6%) at the low level and in 11 (42.4%) at the moderate level. Children were positive for IgM aCL in 1 case (1%) at the low level and for IgA aCL in 1 case (1%) at the moderate level.

When we divided the children into age groups we obtained the following results. In the first group of 14 children aged 6–12 months, IgG aCL were present in 5 cases (35.7%), while IgM and IgA aCL were not detected. In the second group of 43 children aged 1–5 yrs, IgG aCL were found in 14 cases (32.5%), IgM aCL in 1 case (2.3%), and IgA aCL in 1 case (2.3%). In the third group of 31 children 6–10 yrs old, IgG aCL were detected in 6 cases (19.3%). The fourth group of 12 children (11–16 yrs old) had IgG aCL in 1 case (8.3%). The statistical comparison of the frequencies among these subgroups was not significant, so indicating an omogeneous distribution of antibody frequencies in the different pediatric ages.

As showed in Table 1 the statistical comparison of the data obtained from children to those from adults, using the adult cut-off points as reference, revealed a significant prevalence of IgG aCL in children ($P = 0.0001$), with the major contribution from children 6 months–5 yrs old. Moreover, we found in the children a significant low prevalence ($P = 0.041$) of IgA aCL, with the same contribution of all the subgroups. These findings indicate that the adult cut-off points are not appropriate for the pediatric population and that different cut-off points should be calculated for children. Our calculations determined that these should be 27.7 GPL for IgG aCL and 13.8 APL for IgA aCL, respectively. As Table 2 shows, in utilizing the new cut-off levels, IgG and IgA aCL frequencies of children were not significantly different from those of adults.

In the elderly, IgG aCL were positive in 12 cases (12%), in 10 (83.3%) at the low level, and in 2 (16.7%) at the moderate level, respectively. IgM aCL were found in 4 cases (4%), all at the low level. IgA aCL were present in 37 subjects (37%), in 14 (37.8%) at the low level, and in 23 (62.2%) at the moderate level respectively. The elderly were divided into different age subgroups that provided the following re-

Fig. 1. Positivity levels of IgG (a), IgM (b), and IgA (c) aCL in children, adults, and elderly subjects.

sults. In the first group of 13 subjects aged 60–74, IgA aCL were found in 4 cases (30.7%). In the second group of 36 subjects, 75–85 yrs old, IgG aCL were present in 3 cases (8.3%), IgM aCL in 1 (2.7%), and IgA aCL in 10 (27.7%). In the third group of 51 subjects aged >85 yrs, IgG aCL were found in 9 (17.6%), IgM aCL in 3 (5.8%), and IgA aCL in 25 (49%). The statistical comparison of the aCL frequencies among the three different subgroups was not significant, revealing an omogeneous distribution of aCL frequencies in old age. aCL frequencies obtained in elderly subjects and in adults using adult cut-off points as a reference are reported and compared in Table 1, where the significant prevalence of IgA aCL in the elderly ($P = 0.0001$) with the major contribution coming from the oldest subjects may be observed. This result caused us to calculate a new elderly cut-off point for IgA aCL: 51.1 APL. Table 2 shows that, when using the new cut-off level, IgA aCL the frequency of elderly subjects is not significantly different from that of adults.

DISCUSSION

Our study revealed a significant increase of IgG aCL and IgA aCL frequencies in children and the elderly. These in-

creases are not due to APS because all the subjects of this study are healthy and are clearly without the criteria for the diagnosis of APS as venous and/or arterial thrombosis and thrombocytopenia. The increased IgG aCL in children could be considered the epiphenomenon of infectious diseases (11,12) and vaccinations (13) frequently affecting the pediatric population. The high frequency of IgA aCL in the elderly could be attributed to the increased production of IgA, described in elderly subjects (4,5) and it is in keeping with the increased frequency in the elderly of other IgA autoantibodies such as anti-native DNA (14). Moreover in children we have found a significantly low frequency of IgA aCL,

TABLE 1. Comparison of aCL frequencies (%) in children and in elderly subjects with those in adults, using adult cut-off points

	Children (n. 100)	χ^2 (P)	Adults (n. 100)	χ^2 (P)	Elderly subjects (n. 100)	Adult cut-offs
aCL G	26	0.0001	5	n.s.	12	11.6 GPL
aCL M	1	n.s.	2	n.s.	4	7.5 MPL
aCL A	1	0.041	8	0.0001	37	23.9 APL

TABLE 2. Comparison of aCL frequencies (%) in children and elderly subjects with those in adults, using age-related cut-off points

	Children (n. 100)	χ^2 (P)	Adults (n. 100)	χ^2 (P)	Elderly subjects (n. 100)	Age-related cut-offs	
aCL G	6	n.s.	5	n.s.	12	Children	27.7 GPL
						Adults/elderly subjects	11.6 GPL
aCL M	1	n.s.	2	n.s.	4	Children/adults/elderly subjects	7.5 MPL
aCL A	1	n.s.	8	n.s.	2	Children	13.8 APL
						Adults	23.9 APL
						Elderly subjects	51.1 APL

probably due to a low production of serum IgA (3), a characteristic of the pediatric population. Every positive reading was at either a low or moderate level while high antibody levels were never found.

The aim of this study was to determine if adult cut-off points were also fit to be used in studying pediatric and elderly subjects. The different frequencies of IgG and IgA aCL found in pediatric and elderly populations utilizing adult cut-off points led us to modify some cut-off levels relative to age in order to increase sensitivity and specificity for APS of aCL detection. Therefore, to avoid false positive results, we raised the cut-off points of IgG aCL in children and IgA aCL in the elderly, respectively, and to avoid false negative results we decreased the cut-off point of IgA in children. It is interesting to observe that aCL production was omogeneous in all the different age subgroups both for children and elderly. These data indicate that the cut-off levels just established are suitable for all pediatric and elderly subject age subgroups. APS is generally identified in adults (15,16) but it is also described in children (17,18) and the elderly (19,20); thus it is necessary to have a high sensitivity and specificity of the method for aCL detection in all life ages. Our study demonstrates that in order to do so, it is necessary to use aCL cut-off points relative to age.

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