Clin. exp. Immunol. (1989) **76**, 178–183

The specificity of anti-cardiolipin antibodies from syphilis patients and from patients with systemic lupus erythematosus

S. MOURITSEN, M. HØIER-MADSEN, A. WIIK, O. ØRUM & N. STRANDBERG PEDERSEN *Laboratory for Autoimmune Serology, Statens Seruminstitut and World Health Organization Collaborating Centre for Reference and Research on Treponematoses, Department of Treponematoses, Statens Seruminstitut, Amager Boulevard 80, 2300 S, Copenhagen, Denmark*

(Accepted for publication 6 January 1989)

SUMMARY

In order to elucidate the fine specificity of anti-cardiolipin antibodies (ACA) in patients with SLE compared to patients with syphilis (SY) various inhibition experiments were performed. Seven SLE sera and eight SY sera positive for ACA were diluted and preincubated with either cardiolipin VDRL-antigen, mitochrondial particles, dsDNA, ssDNA or dilution buffer. The sera were subsequently assayed for residual ACA activity of IgG or IgM class using a sensitive ELISA technique. Significant inhibition of IgM ACA activity in SLE sera was found with cardiolipin, VDRL-antigen and mitochondrial particles. Cardiolipin inhibited binding to a significantly higher extent than the other antigens. In SY sera significant inhibition of the IgM ACA activity was found with all antigens used. The strongest inhibition was seen using VDRL-antigen. Inhibition of IgG ACA activity could only be clearly estimated in SY sera where VDRL-antigen was found to be a much stronger inhibitor than the rest, purified cardiolipin being the weakest. Only two out of seven SLE sera were IgG ACA positive which made a clear conclusion impossible but a strong inhibitory capability of pure cardiolipin and a weaker inhibition with VDRL-antigen was found. This study disclosed a difference between SLE and SY sera showing strong reactivity of ACA in SLE sera with purified cardiolipin, contrasting to ACA in SY sera which predominantly reacted with cardiolipin in the liposome environment, as found in the VDRL-antigen and in mitochondrial particles.

Keywords SLE anti-cardiolipin antibodies VDRL-antigen syphilis ELISA inhibition studies

INTRODUCTION

Among the many autoantibodies described in patients with systemic lupus erythematosus (SLE), anti-cardiolipin antibodies (ACA) have received increasing attention during the past few years. This is probably due to the serious clinical implications seen in some patients with this serological abnormality, and the recent occurrence of new and more sensitive methods based on solid phase techniques for the detection of ACA (Harris *et al.* 1983; Loizou *et al.*, 1985). There is good evidence that these assays are more sensitive in detecting ACA in SLE patients than standard tests for SY (Harris *et al.*, 1983; Harris, Gharavi & Hughes, 1985) and the antibodies detected correlate to a certain extent with lupus anti-coagulant activity, recurrent cardiovascular events, thrombocytopenia and fetal distress and death (Harris *et al.*, 1983, 1985 a,b,c; Lockshin, 1985; Hansten *et al.*, 1986; Morton *et al.*, 1986).

Correspondence: Søren Mouritsen, Laboratory for Autoimmune Serology, Statens Seruminstitut, Amager Boulevard 80, 2300 S, Copenhagen, Denmark.

Numerous and partly conflicting reports on the cross-reacting properties of ACA with acidic phospholipids other than cardiolipin (CL) and nucleic acids have appeared (Guanieri *et al.*, 1974; Lafer *et al.*, 1981; Koike *et al.*, 1984; Harris *et al.*, 1985a; Janoff & Rauch, 1986). The tests included in the 1982 American Rheumatism Association criteria for SLE are usually based on standard non-treponemal tests used in SY diagnostics (Tan *et al.*, 1982), but recently an ELISA method was described by us (Strandberg *et al.*, 1987) which, when using a mixture of CL, cholesterol and lecithin (1:30:7) as antigens, also suggested differences in specificity of ACA found in SLE patients and in patients with SY. This study indicated that cholesterol and lecithin, although not antigenic by themselves, may change the structure of the epitope on the antigen mixture so that it becomes less recognizable for ACA from patients with SLE. In other words: ACA found in SLE and SY patients seem to recognize pure CL and combinations of phospholipids of a different conformation, respectively. The aim of the present study was to investigate further the nature of this difference by performing various inhibition experiments on ACA positive

sera from patients with SLE and SY. Furthermore we have investigated the prevalence of ACA as determined by ELISA in several groups of patients with connective tissue diseases.

MATERIALS AND METHODS

Antigens

CL was purchased form Sigma and lecithin was purified as described earlier (Strandberg Pedersen *et al.*, 1987). Cholesterol was obtained from Pfannenstiehl Labs, Waukegan, Ill. The purity of all lipids was tested by thin-layer chromatography. The VDRL antigen was prepared in PBS-BS as described previously. Native calf thymus DNA (dsDNA) was purchased from Sigma and single-stranded DNA was prepared from this by boiling for 15 min followed by rapid cooling on ice. Submitochondrial particles (SMP) were prepared from pig heart muscle as described by Mouritsen *et al.* (1985). The SMP preparation was assayed for NADH oxidase and cytochrome C oxidase activity, marker enzymes for the inner mitochondrial membrane, as described by Demant & Jensen (1983).

Sera

Eighty-six sera from patients suffering SLE, 20 sera from patients with rheumatoid arthritis (RA), and 13 patients with progressive systemic sclerosis (PSS) were kindly supplied from Dr Poul Halberg, Department of Rheumatology and Immunology, Hvidovre Hospital, and Dr Henrik Nielsen, Department of Rheumatology, Rigshospitalet, Copenhagen. The diagnoses were based on international, generally accepted diagnostic criteria (Roper *et al.*, 1958; Masi *et al.*, 1981; Tan *et al.*, 1982). Twenty sera from patients with primary Sjøgrens syndrome (SS) were a gift from Dr Rolf Manthorpe, Malmö Almännä Sjukhus, Malmö. The diagnostic criteria for SS were as described by Manthorpe *et al.* (1981). Furthermore we have investigated 20 sera from patients with primary biliary cirrhosis (PBC) from the Department of Hepatology, Rigshospitalet, Copenhagen, based on the diagnostic criteria described by Christensen *et al.* (1980). Finally 35 sera all positive in traditional non-treponemal tests from patients with SY were investigated. The diagnosis of SY was based on extensive centralized, serological and clinical data collected at the Department of Treponematoses, Statens Seruminstitut, Copenhagen. Sera from 100 healthy blood donors came from the Department of Clinical Immunology, Statens Seruminstituit, Copenhagen.

ELISA method for ACA determination

This was performed essentially as described by Loizou *et. al.* (1985) with minor modifications. Briefly, flexible polyvinylchloride microtitre plates (Flow Laboratories) were coated with 30 μl of a solution of 45 μg/ml CL in absolute ethanol. The solvent was evaporated overnight by incubation without lid at 4°C and the plates were blocked for 1 h at room temperature with 150 μl phosphate-buffered saline (PBS), pH 7·38 containing 10% bovine serum (PBS-BS). After four washings with PBS, 100 μl serum diluted 1:100 in PBS-BS were added to each well and incubated for 2 h at room temperature. The washing procedure was repeated and 100 μl horseradish-peroxidase-labelled rabbit anti-human IgG or anti-IgM (Dakopatts, Copenhagen) diluted 1:1000 and 1:5000, respectively, in PBS-BS were added and incubated for 1 h at room temperature. After a thorough washing with PBS, peroxidase binding was detected with ortophenyl diamine and H_2O_2 as the substrates. The optical density was read at 492 nm (OD_{492}) and all estimations were performed in triplicate. On each ELISA microtitre plate one positive laboratory standard and one negative control serum were assayed as well as control wells without serum (background value). To account for day-to-day variations, results were expressed in ELISA units (EU):

$$\frac{OD_{492}\text{ sample} - OD_{492}\text{ background}}{OD_{492}\text{ positive control} - OD_{492}\text{ background}} \times 100$$

Variations of less than 15% were accepted.

Inhibition experiments

Seven SLE sera and eight SY sera, positive for ACA of either IgG or IgM class, were selected for inhibition experiments. Serum dilutions of 1:100 in PBS-BS were preincubated for 2 h at room temperature with dsDNA (250 μg/ml), ssDNA (250 μg/ml), CL(300 μg/ml), SMP or VDRL antigen (corresponding to a CL concentration of 300 μg/ml), before performing the ACA ELISA as described above.

Statistical analysis

The Mann–Whitney–Wilcoxon test for two independent samples was used to estimate differences between SLE and SY sera as regards inhibition of ACA by the various antigens. The Wilcoxon signed rank test for paired samples was used to estimate differences as regards inhibition of ACA by these antigens among the individual SLE sera and SY sera. A confidence level of 95% was chosen.

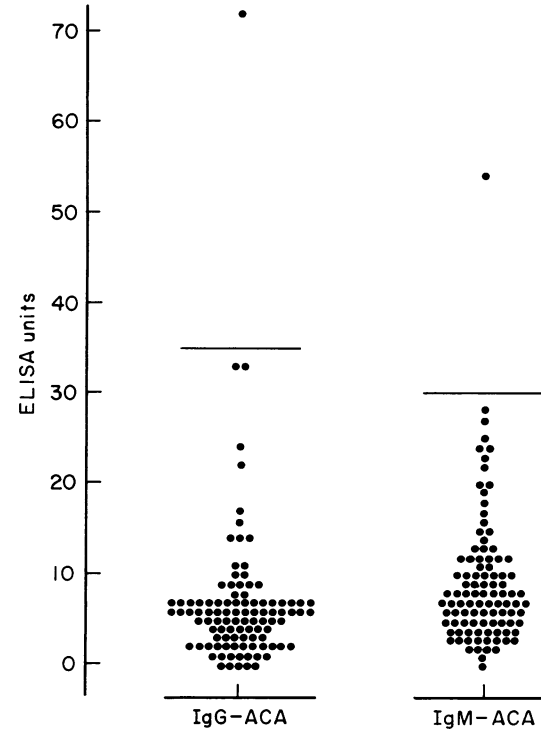

Fig. 1. IgG and IgM ACA values found in healthy control sera. ELISA values are expressed in EU. The 99th percentile is depicted by a horizontal line.

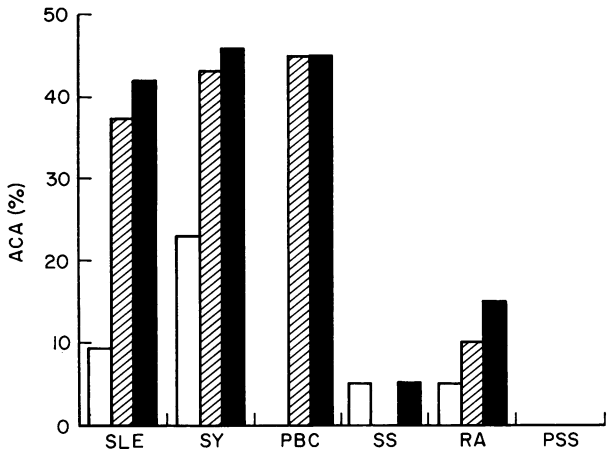

Fig. 2. Prevalence of ACA in different groups of patients with well-defined connective tissue diseases. SLE systemic lupus erythematosus, SY syphilis. IgG ACA (□); IgM ACA (□); Total ACA (■).

RESULTS

ELISA method for ACA determination

The distribution of ACA in 100 healthy blood donors is shown in Fig. 1. When using the 99 percentile of the ACA level in these sera ACA-values > 35 ELISA units (EU) for the IgG class and > 30 EU for ACA of the IgM class were considered positive. The prevalence of ACA among the different patient groups is shown in Fig. 2.

With this ELISA method we found intra-assay variations from 8·8% to 13·4% for high and low IgG ACA titres, respectively, and from 6·4% to 8·8% for the IgM class (data not shown). Inter-assay variations were considerably higher: 19·0–36·5% (IgG) and 15·2–34·9% (IgM) (data not shown). Since these values are higher than usually seen with other ELISA methods, we chose to perform triplicate estimations of each serum.

Inhibition experiments

All ACA positive sera used in these experiments were IgM class positive, but only two out of seven SLE patients and six out of eight SY patients were IgG ACA positive. The results of inhibition were expressed as percentage inhibition of phospholipid binding activity, calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{\text{EU in the presence of inhibitor}}{\text{EU in the absence of inhibitor}}\right) \times 100$$

Previous inhibition experiments using varying antigen concentrations had shown that the amount of antigen used gave maximal competitive inhibition of the ACA activity. The results from the inhibition experiments are shown in Fig. 3a & b. The columns express the mean inhibition value for each of the antigens.

As can be seen in Fig. 3a and Table 1 all compounds but ssDNA and dsDNA were able to inhibit IgM ACA in the SLE sera as compared to PBS. CL inhibited IgM ACA significantly better than did VDRL and SMP. No statistical calculations were performed on the IgG ACA results due to the small number of positive sera available in this study, but, except for the lack of inhibition by SMP, the inhibition profiles were similar. Unexpectedly, preincubation with dsDNA significantly

Table 1. Statistical analysis of the results shown in Fig. 3a

	IgG					
IgM	CL	VDRL	ssDNA	dsDNA	SMP	PBS
CL	—	—	—	—	—	—
VDRL	0·03	—	—	—	—	—
ssDNA	0·05	0·83	—	—	—	—
dsDNA	0·02	0·02	0·03	—	—	—
SMP	0·02	0·93	0·45	0·02	—	—
PBS	0·02	0·02	0·27	0·03	0·02	—

P values using the Wilcoxon signed rank tests for paired samples. No statistical testing performed on IgG ACA since only two positive sera were available.

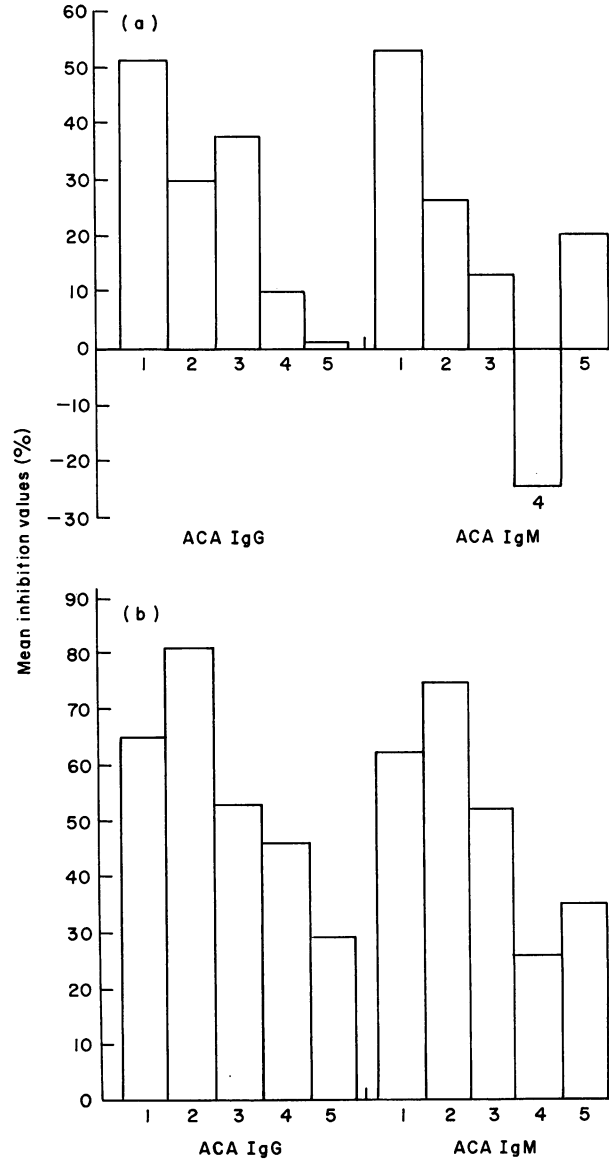

Fig. 3. Mean inhibition values (%) in SLE sera (a) and in syphilis sera (b) by pre-incubation with different antigens before testing ELISA. 1, cardiolipin; 2, venereal disease research laboratory antigen; 3, singles-tranded DNA; 4, double stranded DNA; 5, submitochondrial particles.

Table 2. Statistical analysis of the results shown in Fig. 3b

	IgG					
IgM	CL	VDRL	ssDNA	dsDNA	SMP	PBS
CL	—	0·04	0·04	0·04	0·04	0·04
VDRL	0·16	—	0·04	0·04	0·04	0·04
ssDNA	0·36	0·03	—	0·06	0·04	0·04
dsDNA	0·04	0·01	0·01	—	0·04	0·04
SMP	0·04	0·01	0·36	0·16	—	0·04
PBS	0·01	0·01	0·01	0·04	0·02	—

P-values using the Wilcoxon signed rank tests for paired samples.

Table 3. Differences in IgM ACA specificity between SLE and syphilis sera expressed as differences in ACA inhibition by various inhibitors

CL	VDRL	ssDNA	dsDNA	SMP
0·56	0·005	0·003	0·01	0·04

P-values using the Mann–Whitney–Wilcoxon test for independent samples for differences in inhibition values between IgM ACA from SY and SLE patients.

enhanced (negative mean inhibition value) IgM binding to the solid phase.

In SY sera all antigens were able to inhibit ACA of both Ig classes (Fig. 3b, Table 2). The VDRL antigen was the superior inhibitor, compared to all the other antigens. The difference between CL and VDRL was, however, not statistically significant.

In Table 3 differences in inhibition of IgM ACA activity in SLE and SY sera using different inhibitory compounds are shown. CL was equally potent in inhibiting ACA activity from SLE sera and SY sera, but when present in the same amount in VDRL liposomes, SY sera were inhibited to a significantly higher extent. SsDNA and dsDNA were able to inhibit binding of Ig to the solid phase from SY patients but not from SLE patients. SMP was a more potent inhibitor of SY sera than of SLE sera.

DISCUSSION

We have established an ELISA for detection of ACA and have investigated the prevalence of ACA in sera from patients with well-defined connective tissue diseases and sera from SY patients. Using the 99th percentile of healthy blood donors we found about 42% of SLE patients ACA positive (IgG and/or IgM). This is in agreement with what others have found (Harris *et al.* 1983; Koike *et al.*, 1984; Tincani et al., 1985) although we only found 9·3% to be IgG ACA positive. Most of these sera were only slightly IgG positive compared to IgM ACA. Only one of 20 SS patients, three of 20 RA patients and none of the PSS patients were found ACA positive.

Interestingly 45% of the PBC sera were found IgM ACA positive whereas no sera were IgG positive. To our knowledge this has not been described before. PBC patients are known to possess anti-mitochondrial antibodies (AMA) directed against the inner mitochondrial membrane (Mouritsen *et al.*, 1985), preferentially of the IgG class. One could speculate whether antibodies against CL, which is an inner mitochondrial membrane component, constitute a part of the general autoimmunity to mitochondria in these patients. Whether there are differences in epitope specificity of ACA in PBC, SLE and SY patients should be further investigated. As we have shown earlier (Strandberg *et al.*, 1987) about 45% of the SY sera were ACA positive, the antibodies preferentially belonging to the IgM class.

When sera were found positive for ACA of the IgG class, they generally also contained ACA of the IgM class. On the other hand many patients had IgM ACA in their sera without having any IgG ACA.

In this study we have shown a clear difference between the specificity of ACA found in SY and SLE sera. This is compatible with the fact that most authors have found no correlation between ACA as measured with ELISA technique and the standard VDRL titres (Colaco & Male, 1985; Harris *et al.*, 1985a; Strandberg *et al.*, 1987). In SLE sera CL inhibited ACA activity significantly better than did the VDRL antigen (Fig. 3a and Table 1). In SY sera, the reverse was true but statistical significance was found only for IgG ACA (Fig 3b. and Table 2). Furthermore, although SMP significantly inhibited both kinds of patient sera, SY sera were inhibited significantly more than were SLE sera (Table 3). These observations suggest that the polyclonal ACA response in SY sera may, to a larger extent, be directed against CL placed in a membrane structure whereas ACA in SLE sera are directed more narrowly to pure CL. Inhibition studies by others have shown that this specificity is not necessarily directed against CL but also other acidic phospholipids such as phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol or phosphatidic acid (Lafer *et al.*, 1981; Colaco *et al.*, 1985; Harris *et al.*, 1985a; Janoff *et al.*, 1985).

Harris *et al.* (1988) have recently shown similar differences in antibody specificity between ACA from SY and SLE patients using an ELISA with different phospholipid mixtures with or without cholesterol and lecithin and using a poly-L-lysine precoating layer. Due to the positive net charge of the poly-L-lysine layer one would expect the negatively charged phosphodiester groups to be blocked by the precoating layer. No interference due to this was found, however, when testing SY sera on VDRL-coated wells, but preincubation with VDRL in sera from patients with autoimmune disorders, caused a significant reduction in binding to CL-coated wells despite these sera not binding, to any high extent, to VDRL-coated wells. This further supports that ACA binding in sera from SY patients is highly dependent on non-polar epitopes in the surroundings of the phosphodiester groups, whereas ACA from SLE patients almost exclusively bind to epitopes close to these groups.

There have been conflicting reports concerning the cross-reactivity between ACA and anti-DNA antibodies from SLE patients. Harris *et al.* (1985 b,d) have reported no inhibition of ACA from SLE patients by dsDNA and only occasionally inhibition was seen using ssDNA. These results were obtained using competitive inhibition in a solid phase ACA assay. On the other hand Koike *et al.* (1982), using a solid phase anti-DNA

antibody assay, demonstrated significant competitive inhibition of anti-DNA antibodies from SLE patients by CL.

In our study dsDNA acted as a negative inhibitor of ACA activity in SLE patients (Fig. 3a), i.e. preincubation of sera with dsDNA significantly enhanced IgM binding to CL-coated wells. We believe that the most likely explanation of this was that dsDNA and/or dsDNA containing immune complexes are able to adhere non-specifically to the CL-coated solid phase during the incubation period. The anti-dsDNA antibodies present in the SLE sera will subsequently bind to the immobilized dsDNA thereby enhancing the binding of Ig to the solid phase. This concept is supported by the fact that no such effect was seen in SY sera, which generally are reported not to contain anti-dsDNA antibodies. DsDNA, on the contrary, was found to inhibit ACA activity in these sera. Whether this may be due to steric hindrance by binding of dsDNA to the solid phase or due to ACA from SY patients reacting with dsDNA is unclear. This phenomenon may, however, be the reason why many different results have been published concerning ability of dsDNA to inhibit ACA from SLE patients. It may therefore be difficult to establish, when using a competitive inhibition technique, whether or not dsDNA actually inhibits ACA from these patients.

Like Harris *et al.* (1985a, d), we found no significant inhibition of ACA from SLE sera by ssDNA, whereas this compound was able to inhibit ACA from SY sera. The reason for this is unclear. The hydrophobic, free base pairs and the polar phosphodiester backbone of ssDNA together form a determinant which mimics the antigen structure formed by the apolar cholesterol and lecithin and the polar phosphodiester groups in CL.

We have also tried to preincubate CL-coated microtitre plates with adriamycin, which is known to interact with CL, presumably on its polar head group. This fact has been used to show the specificity of the reaction between complement factor C1q and CL (Kovacsovics *et al.*, 1985). We then tested the above-mentioned sera in the ELISA technique to see whether blocking of this determinant affected the antibody binding. We did not, however, get any clear results from these experiments, presumably because adriamycin is a hydrophobic compound binding non-specifically to polystyrene, immunoglobulin etc.

In conclusion, we have shown with these studies that ACA from SY and SLE sera show very different binding specificities to CL and structurally related compounds. It seems that antibodies from SY patients perhaps recognize CL in context with other cell membrane components whereas ACA from SLE patients might recognize pure CL and structurally similar compounds. This membrane-associated ACA activity found in SY sera perhaps is the natural antibody response towards the treponemal bacteria which also contain CL in the cell membrane.

ACKNOWLEDGMENTS

The excellent technical assistance of Henrik Buch and Anette Østergård is gratefully acknowledged.

REFERENCES

CHRISTENSEN, E., CROWE, J., DONIACH D., POPPER, H., RANEK, L., RODS, J., TYGSTRUP, N. & WILLIAMS, R. (1980) Clinical pattern and course of disease in primary biliary cirrhosis on an analysis of 236 patients. *Gastroenterology*, **78,** 236.

COLACO, C.B. & MALE, D.K. (1985) Anti-phospholipid antibodies in syphilis and a thrombotic subset of SLE: Distinct profiles of epitope specificity. *Clin. exp. Immunol.* **59,** 449.

DEMANT, E.J.F. & JENSEN, P.K. (1983) Destruction of phospholipids and respiratory-chain activity in pig-heart submitochondrial particles induced by an adriamycin-iron complex. *Eur. J Biochem.* **132,** 551.

GUARNIERI. M. & EISNER. D. (1974) A DNA antigen that reacts with antisera to cardiolipin. *Biochem. Biophys. Res. Commun.* **58,** 347.

HANSTEN, A., NORBERG, R., BJÖRKHOLM, M., DE FAIRE, U. & HOLM, G. (1986) Antibodies to cardiolipin in young survivors of myocardial infarction: An association with reccurent cardiovascular events. *Lancet*, **i,** 113.

HARRIS, E.N., GHARAVI, A.E., BOEY, M.L., PATEL, B.M., MACKWORTH-YOUNG, C.G., LOIZOU, S. & HUGHES, G.R.V. (1983) Anticardiolipin antibodies: Detection by radioimmunoassay and association with thrombosis in Systemic Lupus Erythematosus. *Lancet*, **ii,** 1211.

HARRIS, E.N., GHARAVI, A.E. & HUGHES, G.R.V. (1985a) Antiphospholipid antibodies. *Clin. Rheum. Dis.* **11,** 591.

HARRIS, E.N., GHARAVI, A.E., LOIZOU, S., DERUE, G., CHAN, J.K., PATEL, B.M., MACKWORTH-YOUNG, C.G., BUNN, C.C. & HUGHES, G.R.V. (1985a). Crossreactivity of antiphospholipid antibodies. *J. clin. Lab. Immunol.* **16,** 1.

HARRIS, E.N., GHARAVI, A.E., HEGDE, U., DERUE, G., MORGAN, S.H., ENGLERT, H., CHAN, J.K.H. ASHERSON, R.A. & HUGHES, G.R.V. (1985b). Anticardiolipin antibodies in autoimmune thrombocytopenic purpura. *Br. J. Haematol.* **59,** 231.

HARRIS, E.N., ASHERSON, R.A., GHARAVI, A.E., MORGAN, S.H., DERUE, G. & HUGHES, G.R.V. (1985c). Thrombocytopenia in SLE and related autoimmune disorders: Association with anticardiolipin antibody. *Br. J. Haematol.* **59,** 227.

HARRIS, E.N., GHARAVI, A.E., CHAN, J.K.H., ENGLERT, H., MANTELLI, P., ALLEGRO, F., BALLESTRIERI, G. & HUGHES, G.R.V. (1985d). Affinity purified anti-cardiolipin and anti-DNA antibodies. *J. clin. Lab. Immunol.* **17,** 155.

HARRIS, E.N., GHARAVI, A.E., WASLEY, G.D. & HUGHES, G.R.V. (1988) Use of an enzyme-linked immunosorbent assay and of inhibition studies to distinguish between antibodies to cardiolipin from patients with syphilis or autoimmune disorders. *J. Infect. Dis.* **157,** 23.

JANOFF, A.S. & RAUCH, J. (1986) The structural specificity of anti-phospholipid antibodies in autoimmune disease. *Chem. Phys. Lipid.* **40,** 315.

KOIKE, T., TOMIOKA, H. & KUMAGAI, A. (1982) Antibodies cross-reactive with DNA and cardiolipin in patients with systemic lupus erythematosus. *Clin. exp. Immunol.* **50,** 298.

KOIKE, T., SUEISHI, M., FUNAKI, H., TOMIOKA, H. & YOSHIDA, S. (1984) Anti-phospholipid antibodies and biological false positive serological test for syphilis in patients with systemic lupus erythematosus. *Clin. exp. Immunol.* **56,** 193.

KOVACSOVICS, T., TSCHOPP, J., KRESS, A. & ISLIKER, H. (1985) Antibody-independent activation of C1, the first component of complement, by cardiolipin. *J. Immunol.* **135,** 2695.

LAFER, E.M., RAUCH, J., ANDREZEJEWSKI, C., MUDD, D., FURIE, B., FURIE B., SCHWARTZ, R.S. & STOLLAR, B.D. (1981) Polyspecific monoclonal lupus autoantibodies reactive with both polynucleotides and phospholipids. *J. exp. Med.* **153,** 897.

LOCKSHIN, M.D. (1985) Lupus pregnancy. *Clin. Rheum. Dis.* **11,** 611.

LOIZOU, S., MCCREA, J.D., RUDGE, A.C., REYNOLDS, R., BOYLE, C.C. & HARRIS, E.N. (1985) Measurement of anti-cardiolipin antibodies by an enzyme-linked immunosorbent assay (ELISA): Standardization and quantitation of results. *Clin. exp. Immunol.* **62,** 738.

MANTHORPE, R., FROST-LARSEN, K., ISAGER, H. & PRAUSE, J.U. (1981) Sjögren's syndrome. *Allergy*, **36,** 139.

MASI, A.T., RODNAN, G.P. & MEDSGER, T.A. (1981) Preliminary criteria for the classification of systemic sclerosis (scleroderma) *Bull. rheum. Dis.* **31,** 1.

MORTON, K.E., GAVAGHAN, T.P., KRILIS, S.A., DAGGARD, G.E., BARON, D.W., HICKIE, J.B. & CHESTERMAN, C.N. (1986) Coronary artery bypass graft failure—an autoimmune phenomenon? *Lancet*, **ii,** 1353.

MOURITSEN, S., HØIER-MADSEN, M., DEMANT, E.J.F. & PERMIN, H. (1985). Enzyme-linked immunosorbent assay for determination of anti-microchondrial antibodies. *Acta. path. microbiol. immunol. Scand.* (Sect. C.) **93,** 205.

ROPER, M.V., BENNETT, G.A., COBB, S., JACOX, R. & JESSAR, R.A. (1958) Revision of diagnostic criteria for rheumatoid arthritis. *Bull. Rheum. Dis.* **9,** 175.

STRANDBERG PEDERSEN, N., ØRUM, O. & MOURITSEN, S. (1987) Enzyme-linked immunosorbent assay for detection of antibodies to the venereal disease research laboratory (VDRL) antigen. *J. Clin. Microbiol.* **25,** 1711.

TAN, E.M., COHEN, A.S., FRIES, J.F., MASI, A.T., MCSHANE, D.J., ROTHFIELD, N.F., SHALLER, J.G., TALAL, N. & WINCHESTER, R.J. (1982) The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis. Rheum.* **25,** 1271.

TINCANI, A., MERONI, P.L. BRUCATO, A., ZANUSSI, C., ALLEGRI, F., MANTELLI P., CATTANEO, R. & BALESTRIERI, G. (1985) Antiphospholipid and anti-mitochondrial type M5 antibodies in systemic lupus erythematosus. *Clin. exp. Immunol.* **3,** 321.