eral blood stem cell mobilization with etoposide (VP-16) in patients with relapsed or resistant lymphoma who failed cyclophosphamide mobilization. Bone Marrow Transplant 1999; 23:1223-8.
4. Shimazaki C, Oku N, Ashihara E, et al. Collection of peripheral blood stem cells mobilized by high-dose Ara-C plus VP-16 or aclarubicin followed by recombinant human granulocyte-colony stimulating factor. Bone Marrow Transplant 1992; 15:341-6.
5. To LB, Shepperd KM, Haylock DN, et al. Single high doses of cyclophosphamide enable the collection of high numbers of hematopoietic stem cells from peripheral blood. Exp Hematol 1990; 18:442-7.
6. Rosenfeld CS, Shadduck RK, Zeigler ZR, Andrews F, Nemunaitis J. Cyclophosphamide-mobilized peripheral blood stem cells in patients with lymphoid malignancies.Bone Marrow Transplant 1995; 15:433-8.
7. Jagannath S, Vesole DH, Glenn L, Crowley J, Barlogie B. Low-risk intensive therapy for multiple myeloma with combined autologous bone marrow and blood stem cell support. Blood 1992; 80:1660-72.
8. McQuaker IG, Haynes AP, Stainner C, Anderson S, Russell NH. Stem cell mobilization in resistant or relapsed lymphoma: superior yield of progenitor cells following a salvage regimen comprising ifosphamide, etoposide and epirubicin compared to intermediate-dose cyclophosphamide. Br J Haematol 1997; 98: 228-33.
9. Ketterer N, Salles G, Moullet I, et al. Factors associated with successful mobilization of peripheral blood progenitor cells in 200 patients with lymphoid malignancies. Br J Haematol 1998; 103:235-42.
10. Russell NH, McQuaker G, Stainer C, Byrne JL, Haynes AP. Stem cell mobilization in lymphoproliferative diseases. Bone Marrow Transplant 1998; 22:935-40.

Disappearance of factor VIII inhibitors in a severe hemophilia A neonate after steroid treatment correlated with a cytokine shift toward a T-helper 2 pattern

Factor VIII inhibitor developed in a neonate with hemophilia A after a few days of prophylactic therapy with recombinant FVIII. Interleukin-2 and interleukin-6 expression was evaluated in lymphocytes by ELISA and by mRNA *in situ* hybridization. We observed a Th1-type pattern during inhibitor production, which shifted toward a Th2 profile after steroid treatment.

Sir,

We analyzed interleukin-2 (IL-2) and interleukin-6 (IL-6) expression – as a sign of preferential T-helper 1 (Th1) or T-helper 2 (Th2) pattern – in a four-day old boy who was diagnosed as having hemophilia A (FVIII:C <1%/VWF 100%) after hemorrhagic shock due to gastrointestinal bleeding. The family history confirmed congenital hemophilia. After immediate treatment with packed red blood cells and intermediate-purity factor VIII (Hemate-P, Centeon, Marbourg, Germany) 50 U twice a day for 10 days, prophylactic administration of recombinant-FVIII (rFVIII) (Helixate, Centeon, Berkeley, CA, USA) 50 U/kg twice a week was started. Eleven days after FVIII exposure, anti-FVIII antibodies were detected by the Bethesda assay (13.5 BU). rFVIII administration was stopped and, after parents' informed consent, the patient received oral prednisone 2 mg/kg daily for three weeks, with progressive tapering to 7 mg on every other day after five weeks, and reduction of inhibitor to 1 BU.

Intron 22 inversion of the FVIII gene was excluded by polymerase chain reaction (PCR) analysis. Peripheral blood mononuclear cells (PBMC, $2x10^5$/well) were collected 5, 20 and 45 days after the appearance of the inhibitor, then incubated for 5 days in the absence or presence of rFVIII at 2 µg/mL. PBMC from a normal neonate and from a neonate with hemophilia A without inhibitor were tested as controls. IL-2 and IL-6 concentrations were measured in the supernatants by a quantitative *sandwich enzyme immunoassay* (Boehringer Mannheim, Germany) with a sensitivity >20 pg/mL for IL-2 and >10 pg/mL for IL-6. The two cytokines were also evaluated in PBMCs by mRNA *in situ* hybridization. Briefly, probes were obtained by reverse transcription-PCR as described[1] using oligonucleotide primers specific for IL-2 and IL-6. The sequences were the following:

IL-2 : 5'-ATGTACAGGATGCAACTCCTGTCTT-3'
3'-GTCAGTGTTGAGATGATGCTTTGAC-5'
IL-6: 5'-CAAATTCGGTACATCCTCGACGGC-3'
3'-CATCTGGACAGCTCTGGCTTGTTCC-5'

PCR products were reamplified using dNTPs and digoxigenin-coupled dUTP (DIG-11-dUTP) (Boehringer Mannheim, Mannheim, Germany), according to manufacturer's instructions.

In situ hybridization was performed as previously described by incubating overnight at 37°C cytospin of the PBMC with the specific probe.[2] Detection of hybridization was achieved using mouse anti-DIG monoclonal antibodies, followed by peroxidase-coupled rabbit anti-mouse and swine anti-rabbit monoclonal antibodies.

At the time the inhibitor appeared, we found high levels of IL-2 both in the supernatant and on the surface of PBMC cultured with rFVIII, by the double ELISA and *in situ* hybridization (Figures 1a and 2), whereas IL-6 was detected at very low level by the ELISA and was not visible on the surface of PBMC (Figures 1c and 2). After 45 days, when the inhibitor level had markedly reduced to 1 BU, the amount of IL-2 in the supernatant leveled off and was not visible (Figure 1b) by immunohistochemistry whereas the level of IL-6 expression was greatly enhanced (Figures 1d and 2). Cytokine level was unde-

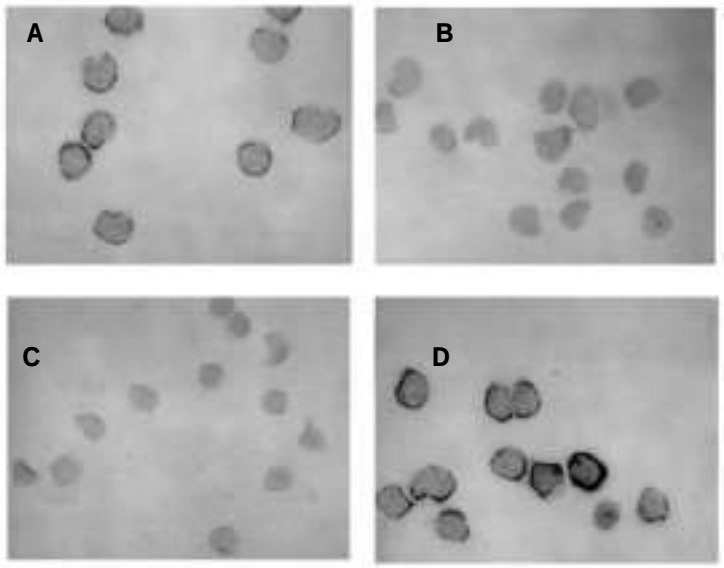

Figure 1. mRNA staining of IL-2 and IL-6 in cytospin peripheral blood mononuclear cells after incubation with rFVIII. The upper panel shows the pattern of cytokines expression for IL-2 (a) and IL-6 (b) before steroid treatment, whereas the lower panel refers to staining for IL-2 (c) and IL-6 (d) at the end of the steroid treatment when the inhibitor had leveled off.

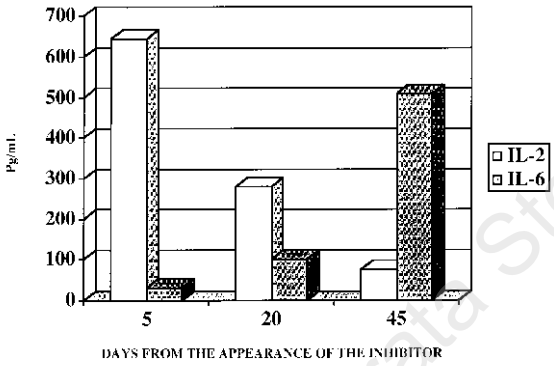

Figure 2. IL-2 and IL-6 supernatant levels in peripheral blood mononuclear cells incubated with rFVIII at different times: at the appearance of inhibitors (5 days), during steroid treatment (20 days) and after the inhibitor had leveled off.

tectable in controls.Twenty months later, B-domain depleted rFVIII (ReFacto, Pharmacia and Upjohn, Sweden) was administered for left gastrocnemius hematoma. The Bethesda test remained negative at day 7 and 15 after rFVIII administration. An *in vivo* recovery evaluation, measured as suggested elsewhere,[3] was normal (86.1%).

This is the first study exploring the cytokine pattern in a neonate with hemophilia A with recent development of FVIII inhibitors. Steroids may inhibit Th1 and enhance Th2 cytokine synthesis,[4] a preferential pattern in models of tolerance induction. A shift toward a Th2 profile is probably more advisable in limiting immune response against FVIII concentrates.

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Key words

Cytokines, hemophilia A, steroids, T-helper, inhibitors.

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References

1. Klein SC, van Wichen DF, Golverdingen JG, Jacobse KC, Broekhuizen R, de Weger RA. An improved, sensitive, non-radioactive in situ hybridization method for the detection of cytokine mRNAs. APMIS 1995; 103:345-53.
2. Denijn M, De Weger RA, Van Unnik JA, Den Otter W, Lips CJ. Detection of calcitonin and calcitonin gene-related peptide mRNA in human medullary thyroid carcinoma. A retrospective study. J Pathol 1993; 169: 53-60.
3. Morfini M, Lee M, Messori A. The design and analysis of half life and recovery studies for factor VIII and factor IX. FactorVIII/Factor IX Scientific and Standardization Committee of the International Society for Thrombosis and Haemostasis. Thromb Haemost 1991; 66:384-6.
4. DeKruyff RH, Fang Y, Umetsu DT. Corticosteroids enhance the capacity of macrophages to induce Th2 cytokine synthesis in CD4+ lymphocytes by inhibiting IL-12 production. J Immunol 1998; 160:2231-7.