Thrombosis and Haemostasis – © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 69 (2) 115–118 (1993)

A Higher than Expected Incidence of Factor VIII Inhibitors in Multitransfused Haemophilia A Patients Treated with an Intermediate Purity Pasteurized Factor VIII Concentrate

Kathelijne Peerlinck, Jef Arnout, Jean Guy Gilles[1], Jean-Marie Saint-Remy[1], and Jos Vermylen

From the Center for Thrombosis and Vascular Research, University of Leuven, Leuven, Belgium and the [1]Allergy and Clinical Immunology Unit, Institute of Cellular and Molecular Pathology, University of Louvain, Brussels, Belgium

Summary

In May 1990, 218 patients with haemophilia A regularly attending the Leuven Haemophilia Center were randomly assigned to a group receiving either of two newly introduced factor VIII concentrates: factor VIII-P, an intermediate purity pasteurized concentrate, or factor VIII-SD, a high purity concentrate treated with solvent-detergent for viral inactivation.

Patients were followed from May 1990 until October 1991. Between August 1991 and October 1991 a clinically important factor VIII inhibitor was detected in five out of the 109 patients receiving factor VIII-P while none of the 109 patients receiving factor VIII-SD developed such antibodies. All patients acquiring an inhibitor had previously been clinically tolerant to transfused factor VIII with 200 to more than 1,000 days of exposure to factor VIII prior to May 1990. Patients with inhibitors were transfused daily with 30 U factor VIII-SD per kg body weight, which was associated with a gradual decline of the inhibitor level. In all patients the antibodies were relatively slow-acting and predominantly directed towards the light chain of factor VIII.

This study demonstrates a higher than expected incidence of factor VIII inhibitors associated with the use of a specific factor VIII concentrate in multitransfused haemophilia A patients. It indicates the usefulness of evaluating newly introduced concentrates in prospective, randomized trials.

Introduction

The occurrence of antibodies to transfused factor VIII (factor VIII inhibitors) remains a major problem in the management of haemophilia A. The percentage of patients confronted with this complication at some time in their life ranges from 3.6 to 25% (1–4). Inhibitors only appear in haemophilia A patients after exposure to exogenous factor VIII and develop mostly in children, usually within a limited number of exposure days (generally less than 50) (1, 3, 4). Inhibitor development in patients previously tolerant to factor VIII is a rare event: in a multicenter study conducted in the United States, an incidence of new inhibitors of 8 per 1,000 patient-years of observation was found (1).

The reason why antifactor VIII antibodies occur in only some patients with haemophilia A is not clear. The formation of inhibitors is not related to the nature of the defect in the factor VIII gene (5), but sibling studies suggest a genetic predisposition (6). That a direct relationship exists between the type of product used and inhibitor formation has not yet been proven (4).

We report here on a clustering of inhibitors within our population of multitransfused haemophilia A patients; this phenomenon was clearly associated with a specific factor VIII preparation.

Correspondence to: Dr. K. Peerlinck, Center for Thrombosis and Vascular Research, K. U. Leuven – Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

Patients and Methods

Patient Population

At the introduction in Belgium in May 1990 of more pure factor VIII preparations to replace the previously used lyophilized heat-treated cryoprecipitate, all haemophilia A patients regularly attending the Leuven Haemophilia Clinic ($n = 234$) were randomly assigned to receive either of two newly introduced products. The study was approved by the Committee of Medical Ethics of this institution. FVIII-P (named factor VIII-CPS-P in The Netherlands) is an intermediate-purity concentrate (specific activity 1.5 IU FVIII/mg protein) produced by controlled-pore silica adsorption, followed by pasteurization (+60° C for 10 h in solution) (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) (7). FVIII-SD, a high-purity concentrate (specific activity 200 IU FVIII/mg protein), is produced by ion-exchange chromatography and treated with solvent-detergent (0.3% tri[n-butyl]phosphate – 1% polysorbate 80) (Biotransfusion, Lille, France) (8). For both products cryoprecipitate from Belgian donors was used as starting material (9). HIV-infected patients ($n = 6$) and patients with an inhibitor known for many years ($n = 11$) were not included in the study. Brothers living in the same household received the same product. Each treatment group thus consisted of 109 patients. Most patients apply home treatment. Follow-up visits were scheduled after 6 months and yearly thereafter.

Laboratory Tests

Blood for factor VIII inhibitor assay was collected in polystyrene tubes on one tenth volume of 0.11 mol/l trisodium citrate. Platelet-poor plasma was obtained by centrifugation at 2,500 × *g* for 15 min at room temperature, carefully pipetted off and stored in a plastic tube at –30° C until analysed.

Factor VIII-inhibitor levels were measured according to the Bethesda method (10). Residual factor VIII levels were determined in a one stage assay (11) adapted to an automated coagulometer ACL-810 (IL, Milan, Italy) using severe haemophilia A plasma and a micronised silica aPTT reagent (IL, Milan, Italy).

The time course of factor VIII inhibition was studied by measuring residual factor VIII at different time intervals in a 1 to 1 mixture of a normal plasma pool and patient plasma as described (12); the time required for inactivation of 50% of FVIII activity (half inactivation time, $t_{1/2}$) was calculated.

To analyse whether the inhibitory activity was linked to IgG or IgM, IgG was purified from factor VIII inhibitor plasma on protein A Sepharose. The specificity of anti-factor VIII antibodies was further determined

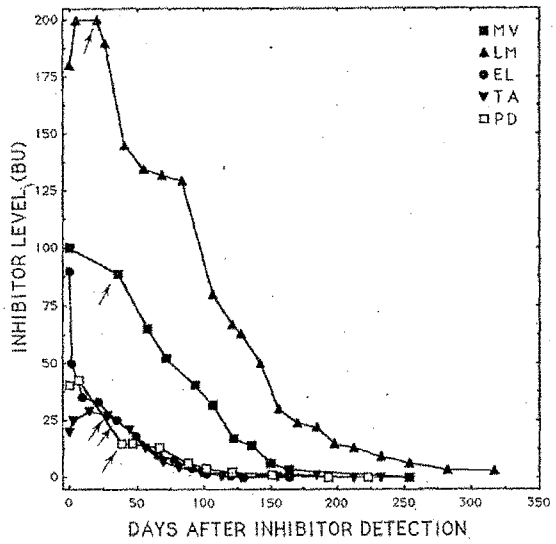

Fig. 1 Time course of the factor VIII-inhibitor levels (BU) in the five patients who developed such an antibody in association with factor VIII-P treatment. Arrows indicate start of daily FVIII-SD transfusion.

using immunoblots in which affinity-purified antibodies were allowed to react with thrombin-digested human recombinant factor VIII (rFVIII) (13). Blotting of rFVIII was performed as follows: 500 µg rFVIII (Baxter, CA) was incubated with 2 U thrombin (Topostatine, Roche, Brussels) for 30 min at 37° C. Digestion was stopped by addition of D-phenylalanyl-L-propyl-L-arginine chloromethyl ketone (Calbiochem, La Jolla, CA) to a final concentration of 0.01 mmol/l. Five µg of thrombin-cleaved rFVIII was applied to a 4 to 15% polyacrylamide gel using the Phast System (Pharmacia, Uppsala, Sweden). rFVIII fragments were then transferred to a 0.2 µm nitrocellulose membrane (Machery-Nagel, Düren, Germany) which was incubated with 3% gelatin for 30 min at room temperature. Specific antibodies were purified by immunoaffinity on insolubilized rFVIII and dilutions ranging from 5 µg/ml to 0.25 µg/ml were incubated with the FVIII nitrocellulose membranes. The binding of antibodies was detected by addition of biotin-labelled anti-human Fcγ goat IgG followed by avidin-peroxidase and chloronaphtol.

Table 1 Classification of patients in the two treatment groups according to severity of haemophilia

Severity of haemophilia	Factor VIII-P	Factor VIII-SD
FVIII:c <1 IU/dl	50 (44)	61 (48)
FVIII:c 1–5 IU/dl	13 (5)	7 (3)
FVIII:c >5 IU/dl	46 (19)	41 (9)
Total	109 (68)	109 (61)

The number of patients screened for inhibitor is given between brackets.

Statistical Evaluation

Incidence rates of inhibitors were calculated as the ratio of the number of inhibitors over the patient-years of observation and confidence intervals (CI95) for the incidence rates were calculated under the assumption of a Poisson distribution. Fisher's exact test was used whenever appropriate.

Results

1. Clinical Outcome

In August 1991 and September 1991 two patients (LM and EL) presented with haemorrhages resistant to factor VIII transfusions and in both a factor VIII inhibitor was detected (200 and 90 Bethesda Units [BU], respectively). An inhibitor of 29 BU was detected in the plasma of a third patient (TA) a few days prior to the development of clinical symptoms. In October 1991 all haemophilia A patients were invited for systematic inhibitor screening; 62% of the patients belonging to the factor VIII-P group were tested and 56% of those from the factor VIII-SD group. Two additional subjects with an inhibitor were found (PD: 42 BU and MV: 100 BU); both patients had noticed that transfusions were less effective in controlling haemorrhages during the previous weeks. In these five patients the plasma factor VIII level 15 min after infusion of 40 U factor VIII per kg body weight rose to 1–19 U/dl, corresponding to a recovery of 0–22%. In addition, a sixth patient, the brother of PD, had an inhibitor of 1.8 BU but no clinical signs; recovery in this patient was 31%. The third brother of this sibship had no factor VIII antibody and a normal recovery. The six patients who developed an inhibitor had been allocated to factor VIII-P treatment; five of them had indeed been treated exclusively with factor VIII-P and one patient (LM) had received small quantities of factor VIII-SD in addition to factor VIII-P. None of the patients randomized to factor VIII-SD treatment developed an inhibitor ($p = 0.02$ considering all inhibitor patients, $n = 6$ or $p = 0.03$ for clinically important inhibitors, $n = 5$; Fisher's exact test). The distribution of factor VIII-P by the Belgian Red Cross was stopped in October 1991 and a warning letter with a summary of these observations was published in the December 1991 issue of Acta Clinica Belgica (14). Factor VIII-P had thus been used for 18 months. Incidence rates of clinically important inhibitors were 31/1,000 patient-years of observation (CI95: 9–71/1,000 patient-years) in the group allocated to factor VIII-P therapy and 0/1,000 patient-years (CI95: 0–22/1,000 patient-years) in the group allocated to factor VIII-SD. The characterization of the two groups of patients and the number whose plasma was screened for inhibitors are shown in Table 1. About 80% of the patients with severe haemophilia were tested in each group. Several patients with mild haemophilia had not received factor VIII transfusions during the observation period.

Characteristics of the patients who developed a high titer inhibitor are shown in Table 2. None of these patients had a

Table 2 Characteristics of inhibitor patients

Patient initials	Age (years)	Severity of haemophilia (FVIII:c)	Exposure to factor VIII prior to factor VIII-P administration (days)	Exposure to factor VIII-P prior to detection of inhibitor (days)	Date of last negative inhibitor test (month–year)	Date of inhibitor detection (month–year)	Maximum titer (BU)
LM	25	<1 IU/dl	>1,000	150	5–91	8–91	200
EL	27	<1 IU/dl	>1,000	120	10–90	9–91	90
TA	12	<1 IU/dl	> 300	98	10–90	9–91	29
PD	14	<1 IU/dl	> 500	130	10–88	10–91	42
MV	10	<1 IU/dl	> 200	170	3–91	10–91	100

Table 3 Factor VIII inhibitor properties

Patient initials	Factor VIII half inactivation time ($t_{1/2}$) (min)	Specific inhibitory activity (BU/mg IgG)	Specificity for factor VIII fragments*
LM	32	20.4	72 (54, 44)
EL	19	9.6	72 (54, 44)
TA	35	2.4	72 (44)
PD	30	3.5	72 (54)
MV	21	10.2	72 (54, 44)

* Figures between brackets represent factor VIII fragments exhibiting weak reactivity with the affinity purified antibodies.

family history of inhibitor formation. All five subjects had antibodies to hepatitis C, as have more than 90% of our haemophilia patients. One of the patients (PD) was HbsAg positive and had recurrent bouts of malaria contracted during a prolonged stay in Zaïre; although in this patient no inhibitor test was performed immediately prior to entry in the study nor after 6 months, we found it justified to include him in the present analysis since his clinical response to infused factor VIII was unaltered up to 3 to 4 weeks before the inhibitor was detected. In four of the patients bleeding episodes were initially treated with FEIBA (activated prothrombin complex concentrate, Immuno, Brussels), with a satisfactory clinical response.

Induction of immune tolerance was attempted in the five patients with a clinically important inhibitor. Treatment consisted of daily transfusions of 30 U factor VIII-SD per kg body weight. No major bleeding episodes occurred after starting this treatment regimen. In all patients a gradual decline of the inhibitor titer was observed (Fig. 1). In four patients (EL, TA, PD, MV), the inhibitor disappeared after 120, 128, 193 and 223 days respectively, with an in vivo recovery of infused factor VIII returning to normal. One of these has since undergone uneventful orthopedic surgery.

2. *Inhibitor Characterization*

To investigate whether these antifactor VIII antibodies had specific properties, they were partially characterized. Factor VIII half inactivation times ranged from 19 to 35 min (see Table 3) as compared to less than 8 min when measured with plasma from five haemophilia patients with stable inhibitors adjusted to comparable levels. These inhibitors can therefore be considered as relatively slow-acting. Specific inhibitory activity of the purified IgG fractions isolated on protein A Sepharose ranged from 2.4 to 20.4 BU/mg IgG. From this it was calculated that IgG accounted for at least 85% of the inhibitory activity.

Anti-factor VIII antibodies were directed towards epitopes on both heavy and light chains in all five patients (see Table 3). Upon dilution of the affinity purified IgGs, the reactivity towards the 72 kDa fragment was the only one to persist, suggesting that a majority of the anti-factor VIII antibodies were directed towards the factor VIII light chain.

Discussion

About 15 months after the introduction in Belgium of two new factor VIII-concentrates, a clustering of factor VIII inhibitors was found in the patient group randomly assigned to receive the intermediate purity factor VIII-P. All patients developing an inhibitor in this series were multitransfused subjects with severe haemophilia A. Although the occurrence of a factor VIII inhibitor is a well-known complication of haemophilia A treatment, most inhibitors develop early (generally within 50 exposure days) (3, 4) after the start of replacement therapy. The occurrence of an inhibitor in multitransfused, tolerant patients is a rare event: in the American Cooperative Study (1), new clinically important inhibitors were detected in 24 of 1,306 patients during a 4-year follow-up period (6.2 per 1,000 patient-years, CI95: 3.9–9.2 per 1,000 patient-years) [calculated from (1)]. In a study with human recombinant factor VIII only 1 of 81 previously multitransfused patients developed an inhibitor (14). In the present study 5 out of the 109 patients assigned to the group on factor VIII-P therapy developed a clinically important inhibitor within 18 months of follow-up (31 per 1,000 patient-years of observation). If only patients with severe haemophilia are considered, this figure becomes even more striking (5 out of 50; 66 per 1,000 patient-years of observation). No inhibitors were found during the same time period in the group randomized to FVIII-SD ($p = 0.03$ by Fisher's exact test). Even though not all patients responded to our invitation for inhibitor testing, the chance that clinically important inhibitors would have been missed in the FVIII-SD group is remote, since it is unlikely that patients suffering from clinical complications due to a high titer inhibitor would not have contacted our Haemophilia center, in particular after having been called in for inhibitor screening. Moreover, in the group most at risk for inhibitor development, the patients with severe haemophilia, the response rate was 80%.

It is important to point out that in our study inhibitors developed exclusively in patients exposed to infused factor VIII for more than 200 days, whereas in the American Cooperative Study (1) 92% of clinically important inhibitors developed within 200 days of exposure to factor VIII.

The present data demonstrate the association between the appearance of inhibitors and factor VIII-P infusion in previously multitransfused patients. In further support of this, a national study recently conducted in The Netherlands after a high incidence of inhibitors was reported, led Dutch investigators to the same conclusion (16). Remarkably, the number of exposure days to factor VIII-P before the detection of inhibitor ranged from 98 to 170 days (mean 134) (Table 2) which is longer than what has been reported for previously untreated patients (3, 4). A bias in detection, e. g. due to infrequent inhibitor testing, is unlikely since 4 of the 5 patients (LM, EL, TA and MV) had a negative inhibitor test after 45, 60, 80 and 110 exposure days to factor VIII-P respectively. The relatively late appearance of these inhibitors and the fact that they all were detected within 2 months, suggest that the conditions used for pasteurization could have rendered only some batches of factor VIII-P immunogenic; evidence for this is currently lacking and no single batch had been used by all five patients.

In vitro characterization of the inhibitors showed a rather uniform reactivity: a predominant recognition of the FVIII-light chain and a relatively slow inhibitory action. Whether this slow inhibitory action is specific for this group of inhibitors or is determined by the point in time during inhibitor development cannot be answered at this moment, since no data are available in the literature.

In an attempt to induce immune tolerance, all five inhibitor patients were treated with daily transfusions of 30 U factor VIII-SD per kg body weight. This treatment was associated with a gradual decline of inhibitor levels, which was not preceded by an initial rise. It is however presently unclear whether this decrease in inhibitor levels represents an actual induction of immune tolerance or mere catabolism of IgG antibodies.

In conclusion, this study shows for the first time the association between the development of FVIII inhibitors and a specific

factor VIII-concentrate and emphasizes the usefulness of evaluating newly introduced concentrates in a prospective, randomized fashion.

Acknowledgements

The authors thank Dr. F. R. Rosendaal, Departments of Clinical Epidemiology and Hematology, University Hospital Leiden, The Netherlands, for his advice. The National Health Council (Hoge Gezondheidsraad) gave its approval to this randomized evaluation.

REFERENCES

1. McMillan CW, Shapiro SS, Whitehurst D, et al. The natural history of factor VIII:c inhibitors in patients with hemophilia A: a national cooperative study. II. Observations on the initial development of factor VIII:c inhibitors. Blood 1988; 71: 344–8.
2. Rasi V, Ikkala E. Haemophiliacs with factor VIII inhibitors in Finland: prevalence, incidence and outcome. Br J Haematol 1990; 76: 369–71.
3. Schwarzinger I, Pabinger I, Korninger C, et al. Incidence of inhibitors in patients with severe and moderate hemophilia A treated with factor VIII concentrates. Am J Hematol 1987; 24: 241–5.
4. Ehrenforth S, Kreuz W, Scharrer I, et al. Incidence of factor VIII and factor IX inhibitors in haemophiliacs. Lancet 1992; 339: 594–8.
5. White GC, Shoemaker CB. Factor VIII gene and hemophilia A. Blood 1989; 73: 1–12.
6. Frommel D, Allain JP. Genetic predisposition to develop factor VIII antibody in classic hemophilia. Clin Immunol Immunopathol 1977; 8: 34–8.
7. Hiemstra H, Nieuweboer CEF, Idoe MA, et al. Evaluation of a wet pasteurization of a factor VIII concentrate produced by controlled-pore silica adsorption. Folia Haematol (Leipzig) 1990; 117: 557–63.
8. Burnouf T, Burnouf-Radosevich M, Huart JJ, Goudemand M. A highly purified factor VIII:c concentrate prepared from cryoprecipitate by ion-exchange chromatography. Vox Sang 1991; 60: 8–15.
9. Peerlinck K, Arnout J, Tamise M, Vanherle A, Fondu P, Vermylen J. In vitro and in vivo evaluation of two factor VIII concentrates virally inactivated by solvent-detergent or by pasteurization. Acta Clin Belg 1991; 46: 298–304.
10. Kasper CK, Aledort LM, Counts RB, et al. A more uniform measurement of factor VIII inhibitors. Thromb Diath Haemorrh 1975; 34: 869–72.
11. Langdell RD, Wagner RH, Brinkhous KM. Effect of antihemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a simple one-stage antihemophilic factor assay procedure. J Lab Clin Med 1953; 41: 637–47.
12. Biggs R, Austen DEG, Denson KWE, Rizza CR, Borrett R. The mode of action of antibodies which destroy factor VIII. I. Antibodies which have second-order concentration graphs. Br J Haematol 1972; 23: 125–35.
13. Fulcher CA, de Graaf Mahoney S, Roberts JR, Kasper CK, Zimmerman TS. Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments. Proc Natl Acad Sci USA 1985; 82: 7728–32.
14. Vermylen J, Peerlinck K. Epidemic of factor VIII inhibitors linked to factor VIII-P. Acta Clin Belg 1991; 46: 419–20 (Letter).
15. Schwartz RS, Abildgaard CF, Aledort LM, et al. Human recombinant DNA-derived antihemophilic factor (factor VIII) in the treatment of hemophilia A. N Engl J Med 1990; 323: 1800–5.
16. Rosendaal FR, Nieuwenhuis HK, van den Berg HM, et al. A sudden increase in factor VIII inhibitor development in multitransfused hemophilia A patients in the Netherlands. Blood 1993; in press.

Received August 31, 1992 Accepted after revision October 13, 1992