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MATTERS ARISING

Antiphospholipid antibodies and rheumatoid arthritis

We read with interest the letter entitled “Antiphospholipid antibodies and RA: presence of β_2GP1 independent aCL” by Bonnet *et al* published in the *Annals* in March 2001.[1] We believe that the letter needs additional clarification owing to inconsistencies in the terminology, methodology of antiphospholipid antibody (aPL) detection, and determination of positive values.

The use of the term “anticardiolipin antibodies” was somewhat misleading. The term was introduced and abbreviated as “aCL”, a group of antibodies detected in many conditions, but the β_2 glycoprotein 1 (β_2GP1) dependence of the aCL was not defined, even though the authors focused on β_2GP1 independent aCL. It is generally agreed that the term aCL, if not stated otherwise, defines the antibodies detected by the classical aCL enzyme linked immunosorbent assay (ELISA),[2 3]—that is, both β_2GP1 dependent and β_2GP1 independent antibodies.

There were some potential methodological errors in determining β_2GP1 independent aCL. It was shown that antibodies against β_2GP1 (anti-β_2GP1) from patients with the antiphospholipid syndrome (APS) have the ability to bind β_2GP1 in complexes with cardiolipin only if the β_2GP1 concentration in solution is high enough. The threshold concentration of β_2GP1 was found to be just about 2 μg/ml, because no binding of anti-β_2GP1 was seen when serum samples were diluted 1:200 or more.[4] As the physiological concentration of β_2GP1 in human serum is approximately 200 μg/ml, the threshold binding concentration is reached at a serum dilution of 1:100. In the presence of a relatively high concentration of endogenous β_2GP1, the statement that antibodies detected by this method are exclusively β_2GP1 independent is unjustified, as the sera containing high titres of anti-β_2GP1 might have yielded positive results by the method described in the letter.

The definition of antibody units in the letter is not clear and using Harris’s standards for β_2GP1 independent aCL is not appropriate. With the use of Harris’s standards,[5] the units should be abbreviated as GPL (for IgG) and MPL (for IgM) as previously defined.[5] However, Harris’s standards were designed for use in the classical aCL ELISA and were prepared by pooling serum samples from patients with APS. Therefore, they contain mainly, or predominantly, β_2GP1 dependent aCL. β_2GP1 independent aCL were not defined in those standards and they were not meant as standards for β_2GP1 independent assays.

The interpretation of anti-β_2GP1 ELISA as a method to detect β_2GP1 dependent aCL may not be valid in all cases. It was shown that not all anti-β_2GP1 binding β_2GP1 adsorbed on polystyrene high binding plates also recognise β_2GP1 associated with cardiolipin. We reported this binding pattern for anti-β_2GP1 in children with atopic dermatitis,[6] and the same was shown also for some patients with autoimmune diseases, including APS.[7]

The method for purification of β_2GP1 was not described. Because the authors focused on patients with rheumatoid arthritis (RA), it should be ensured that immunoglobulins were specifically removed from the β_2GP1 preparation. If this purification step was not carried out, traces of immunoglobulins in the β_2GP1 preparation might have yielded positive results for sera containing high titres of rheumatoid factor (RF). In fact, all sera containing IgM anti-β_2GP1 also had RF and the authors already suspected that this might be due to non-specific binding involving RF.

The method for determining cut off values was not explained and the number of normal human sera (NHS) included in the study as negative controls was not given. From the data presented in the letter, one may conclude that the cut off values were arbitrarily set at 20 units both for IgG and IgM isotypes of β_2GP1 independent aCL and for anti-β_2GP1. We recently compared the sensitivity of anti-β_2GP1 ELISA and classical aCL ELISA. The results showed great differences between their sensitivities and therefore also between the cut off values calibrated by the same standards.[8] In addition, the authors did not report the proportion of NHS positive for each assay and the values of positive samples compared with patients with RA. Instead, they just referred to one study,[9] which is only one of the several published estimations of aPL in healthy subjects.

We would like to support our criticism by adding some data about aPL in our patients with RA. We randomly selected 53 serum samples from patients fulfilling the ARA criteria for RA and 53 NHS as negative controls. The samples were tested for anti-β_2GP1, β_2GP1 dependent aCL, and β_2GP1 independent aCL. The assays were calibrated with β_2GP1 dependent monoclonal aCL (IgG and IgM anti-β_2GP1 ELISA and β_2GP1 dependent aCL ELISA) and positive in-house standards (all IgA assays and β_2GP1 independent aCL). The cut off values for anti-β_2GP1 were set as described[8] by calculating the mean + 2 SD of logarithms of absorbance values for NHS and the 95th centile value of 32 NHS for both β_2GP1 dependent and β_2GP1 independent aCL. For the anti-β_2GP1 determination, we used affinity purified β_2GP1 adsorbed on Costar high binding plates as previously described.[8] The β_2GP1 preparation did not contain any immunoglobulins. β_2GP1 independent aCL were tested as described in the letter, but the sera were diluted 1:200. Serum samples were tested simultaneously for β_2GP1 dependent aCL on the same plate by adding β_2GP1 in parallel duplicate wells. The final concentration of β_2GP1 was 10 μg/ml. This experimental design enabled direct comparison of binding to cardiolipin coated wells in the presence and absence of β_2GP1. For the final determination of β_2GP1 dependent binding, the values obtained in wells without β_2GP1 were subtracted from the values measured in wells with added β_2GP1. The patients’ histories were evaluated for the occurrence of arterial or venous thrombosis and recurrent fetal loss. Statistical analysis was performed with the χ^2 test where appropriate.

Table 1 presents the frequency of positive sera in each group (NHS, RA, RA-RF positive, and RA-RF negative). The frequency of increased anti-β_2GP1, β_2GP1 dependent aCL, and β_2GP1 independent aCL was higher in patients with RA than in controls, but the difference was significant only for anti-β_2GP1. There were no differences in the frequency of

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Table 1 Frequency of anti-β_2GPI, β_2GPI dependent aCL, and β_2GPI independent aCL in patients with rheumatoid arthritis (positive or negative for RF) and normal controls

No of positive samples:	Anti-β_2GPI*								β_2GPI dependent aCL†								β_2GPI independent aCL†							
	IgG		IgM		IgA		Any Ig		IgG		IgM		IgA		Any Ig		IgG		IgM		IgA		Any Ig	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
NHS (n=53*; n=32†)	1	2	2	4	1	2	4	8	1	3	2	6	1	3	3	9	2	6	2	6	2	6	5	16
RA (n=53)	3	6	6	11	4	8	11	21	2	4	6	11	2	4	9	17	4	8	4	8	9	17	12	23
RA - RF+ (n=36)	2	6	4	11	3	8	8	22	2	6	3	8	2	6	6	17	2	6	4	11	7	19	9	25
RA - RF– (n=17)	1	6	2	12	1	6	3	18	0	0	3	18	0	0	3	18	2	12	0	0	2	12	3	18

aCL, Anticardiolipin antibodies; β_2GPI, β_2 glycoprotein I; NHS, normal human sera; RA, rheumatoid arthritis; RF, rheumatoid factor.

any type of antibodies between the RF positive and negative patients. One patient (a male, 66 years old) had a history of deep venous thrombosis and pulmonary embolism together with positive anti-β_2GP1 and β_2GP1 dependent aCL of IgA isotype. Interestingly, 5/11 RA sera which showed binding to β_2GP1 adsorbed on a high binding plate did not recognise β_2GP1 associated with cardiolipin, as already reported.[6 7] In contrast, 3/9 RA sera binding β_2GP1 complexed with cardiolipin did not recognise β_2GP1 adsorbed on the surface of high binding plates. This phenomenon probably reflects the heterogeneous nature of anti-β_2GP1 in RA, which may differ in fine specificity from anti-β_2GP1 in APS.

The sera from our patients with RA exhibited an even higher frequency of β_2GP1 independent aCL than that reported in the letter. As expected from reported data, the presence of β_2GP1 independent aCL was not associated with signs of APS in our patients. We also found that the addition of β_2GP1 (10 μg/ml) lowered the binding of β_2GP1 independent aCL by about 50%, most probably owing to the competition between β_2GP1 independent aCL and β_2GP1 for the same binding sites on cardiolipin.

In conclusion, patients with RA may have anti-β_2GP1 and β_2GP1 dependent aCL, which might be associated with the signs of APS. The importance of distinguishing β_2GP1 independent aCL has not been fully clarified. It seems that β_2GP1 independent aCL do not confer an increased risk for APS in RA.

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Authors' reply

In response to the comments of Ambrozic *et al* we would like to add some information to the data published earlier in the *Annals*.[1]

The term "anticardiolipin antibodies" (aCL) is classically used to designate antibodies directed against the cardiolipin antigen and detected in sera. Commonly, the dependence of aCL on β_2 glycoprotein 1 (β_2GP1) is assessed by an enzyme linked immunosorbent assay (ELISA) test using exogenous β_2GP1 in blocking buffer (containing fetal calf sera or bovine sera). In our study, the blocking solution did not contain bovine or calf sera but only purified bovine serum albumin. So, this method was adapted to detect antibodies directed against cardiolipin antigen alone and not against the complexes of cardiolipin bound to exogenous β_2GP1. This method justified the terminology of β_2GP1 independent aCL for sera containing aCL without anti-β_2GP1 antibodies; the absence of anti-β_2GP1 antibodies was shown by another ELISA test specific for the detection of these antibodies. Both ELISAs were used to screen all sera.

The concentration of endogenous β_2GP1 contained in human serum is not significant at a 1/100 dilution (the dilution employed to screen our sera), in comparison with the 10% of calf sera added to the test as source of exogenous β_2GP1 in the assays used for the detection of β_2GP1 dependent aCL. In addition, the sera containing aCL (detected by an ELISA without addition of exogenous β_2GP1) did not react with the purified β_2GP1 in the other ELISA test specifically designed to detect anti-β_2GP1 autoantibodies, and therefore which could detect hypothetically high titres of anti-β_2GP1 antibodies contained in these sera.

Harris's standards were used after calibration of our positive control sera from patients with proven antiphospholipid syndrome (APS), which were used as positive controls in every microtitration plate. We used these for the detection of aCL in our previous studies employing ELISA test without bovine or calf sera.[3 4] The antiphospholipid antibodies, including aCL, are directed against several antigenic targets. Among them, some epitopes are located on the cardiolipin alone. These data were described by Harris when aCL were first characterised in systemic lupus erythematosus sera reacting in a VDRL test. By radioimmunoassay, he showed that antibodies contained in these sera were directed against cardiolipin contained in liposomes used as a reagent of the VDRL test.[2] These reagents were constituted by lipids alone without any other cofactor such as β_2GP1. So, Harris's standard can also be used to detect aCL directed only against phospholipid and not against the complex β_2GP1-cardiolipin. In addition, the use of Harris's standards seems to be better adapted to the detection of polyclonal antiphospholipid antibodies, than monoclonal human aCL used as internal controls.

The β_2GP1 used in our assay was provided by Stago laboratories (Asnière, France) and was purified from human sera. We used sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blotting to ensure that this purified protein was not contaminated

For every antibody determination, aCL and anti-β_2GP1 autoantibodies, normal levels were established from studies of a large number of normal subjects (blood donors) as previously described.[3 4] In this study, 50 serum samples, provided by consenting healthy donors, were tested as controls.

Cut off values were determined as the mean and two standard deviations of the arbitrary units obtained by reference to positive and negative internal standards. For every serum, we defined the corrected optical density (OD) (that is, the mean OD obtained in three coated wells minus the OD corresponding to non-specific binding of each serum, obtained in three uncoated wells). The cut off values defined for anti-β_2GP1 and anti-cardiolipin ELISA were 20 units in both tests. The standards for the anti-β_2GP1 test corresponded to positive controls from patients with APS and were used according to previous studies.[3 4]

In contrast with the report of Ambrozic *et al*, we did not find raised levels of aCL or anti-β_2GP1 antibodies in normal sera; the percentage of positive normal serum samples was <3%. These differences between our results and those of Ambrozic *et al* are probably associated with a differing sensitivity and specificity of the methods between the two laboratories.

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Methotrexate and postoperative complications

Grennan *et al* report the safety of continued methotrexate in the perioperative period.[1] Previous investigators have despaired of answering this question definitively owing to the difficulty in recruiting subjects.[2] It is reassuring to see that methotrexate use throughout the postoperative period does not interfere with wound healing or increase the incidence of early complications.

Despite this important finding, we believe that the results of this study should be regarded with some reservation: continuation of methotrexate throughout the perioperative period should be accompanied by significant caution. The elderly and those with renal impairment are at increased risk of methotrexate related pancytopenia.[3–5] Indeed, in a community based, observational study of methotrexate use in 460 patients we found the