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Platelet function in patients with lupus anticoagulant and thrombosis.

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Abstract. In a group of 337 patients with a history of thrombotic episodes, pregnancy losses and/or thrombocytopenia, 66 cases of lupus anticoagulant (LA) were found. Spontaneous platelet aggregation and the aggregatory responses of platelet rich plasmas (PRP) from 14 patients, with a history of thrombotic episodes, with anticardiolipin (ACA) levels above 21 IgG antiphospholipid antibodies units and normal platelet counts were studied and compared with those of 8 patients with history of thrombosis and negative LA and ACA (controls). Epinephrine, adenosine diphosphate, collagen and ristocetin were used as platelet aggregation inducers. Early collagen-whole blood interaction (BASIC WAVE), as a measure of platelet recruitment, and the levels of von Willebrand factor were also determined. The results of each test were compared with those of nine patients, used as controls, with thrombotic antecedents but negative LA and ACA. None of the patients with LA, or the control group, showed spontaneous platelet aggregation. The aggregatory responses, when epinephrine, ADP or collagen were added to the patient's PRP, were within normal range in most cases (64.2%, 52% and 72% respectively). The highest rate of hyperaggregation after the above mentioned inducers, was 12% and corresponded to the response to collagen. On the contrary, platelet aggregation rate with ristocetin was higher than 100% in 61.0% of the problem group, with no significative difference from the controls. The BASIC WAVE was of low rate and similar in the two groups studied. The von Willebrand factor was significantly higher (150 ± 55%) in the problem group than in the controls (98± 25.6%) ($p < 0.01$). The present results do not support the theory that platelet hyperactivity plays a significant role in the development of thrombotic complications in patients with LA. The increased von Willebrand factor points toward endothelial cell damage caused by the LA antibodies, with activation of the extrinsic pathway. However, the measurement of other platelet activation

markers, would help in establishing the extent of platelet activation and its role in the pathogenesis of thrombosis in patients with LA.

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Palabras claves: Anticoagulante lúpico, anticuerpos antifosfolípidos, agregación plaquetaria, trombosis.

Resumen. Se determinaron las principales manifestaciones clínicas y la función plaquetaria de 66 pacientes con anticoagulante lúpico (AL). Las pruebas de funcionalismo plaquetario consistieron en la determinación de agregación espontánea y después de la adición de agonistas plaquetarios al plasma rico en plaquetas (PRP) de 14 pacientes con antecedentes trombóticos y anticardiolipinas (ACA) con títulos superiores a 21 unidades GPL y número normal de plaquetas y de 8 pacientes que tenían antecedentes trombóticos pero no tenían AL ni ACA. Además se estudió la interacción temprana colágeno-sangre total (ONDA BASIC), como medida de reclutamiento plaquetario y se determinaron los valores de factor von Willebrand (FvW) en plasma. Los resultados de cada prueba fueron comparados con los de 9 personas con antecedentes trombóticos sin AL y ACA. No se observó agregación espontánea en ninguno de los casos estudiados y la agregación después de añadir epinefrina al PRP, fue normal en el 64,2% de los pacientes, en el 52% después de adenosín difosfato y en el 72% después de colágeno. No se hallaron diferencias estadísticamente significativas con los resultados del grupo sin AL o ACA. Sin embargo la mayoría de los pacientes mostró una hiperrespuesta a la adición de ristocetina en el 61,0% de los casos sin diferencia significativa con el grupo control. La ONDA BASIC fue de baja magnitud y similar en ambos grupos. Por el contrario, el FvW estuvo significativamente más elevado (155 ± 55%) en el grupo problema que en el grupo control (98 ± 26%), ($p < 0,01$). Los resultados obtenidos no demuestran que exista una activación plaquetaria aumentada *ex vivo* en pacientes con AL y ACA. El aumento del FvW, sugiere la existencia de daño de la célula endotelial con la liberación de sustancias trombogénicas. Sin embargo se hace necesario el estudio de otros marcadores de la activación plaquetaria, a fin de esclarecer la existencia o no de hiperactividad plaquetaria como factor importante en la génesis de trombosis en pacientes con anticuerpos antifosfolípidos.

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INTRODUCTION

The presence of lupus anticoagulant (LA) has been widely associated with the development of arterial and venous thrombosis, recurrent pregnancy losses, and thrombocytopenia (2, 3, 10). Several possible causes of these complications have been postulated, among them, increased formation of thromboxane-B_2 and decreased prostacyclin release with the consequential hyperaggregability of platelets (3, 4, 8). In other reports, no evidence of platelet hyperactivity has been found, even when the union of the antibodies to the platelet membrane was demonstrated (9, 12). The aim of the present work is to report the frequency of associated pathology and the results of platelet function studies in patients with LA and anticardiolipin antibodies (ACA), and to compare them to patients with history of thrombotic episodes and negative LA and ACA.

MATERIAL AND METHODS

Three hundred and thirty seven patients with a documented history of thrombotic episodes, pregnancy losses, and/or thrombocytopenia, were studied. Previous to blood extraction, the patients were instructed to refrain from taking any medication during 11 days.

Blood from each patient was withdrawn early in the morning, after overnight fasting, utilizing a double plastic syringe technique with a 21 gauge butterfly needle. Blood from the first syringe was destined for routine hematological studies and for the obtention of serum, and the content of the second syringe was placed in poliethylene tubes containing one part of 3.8% sodium citrate for nine parts of blood. Platelet poor plasma (PPP) was obtained by centrifugation at 2,400 g during 20 minutes at 4° C. The plasmas to be used for the determination of LA were recentrifuged at 12,400 g for the same period of time and temperature. When LA assaying was not done immediately, plasmas were kept frozen at -70° C until used. Platelet rich plasma (PRP) was prepared by centrifugation at 180 g during 10 minutes at room temperature.

Activated partial thromboplastin time (aPTT) and prothrombin time (PT) were performed according to standard techniques using Thrombofax and tissue brain thromboplastin, respectively (Ortho Diagnostics). In cases with prolonged coagulation times, the tests were repeated after mixing equal parts of patient's plasma and normal plasma pool obtained from sixteen normal donors and kept divided in aliquots at -70° C. However, in disregard of the results, the presence of LA was also determined according to Thiagarahan et al. (14) utilizing diluted Russell's viper venom (Sigma Laboratories). The neutralization procedure for positive plasmas consisted in the addition of

platelet concentrate according to Tripplet *et al.* (15).

Antiphospholipid antibodies (anticardiolipins), were measured in 51 patients with positive LA utilizing an enzyme linked immunoabsorbent assay (ELISA), (Hemagen Diagnostics, Inc.). Patients were considered positive when the immunoglobulin concentrations were more than 11 units of IgG antiphospholipid antibody (GPL) or IgM antiphospholipid antibody (MPL).

Individuals with a history of thrombosis, positive LA and more than 21 GPL units of ACA (medium positive) were considered as patients and those with negative LA and less than 11 GPL units of ACA but with history of thrombotic episodes, were considered as controls.

Platelet aggregation was studied in PRP from 14 patients and 8 controls with normal platelet counts, after adjusting the platelets to 250.000/uL with the patients own PPP. The PRP was placed in an aggregometer (Chrono-Log, model 440) and spontaneous aggregation was tested during 15 minutes. Platelet aggregation was studied after addition of agonists at final concentrations of $3x\ 10^{-3}$ mm for epinephrine, $3x\ 10^{-3}$ mm for adenosine diphosphate , 3ug/mL for collagen (Horm Laboratories, Germany) and 2.2 mg/mL for ristocetin (Lundbeck, Denmark). Aggregations of less than 60% were considered low, those between 60 and 100% were considered normal and those above 100% were considered high.

The early interaction of collagen with the components of whole blood (BASIC WAVE) was determined in 9 patients and 9 controls, according to Pérez-Requejo et al. (10).

The quantity of von Willebrand factor was determined in 18 patients and 17 controls by immunoelectrophoresis according to Laurell (7).

The statistical analysis of the results consisted in the calculation of probability by the X^2 and the Student's t tests.

RESULTS

Lupus anticoagulant was present in 66 patients out of the 337 persons studied (19.6%), of which 55 were females. Thirty four (51.5%) met the requirements for the diagnosis of systemic lupus erythematosus, seven (10.6%) had chronic immune thrombocytopenic purpura (ITP), 14 cases (21.2%) had no evidence of underlying diseases and were classified as "Primary antiphospholipid syndrome" (PAPS) and the rest of the patients suffered from varied diseases such as rheumatoid arthritis (2 cases), mitral valvular disease with *livedo reticularis* and thrombosis (Sneddon's syndrome), type II Diabetes, chronic renal failure, chronic hepatitis, nocturnal paroxysmal hemoglobinuria, bone marrow hypoplasia and myeloproliferative syndrome.

Nineteen patients (28.78%) had had previous thrombotic episodes, mainly deep vein thrombosis

(52.6%) and cerebrovascular thrombosis (31.6%). Two of these patients also had ITP, one with less than 15,000 platelets/uL. Twenty four patients (36.6%) including those with ITP, presented thrombocytopenia and twelve women (21.8% of females) had a history of one or more pregnancy losses.

Anti IgG or IgG and IgM antibodies (ACA) were found in 89.4% of patients with LA and thrombosis. Isolated IgM antibodies were present in two cases, but none of them had had previous thrombotic events (Table I).

No spontaneous aggregation was observed in any of the 14 PRPs studied. Sixty four per cent of the PRPs from patients with LA and a history of thrombosis, registered aggregations within normal range after the addition of epinephrine, 52% when the inducer was ADP and 72% after collagen. However, a high aggregatory response (100%), was evidenced in 61% of the cases after the addition of ristocetin. These results do not statistically differ from those of 8 controls with a history of thrombosis in which no LA or ACA were found (Table II). No differences were found in the intensity of the BASIC WAVE in the two groups studied (Table III).

The von Willebrand factor was significantly higher in the group with LA and thrombosis (150 ± 55%) than in the group with thrombosis with no LA (98± 25.6%), ($p < 0.01$).

TABLE I

PREVALENCE OF THROMBOSIS AND ITS RELATIONSHIP WITH ANTICARDIOLIPIN ANTIBODIES IN PATIENTS WITH LUPUS ANTICOAGULANT

Diagnosis (Number of cases)	Number of Cases with Thrombosis	Anticardiolipin immunoglobulins IgG	IgG - IgM	IgM
SLE * (34)	6 (21%)	2	4	0
ITP** (17)	2 (12%)	0	2	0
PAPS *** (14)	8 (50%)	5	3	0
OTHER (11)	3 (28%)	0	3	0
TOTAL	19	7	12	0

* Systemic Lupus Erythematosus
** Inmune Thrombocytopenic Purpura
*** Primary antiphospholipid syndrome
( ) Correspond to number of cases

TABLE II

PLATELET AGGREGATION FROM PATIENTS WITH THROMBOTIC HISTORY. ITS RELATIONSHIP WITH THE PRESENCE OF LUPUS ANTICOAGULANT (L.A.)

Inducer	Low		Medium		High	
	L.A. Positive	L.A. Negative	L.A. Positive	L.A. Negative	L.A. Positive	L.A. Negative
	%	%	%	%	%	%
Epinephrine	28.0	0.0	64.0	100.0	7.0	0.0
A.D.P.	40.0	25.0	52.0	75.0	8.0	0.0
Collagen	16.0	0.0	72.0	75.0	12.0	25.0
Ristocetin	11.0	12.5	28.0	25.0	61.0	62.5

Low aggregation 60%
Normal aggregation 60% - 100%
High aggregation 100%
Number LA Pos. cases: 14
Number LA Neg. cases: 8

TABLE III

EARLY COLLAGEN-WHOLE BLOOD INTERACTION (BASIC WAVE) IN PATIENTS WITH THROMBOTIC HISTORIES. ITS RELATION WITH THE PRESENCE OF LUPUS ANTICOAGULANT

Group	Basic Wave Agregation %
Positive L.A.	17.5 ± 7.9 (9)
Negative L.A.	17.0 ± 12.1 (9)

( ) Number of cases

DISCUSSION

The associated pathology found in 66 patients with LA is in agreement with what has been already reported *v.g.* thrombosis, thrombocytopenia and recurrent abortions or fetal deaths. When patients with ITP were included, thrombocytopenia was the most frequent fea-

ture (36.3%). But, when ITP cases were excluded, thrombocytopenia was only encountered in 21.2% of patients, thus making thrombotic episodes the complication most frequently found. The omnipresence of IgG antibodies in patients with thrombosis was also found, and only two patients had isolated IgM isotype, although none of them had experienced thrombosis. Systemic lupus erytematosus was the most frequent disease associated with LA, followed by cases where no underlying disease was demonstrated, but with histories of thrombosis or fetal wastage (primary antiphospholipid syndrome).

While no spontaneous platelet aggregation could be demonstrated, the responses after epinephrine, ADP, or collagen were within normal limits in most cases, with no statistical difference from a group that also had a history of thrombosis but no LA. Sixty one per cent of patients showed enhanced aggregation after ristocetin was added to their PRP, a finding that was also similar to the results in the group with negative LA. These results agree with the findings of Out *et al* (9) who, after demonstrating the union of the antiphospholipid antibodies to the platelet surface did not observe platelet activation and aggregation was not impaired. Schorer *et al* (12), found defective endothelial prostacyclin release and diminished platelet aggregation after stimulation with thrombin. However, Wiener *et al* (16), found microscopic spontaneous platelet aggregation in all patients with LA and Carreras *et al* (3), reported increased TxB_2 production in patients under the same clinical condition. More recently, Martinuzzo *et al* (8) found increased platelet aggregation and serotonin C^{14} release, after the addition of F (ab)'$_2$ fragments of IgG from patients with antiphospholipid antibodies with a stronger response after stimulation with low dose of thrombin. However, the lack of increased early collagen interaction with the components of total blood in relation to patients without LA, is against the theory that platelet hyperactivity plays an important role in the development of thrombosis in patients with LA. The fact that thrombosis may be present in patients with very low platelet counts goes against this assumption.

According to Zwaal (17), the complete externalization of anionic phospholipids occurs after platelet activation with strong agonists such as collagen and thrombin. In vivo, the interaction of platelets with collagen occurs after endothelial damage when the collagen from the subendothelial layer is exposed. The same endothelial damage is responsible for increased tissue factor in plasma with activation of the extrinsic pathway and the consequent generation of thrombin (5). This has been confirmed by Bertrand *et al* (1) in tissue cultures, where they found that IgG antiphospholipid antibodies caused increased tissue factor activity. Increased von Willebrand factor in the plasma of patients with LA supports this mechanism. Under

this assumption platelet activation would be the result of endothelial cell damage caused by the LA antibodies or immune complexes with exposure to collagen and thrombin. Kornberg *et al* (6), have demonstrated that monocytes generate a potent procoagulant activity after stimulation with murine monoclonal ACA. This activity simulates that of tissue factor in its dependency of factor VII. Although these were in vitro studies utiliziyng murine ACA, the results imply that monocytes play an important role in the development of thrombosis in patients with ACA. The above results , present evidence that several mechanisms are involved in the production of thrombi in these patients, probably some have a more important role than others, and as Schorer *et al* signify (13), not all the antiphospholipid antibodies produce the same effect in hemostasis.

The controversial findings regarding the effect of these antibodies on platelets, demonstrate that further studies are necessary to clarify whether platelet activation plays an important role in the pathogenesis of thrombosis in patients with ACA. The determination of other platelet activation markers, would be of help in establishing the extent of platelet activation in these patients.

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BIBLIOGRAPHY

1- BERTRAND J.J., ARNOUX D., SANYD J.: Induction of tissue factor by IgG isolated from patients with lupus anticoagulant. Thromb Haemost (Abstract) 69:594, 1993.

2- BOWE B.W., THOMPSON J.H., PASCUZZI C.A., OWEN G.A.: Thrombosis in SLE despite circulating anticoagulants. J Lab Clin Med 62:416-430, 1963.

3- CARRERAS L.O., MACHIN S.J., DEMAN R., DEFREYN G., VERMYLEN J., SPITZ B., VAN ASSHE A.: Arterial thrombosis, intrauterine death and "lupus anticoagulant": detection of immunoglobulin interfering with prostacyclin formation. Lancet 1:244-246, 1981.

4- CARRERAS L.O., VERMYLEN J.G.: "Lupus anticoagulant" and thrombosis, possible role of inhibition of prostacyclin formation. Thromb Haemost 48:28-40, 1982.

5- CINES D.B., TOMASKY A., TANNENBAUN S.: Immune endothelial cell injury in heparin associated thrombocytopenia. N Engl J Med 316:581-589, 1987.

6- KORNBERG A.. BLANK M., KAUFMAN S., SHOENFELD Y.: Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. J Immunol 153:1328-1332, 1994.

7- LAURELL C.B.: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Ann Biochem 15:45-52, 1966.

8- MARTINUZZO M.E.,MACLOUF J., CARRERAS L.O., LEVY-TOLEDANO S.: Antiphospholipid antibodies enhance thrombin-induced platelet activation and thromboxane formation. Thromb Haemost 70:667-671, 1993.

9- OUT H.J., DE GROOT P.G., VAN VLIET M., DE GAST G.C., NIEUWENHUIS H.K., DERKSEN R.H.W.M.: Antibodies to platelets in patients with antiphospholipid antibodies. Blood 77:2655-2659, 1991.

10- PEREZ-REQUEJO J.L.,AZNAR J., SANTOS M.T., VALLES J.: Early platelet collagen interactions in whole blood and their modifications with aspirin and dipyridamole evaluated by a new method (Basic Wave). Thromb Haemost 54:799-803, 1985.

11- SCHLEIDER M.A., NACHMAN R. L., JAFFE E. A., COLEMAN M.: A clinical study of lupus anticoagulant. Blood 48:499-509, 1976.

12- SCHORER A.E., WICKHAM N.W.R., WATSON K.V.: Lupus anticoagulant induces a selective defect in thrombin-mediated endothelial prostacyclin release and platelet aggergation. Brit J Haematol 71:399-407, 1989.

13- SCHORER A.E., DUANE P.G. WOODS V.L., NIEWOEHNER D.E.: Some antiphospholipid antibodies inhibit phospholipase A_2 activity. J Lab Clin Med 120:67-77, 1992.

14- THIAGARAJAN P., PENGO V., SHAPIRO S.S.: The use of the diluted Russell viper venom time for the diagnosis of lupus anticoagulants. Blood 68:869-874, 1986.

15- TRIPPLET D.A., BRANDT J.T., KACZOR D., SCHAEFFER J.: Laboratory diagnosis of lupus inhibitors: a comparison of the tissue thromboplastin inhibition procedure with a new platelet neutralization procedure. Am J Clin Path 79:678-682, 1983.

16- WIENER H.M., VARDINON N., YUST I.: Platelet antibody binding and spontaneous aggregation in 21 lupus anticoagulant patients. Vox Sang 61:111-121, 1991.

17- ZWAAL R.F.A.: Membrane and lipid involvement in blood coagulation. Biochim Biophys Acta 515:163-205, 1978.