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Blastogenic response of activated human umbilical cord blood T-lymphocytes

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Donor T-lymphocytes are thought to play a crucial role in both acute and chronic graft-versus-host disease (GvHD), pathological conditions that frequently complicate allogeneic bone marrow transplantation.[1] These diseases are described as occurring with a lower incidence and lesser severity when human umbilical cord blood (HUCB) cells, which have recently emerged as a potential source of hematopoietic progenitors, are used for transplantation.[2-6] This condition is probably related to the immaturity of neonatal HUCB T cells.[7,8] Lymphocyte blastogenic response to phytohemagglutinin (PHA), evaluated by means of flow cytometry, is a useful tool for testing the functional ability of T-cells to display an immune response against allo-antigens, reproducing *in vitro* the *in vivo* mechanism of activation. This study was designed to verify whether an impairment in HUCB T-cell ability to undergo an *in vitro* blastogenic response to mitogens could explain their reduced *in vivo* ability to induce GvHD.

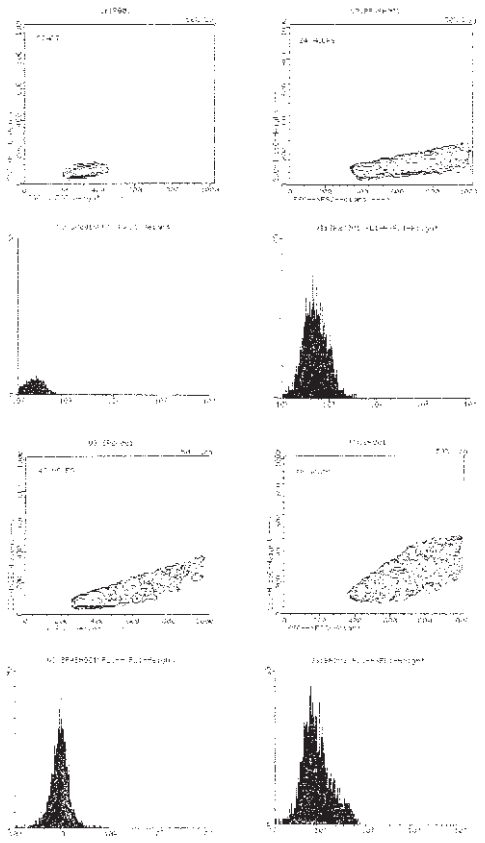
Lectins, such as PHA, are proteins and glycoproteins able to bind specifically to certain carbohydrate structures on cell surfaces.[9] In lectin-mediated lymphocyte activation the first step is probably the cross-linking of membrane proteins such as T3 complex which is now recognized as the probable transducer of antigenic signals in T-cell activation.[10,11] Cellular DNA staining with intercalating dyes of stimulated peripheral blood (PB) lymphocytes has commonly been used to test cell-mediated immunity.[12] PHA predominantly stimulates T-lymphocytes. Lymphocyte blastogenesis is induced in cultures and evaluated by flow cytometric cell-cycle analysis after propidium iodide (PI) staining of the nuclei. PI binds to DNA in cells at all stages of the cell cycle, and the nucleus light emission intensity is directly proportional to its DNA content. Stimulated and activated cells entering the cell cycle pass through the S-phase and synthesize DNA. Furthermore, activated T-cells co-express HLA-DR and CD25 glycoproteins. On these bases, in our study we used flow cytometry as a tool to verify whether differences in allo-activation ability of T-lymphocytes of cultured HUCB and PB samples exist.

PB mononuclear cells were obtained from 20 HUCB and 20 healthy adult PB heparinized samples by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation. Eight aliquots of 200 µL each containing 2×10^9 cells were obtained from the pellet. Six aliquots were cultured in 6 flasks containing 5 mL of RPMI 1640 medium with 100 µg/mL PHA. Cultures were placed in a 37°C incubator gassed with 5% CO_2 in air. From the seventh aliquot, 50 µL were stained with CD3-phycoerythrin (PE)/HLA-DR-fluorescein isothiocyanate (FITC), and 50 µL with CD3-PE/CD25-FITC monoclonal antibody combinations, respectively, and evaluated by means of flow cytometry following incubation for 30 minutes at 4°C in the dark. Aliquot number 8 was immediately stained with PI, as follows: to 2×10^6 cells, 2 mL of PI solution (50 µg/mL) + 25 µL of RNAse solution (0.5 mg/mL: 100 U) + 25 µL of Nonidet P40 0.1% solution were added. Samples were incubated at room temperature in the dark for 30 minutes. These reagents are able to isolate and stain the cell nuclei from blood cells. Samples were then filtered through a 40 µm nylon mesh just before analysis. For each sample a minimum of 50,000 events were acquired in list mode data files on a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems, BDIS, San José, CA, USA) equipped with a 15

Table 1. Stimulation of mononucleated HUCB and PB samples with PHA.

Hours	*HUCB*	*PB*	*p*
$CD3^+HLA\text{-}DR^+$ cells (%)			
0	3.6±0.5	3.6±0,9	ns
24	34.6±12.7	38.6±10.3	ns
48	40.3±10.2	42.1±5.5	ns
72	58.2±12.1	59.4±5.6	ns
$CD3^+CD25^+$ cells (%)			
0	3.9±0.5	5.1±0.3	ns
24	45.2±9.6	42.4±14.9	ns
48	44.3±15.8	55.8±16.5	ns
72	64.6±7.4	70.1±14.6	ns
S-phase cell number (%)			
0	1.7±1.2	1.4±0.4	ns
24	6.1±1	4.4±1.9	ns
48	10.9±9	9.2±5.3	ns
72	24±12.8	25.2±9.8	ns

Data are expressed as mean percentage cell number±standard deviations. Statistical comparisons were carried out using Student's t-test.

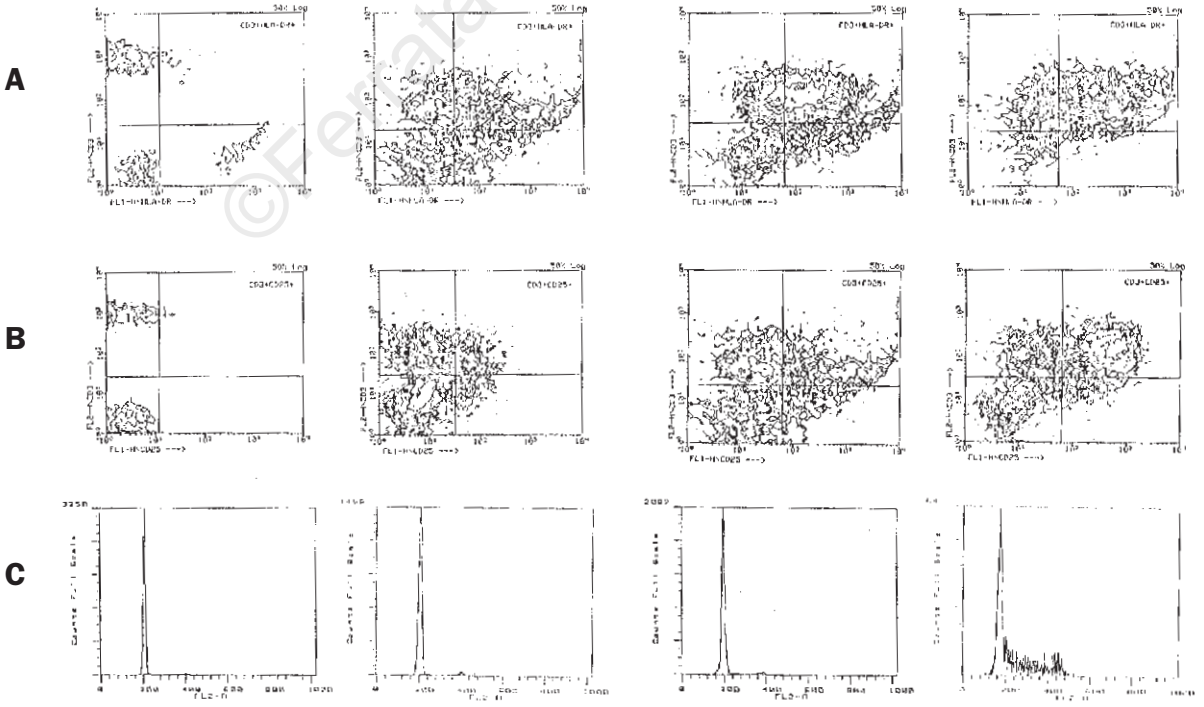


mW argon laser emitting at 488 nm and Lysis II software. All channels were set for acquisition in the logarithmic mode. An acquisition gate was set according to side and forward light scattering cell properties to collect only the lymphoid population. Cells were then analyzed for two-color fluorescence. In addition, for each DNA histogram, the cell cycle distribution was calculated using the CellFIT software program (BDIS). The above mentioned procedures for either CD3/CD25 or CD3/HLA-DR co-expression and DNA analysis were used for the cultured samples after two washings 24, 48, and 72 hours after starting the incubations. Cell proliferation was evaluated comparing S-phase percentage cell number and $CD3^+CD25^+$ and $CD3^+HLA\text{-}DR^+$ percentage cell number at time 0 (just before culturing) with the same values obtained 24, 48, and 72 hours after starting the culture.

Both cultured HUCB and PB mononuclear cells show increased cell size, granularity and autofluorescence intensity due to PHA stimulation. Figure 1A

Figure 1A (left). Variations in light scattering properties of an HUCB sample for both cell size and granularity (contour plots) and autofluorescence (histograms) from time 0 (just before culturing) to 72 hours after culture commencement.

Figure 1B (below). Variations of HLA-DR (a) and CD25 (b) co-expression by T-cells ($CD3^+$) and S-phase cycle cell number (c) of the same HUCB sample from time 0 to 72 hours later.



portrays the typical variations in cell light scattering properties of a HUCB sample from time 0 (just before culturing) to 72 hours after culture commencement. Table 1 gives the results obtained, clearly showing that there were no differences in PHA's capacity to stimulate T-lymphocytes between the HUCB and PB samples tested, as evaluated in terms of $CD3^+CD25^+$ and $CD3^+HLA\text{-}DR^+$ co-expression and in terms of percentage of cell number in S-phase. Finally, Figure 1B shows the pattern of change in HLA-DR and CD25 co-expression by $CD3^+$ T-cells and in the S-phase of the cell cycle at time 0 and throughout the 72 hours of culturing.

As shown, no differences were revealed between HUCB and adult PB functional assays, suggesting that HUCB lymphocyte functional immaturity could reside in other immunologic mechanisms, thus needing further investigation.

Key words

Human umbilical cord blood, lymphocyte blastogenesis, flow cytometry.

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A patient homozygous for mutation 20210 A in the prothrombin gene with venous thrombosis and transient ischemic attacks of thrombotic origin

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It is well established that genetic disorders interact with environmental factors to cause thrombotic diseases.[1] Therefore, antithrombin, protein C, protein S deficiencies and the more recently described factor V Leiden and prothrombin mutations are currently been investigated to explain some thrombophilic states. We report the case of a 63-year-old man who developed two transient ischemic attacks and two years later an extensive femoro-iliac venous thrombosis. He was genotyped as FV R506Q negative and FII G20210A positive in homozygous state (FII 20210AA).

A polymorphism in the 3'-UTR (untranslated region) of the prothrombin gene, due to a G to A transition at nucleotide 20210 has been recently described.[2] This anomaly was associated with elevated plasma prothrombin (factor II) levels and with an increased risk of venous thrombosis.[2] This situation is not uncommon. The variant (A allele) was identified in 2.3% of healthy controls and in 6.2% of unselected patients with venous thrombosis, in the original paper which describes the Dutch population. It was established that there was a 2.8-fold increased risk of venous thrombosis in the carriers.

Subsequently, other reports described different populations with similar frequencies: 2.6% in healthy donors and 5.0% in patients with venous thromboembolism, in the Cambridge report[3] and 1.8% in the control group versus 7.1% in patients from Sweden.[4]

The first homozygous patient was mentioned in the original paper.[2] Specifically, the first report was made on a young Mexican male (24 years old) who had a myocardial infarction (MI) and subsequently iliofemoral venous thrombosis and massive pulmonary embolus. Although the patient was found to be a factor V Leiden carrier,[5] an association between the arterial thrombotic events and the prothrombin muta-