Thrombosis

An improved method for lupus anticoagulant detection

The diagnosis of lupus anticoagulant (LA) becomes difficult when there is a weak titer of LA antibodies. This study demonstrates that 20 minutes' incubation increases the sensitivity of the mixing test to diagnose LA by 23% and to suspect the antibodies by 66%.

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The criteria to define LA are based on different tests. In the mixing test (MT), inhibitory activity is measured in a 50:50 mixture of patient's plasma and normal pooled plasma. However, approximately 15% of LA have time-dependent inhibitory activity and the MT may show complete correction if tested immediately after mixing, especially when the titer of LA antibodies is low.[1,2] Nevertheless, no correction is made when the test is done after incubating the plasma for 1 hour at 37°C.[3,4] The current ISTH recommendations are ambivalent about the need for time-dependent LA MT assays[5,6] Recently, it has been shown that a drift to higher pH levels after incubation of the plasmas for one hour led to unreliable results.[7,8] The aims of our work were, firstly, to determine the optimal conditions of the MT incubation and, secondly, to determine whether incubation of the plasma mixture increases the sensitivity of the PTT-LA MT (Stago®) to diagnose LA.

First, we determined whether incubating the plasma produced significant changes in pH, which could explain an eventual prolongation of the MT. The PTT-LA MT and pH were measured after 0', 20' and 60' of incubation in closed cuvettes on 15 negative and 10 doubtful LA plasmas divided into 5 different groups: control plasma, patients' plasma, mixed plasma (50:50), mixed plasma + Owen's buffer, mixed plasma + HEPES buffer. Although the pH increase was statistically significant for all groups at 20' and 60' of incubation, the PTT-LA MT times were not significantly prolonged for any group, either after 20' or after 60' of incubation. The addition of Owren or HEPES buffer to the MT did not improve the results of the PTT-LA MT. For the subsequent tests we chose 20' of incubation of the simple PTT-LA MT at 37°C since this period seemed enough to improve the sensitivity of the MT without the test being influenced by any change in pH.

We then studied 199 patients registered in our hospital during 2000-2001 with a positive (n=77) or a doubtful (n=122) diagnosis of LA. Tests performed in all 199 patients were: aPTT, PT, TT, fibrinogen, aPTT MT, PTT-LA, TIT (thromboplastin inhibition test 1:200; recombiplastin hemoliance®), PTT LA MT 0', 20' and PNP (platelet neutralization procedure). Exclusion criteria were: TT>30”, liver diseases, anticoagulant treatment, hemophilia and other coagulation factor deficiencies. The diagnosis of LA was considered positive if both the PNP and the PTT-LA MT at 0' and/or at 20' were positive and was doubtful when either the PNP or the PTT-LA MT was negative. The clinical histories of 113 of the 199 patients were reviewed (Table 1). A total of 60 (53%) of the 113 patients had clinical features compatible with the presence of the LA and 13 of them had a positive Staclot in addition to the current LA tests performed. PTT-LA MT times after 20' of incubation were statistically longer than those before incubation for both the patients with a positive and a doubtful diagnosis of LA (Table 2). The mean PTT-LA MT increased by 8' after incubation among the patients with a positive diagnosis of LA. The incubation increased the sensitivity of the MT for LA diagnosis by 23%. The TIT was positive for 71% of positive patients and 87% of them had a positive immediate MT. Seventy-one percent of positive patients had compatible clinical features. Among the patients with a doubtful diagnosis of LA, 119 of 122 had a positive PTT-LA MT and a negative PNP. PTT-LA MT increased by 10" after incubation. The PTT-LA MT was the main test raising the suspicion of the presence of LA and incubation increase the sensitivity of this test to 60%. The TIT was positive for 60% of patients with a doubtful diagnosis and for 23% of negative ones. Incubation did not modify the rate of TIT positivity: 45% of plasmas with a positive immediate MT and 55% of plasmas with a positive MT only after incubation. Sixty-three percent of patients with a doubtful diagnosis had a compatible clinical history. All these facts reinforced the idea that the increase in the sensitivity of the PTT-LA MT after 20' incubation was well related to the presence of LA. In conclusion, incubating the mixtures in closed cuvettes at 37°C for 20 minutes seems to be enough to improve the sensitivity of PTT-LA of MT considerably. Changes in pH after incubation cannot explain the prolongation of the MT. The increase in the sensitivity of the MT has important clinical consequences given the well-known high risk of thrombo-embolic complications in patients positive for LA. A doubtful diagnosis of LA with a positive PTT-LA MT only after incubation needs attention and should be integrated in the clinical history of the patient.

Table 1. Clinical events related to the presence of LA and positive Staclot tests for patients with a positive or doubtful diagnosis of LA.

Clinical events	*Positive LA (n=34)*	*Doubtful LA (n=79)*	*Total (n=113)*
Thrombosis	17	19	36
Fetal losses	0	3	3
Autoimmune diseases	3	18	21
Staclot	4	9	13
MT positive at 0'	22	16	38
MT positive at 20'	2	28	30

Table 2. Percentage of positive PTT-LA MT at 0' or at 20' and statistics for patients with a positive or doubtful diagnosis of LA.

	PTT-LA MT positive at 0'	*PTT-LA MT positive only at 20'*	*PTT-LA 0' MT mean±SD*	*PTT-LA 20' MT mean±SD*	*p*
Positive LA (n=77)	59 (77%)	18 (23%)	64"±18.0	71"±20.1"	<0.05
Doubtful LA (n=119)	41 (34%)	78 (66%)	50"±9.9"	60"±9.6"	<0.05

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Stem Cell Transplantation

Long-term follow-up of lymphocyte populations and cellular cytokine production in patients with chronic graft-versus-host disease treated with extracorporeal photopheresis

We studied lymphocyte populations and cytokine-expression profiles of ten patients with chronic graft-versus-host disease who at least transiently responded to photoimmunotherapy. The numbers of lymphocytes, monocytes and dendritic cells rose in most cases. Th1 cells always increased during therapy, supporting the hypothesis that a more favorable immune balance contributes to clinical responses.

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Extracorporeal photoimmunotherapy (ECP) is partially effective in controlling cutaneous and visceral chronic graft-versus-host disease (GVHD).[1-3] The mechanisms of action include T-cell apoptosis and modulation of dendritic and regulatory cells and cytokine production.[4-6] A Th1-polarization induced by ECP has been suggested;[7,8] this, and a Th2-skewed cytokine profile found in some patients with chronic GVHD,[9] prompted us to evaluate Th1/Th2 cytokine-expression profiles and circulating cell compartments in 10 patients (8 men, 2 women, median age 35 years, 16-61) with steroid-refractory, extensive chronic GVHD under ECP. Four patients had acute lymphoblastic leukemia, six had chronic myeloid leukemia and all had received bone-marrow and/or donor-lymphocyte infusions from identical-sibling donors. ECP was started a median of 31 (5-124) months after the onset of chronic GVHD, when ocular and/or extensive cutaneous disease, refractory to at least two immunosuppressants was present. The UVAR XTS System (Therakus, Johnson & Johnson) was used as described previously.[1] Treatment was given on two consecutive days every 2-4 weeks for 3-6 months and then reduced according to the clinical response. Peripheral blood samples were collected before the first and after the second ECP in each therapeutic session. Phenotypic analysis for surface markers and intracellular cytokines[10] employed phycoerythin (PE)-conjugated interleukin (2), IL4 and IL10 and fluorescein isothiocyanate (FITC)-conjugated interferon-γ (Pharmigen, S. Diego, CA, USA); FITC-conjugated CD3, CD8, CD14, CD19 and CD56; PE-conjugated CD4, CD56 and CD80; HLA-DR and peridin chlorophyl protein (PerCP)-conjugated HLA-DR and CD3 (BDIS, San José, CA, USA). Cell populations were compared before and at different time-points during photoimmunotherapy in each patient, and between patients exhibiting complete or partial responses (*good-responders*) and patients without response in at least one organ or dependent on frequent ECP for control of their GVHD (*bad-responders*). The Wilcoxon and Student' t tests were used to compare cell populations in each patient and between groups; the null hypothesis was rejected for $p<0.05$.

Most patients had oral and cutaneous chronic GVHD. Intestinal, hepatic, ocular and lung involvement was present in five, three, two and two patients, respectively. With a median follow-up of 36 (26-38) months after starting ECP, all patients had at least a partial response.[3] Major improvements occurred in cutaneous and ocular disease; pulmonary disease did not respond to ECP. Seventy-percent of patients were *good-responders* (binomial exact confidence interval 34.8%-93.3%); three patients were *bad responders*.

All patients were profoundly immunosuppressed before ECP, with T, B and NK-cell counts well below normal (Figure 1). The numbers of T ($CD4^+$ and $CD8^+$), B and NK-cells, monocytes and dendritic cells did not change significantly after each session; the small increments detected did not persist until the next treatment (*data not shown*). However, after at least 9 months of therapy, the T-cell count had increased a median of 1.65-fold; $CD4^+$-cells increased in eight patients, although only five reached the normal range and the number of $CD8^+$-cells did not change significantly in the majority of patients. B and NK cells were normal in seven and eight patients, respectively, at last follow-up (Figure 1). Monocytes and dendritic cells increased in most cases (median increases 1.9 and 11.4-fold, respectively). The changes in lymphocyte populations were similar in *good* and *bad-responders* ($p>0.05$). T cells (particularly $CD4^+$) increased significantly with photopheresis ($p=0.01$) and were higher after ECP in *good-responders* ($p=0.02$); no differences were found in other cell populations before or after treatment. Th1 and Th2 cells were almost undetectable before ECP. An immediate, transient increase in circulating Th1 cells after ECP occurred in ≤20% sessions. However, in the long-term photoimmunotherapy consistently increased the number of Th1-producing cells in all patients (Figure 2). IFN-γ and IL2-producing T-cell numbers were normal in all but one case at the last follow-up (median fold-increase of 92.6, 171.5 and 70.7 for IFN-γ producing $CD3^+$, $CD4^+$ and $CD8^+$-cells, respectively, and 87.4, 122.3 and 25.4 for IL2-producing $CD3^+$, $CD4^+$ and $CD8^+$-lymphocytes, respectively). *Bad* and *good-responders* had similar increases ($p>0.05$), possibly because all had at least a partial response to therapy. ECP did not modify Th2-cell percentages. In conclusion, only transient modifications in immune cells and cytokine-expression profiles occurred shortly after ECP, but prolonged therapy consistently increased the numbers of $CD4^+$ T cells (without significant changes in $CD8^+$ counts), NK cells and B cells in ≥50% cases and Th1-producing cells in

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