IMMUNE TOLERANCE IN HEMOPHILIA
AND THE TREATMENT OF HEMOPHILIACS
WITH AN INHIBITOR

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Forthcoming Workshop
The fourth Workshop on Immune Tolerance will take place in Bonn, Germany, Thursday August 30th through September 1st 2001.
Information concerning the site and the programme will be circularized later

Foreword

Inhibitor appearance in haemophilia is considered by far the severest problem of hemophilia treatment, being associated with increased morbidity if not mortality. The presence of an inhibitor, especially if high-titre, leads to a clinical setting which is comparable to that of the early years of haemophilia treatment where bleedings were difficult to treat and arthropathy was rapidly progressing towards disability.

However, in the recent years, new drugs and treatment modalities have been proposed which modified this otherwise gloom scenario. In addition, the scientific knowledge on the matter has grown in a tumultuous way and important fall out concerning the management of patients with inhibitors to FVIII are foreseen in the forthcoming years. Among the many issues of interest, we are understanding more about the humoral and cellular mechanisms of inhibitor formation and some of the processes underlying the return to a tolerant status towards FVIII.

This issue of Haematologica, a fastly growing international journal of haematology, contains selected papers from the III Workshop on Immune Tolerance which took place in Palermo, Italy, October 1999. The discussion developed during the meeting has been included, which has involved a major editorial effort. All things considered, this volume contains a great deal of information valuable for the researchers and the treaters.

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Factor VIII inhibitors with recombinant products: prospective clinical trials

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Abstract

Background and Objectives. During the late 1980s, short-term hepatitis safety trials with certain new high purity, virally attenuated plasma derived factor VIII (FVIII) concentrates showed a higher than anticipated percentage of inhibitors in previously untreated patients (PUPs). Thus, the design of pre-licensure clinical trials with each of the recombinant (r) FVIII products included prospective evaluations for inhibitors at well-defined time points, to see whether treatment with rFVIII resulted in a higher incidence of inhibitors.

Design and Methods. Each of several prospective clinical trials with rFVIII preparations is described and analyzed in terms of inhibitor development, patient demographics, and current prevalence of inhibitors in the cohort.

Results. In prospective trials with rFVIII preparations (both full length and B domain deleted rFVIII), the percentage of PUPs with severe hemophilia A who developed FVIII inhibitors has varied between 28.3 and 30.6%. Many of the inhibitors have been transient, disappearing while the patient was receiving episodic (***on demand***) treatment with rFVIII, while others have responded to immune tolerance induction regimens with rFVIII alone. However, many other have persisted. Genetic influences (underlying gene defect and race) were shown to play a role in inhibitor development. In contrast to the experience in the PUPs, in trials with rFVIII preparations in previously treated patients (PTPs), only 0-1 subject per trial developed an inhibitor.

Interpretation and Conclusions. While the percentage of PUPs developing inhibitors in each of the prospective clinical trials with rFVIII preparations appears high, published data from similarly conducted studies with plasma-derived FVIII products show very similar results. Additionally, only an occasional PTP on rFVIII developed an inhibitor. Thus, none of the rFVIII preparations studied to date appears to be more immunogenic than plasma-derived FVIII.
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Key words: recombinant (r) FVIII, B domain deleted rFVIII, recommendations of the FVIII/FIX subcommittee, transient inhibitors, genetic influences

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With the introduction of high purity, virally attenuated plasma derived factor (F) VIII concentrates in the late 1980s, there was some concern that such manipulations might render FVIII more immunogenic. Thus, the hepatitis safety trials conducted in previously untreated patients (PUPs) with these new products included inhibitor assays. While these short-term trials were aimed at assessing hepatitis safety[1] and usually included only a few inhibitor assays done at 6 month intervals, a surprisingly high percentage of PUPs in one prospective study developed inhibitors early in the trial.[2,3] Reports of higher than expected inhibitor development in other small pediatric patient cohorts[4] also raised concerns. Thus when recombinant (r) FVIII products became available for human use in 1987 and 1988, the design of pre-licensure clinical trials included inhibitor assays every 3 months (or sooner if an inhibitor was suspected clinically).

Design and Methods

We looked at inhibitor development in the prospective clinical trials with each of the currently available rFVIII preparations (Bayer's Kogenate®, Baxter's Recombinate®, Genetics Institute's ReFacto®, and Bayer's Kogenate®FS). The trials with the first two of these products have been completed, while the ReFacto, and Kogenate®FS are closed to patient entry but are still accruing data.

Demographics, and genetic factors (when available) are described for each cohort, and for those who did or did not develop inhibitors. The number of high and low titer inhibitors, transient inhibitors, and current inhibitor prevalence in each cohort was also looked at.

Prospective clinical trials in previously untreated patients with "first generation" full-length rFVIII concentrates

The Kogenate® PUP Trial

The first rFVIII trial in previously untreated patients (PUPs) began in January, 1989, with Bayer's Kogenate®. This multinational, multicenter trial enrolled not only severely affected

PUPs with hemophilia A (defined here as having baseline FVIII values of < 2%), but some with moderate (2-5% baseline FVIII) and mild (> 5% FVIII) hemophilia A as well.[5] Patients were enrolled between January, 1989 and April, 1992; 101 patients were entered, treated and evaluable. Sixty-four of the 101 had severe hemophilia A. Each patient received the study product (Kogenate®) only, for all bleeding episodes and/or prophylaxis. Following each PUPs first treatment with Kogenate®, inhibitor assays were done every 3 months (or sooner, if inhibitor development was suspected clinically).[5] The study cohort included 92 Caucasians and "others", and 9 Blacks (of African descent). Thus, 19 of 64 (29.7%) severe hemophilia A PUPs developed inhibitors.

During the course of the study, 21 of 101 (20.8%) patients developed inhibitors after a median of 9 exposure days (ED) to rFVIII (range, 3-51ED). Nineteen of the 21 inhibitors developed in severely affected patients, and 2 in moderately affected patients. Ten of the 19 were high titer (> 10 Bethesda Units [BU]) and 9 low titer < 10 BU). Eight of the inhibitors were transient, disappearing while the patient received episodic (*on demand*, or, as necessary) treatment with Kogenate®.[6] During the study period, 8 inhibitor patients were started on immune tolerance (ITI) regimens with Kogenate®, alone, and five were successfully tolerized while two others remained on ITI at the conclusion of the Kogenate®, PUP trial. At that time, 8 patients (8 of 101, or 7.9%) still had an inhibitor, 6 of which were high titer and 2 low titer.

Of 9 Black children in this cohort, 4 developed inhibitors, all of which were high titer.

The Recombinate® PUP Trial

The PUP trial with Baxter's Recombinate®, began in July, 1990; patient enrollment was closed in March, 1992, and the last patient received his first treatment with Recombinate in August, 1993. Although the trial design was quite similar to that of the Kogenate® trial, the Recombinate® trial enrolled only severely affected PUPs.[7,8] Of 72 treated and evaluable PUPS, 22 (31%) developed inhibitors after a median number of 10 ED. Nine were high (> 5 BU) and 13 low titer (≤ 5 BU); 12 were transient, having disappeared on episodic treatment (7 patients) or while receiving regular (e.g. twice weekly) infusions of Recombinate® (5 patients).[9] In this cohort, 6 patients with high responding inhibitors were started on ITI with Recombinate® alone, and only one of these was tolerized by study exit.[9]

Nine of the 72 Recombinate® PUPs were Black children; 5 of 9 (55%) developed inhibitors, all

of which were low titer. In contrast, 17 of 63 (27.4%) Caucasians and "others" developed inhibitors.

By 1998, the prevalence of inhibitors was 14% (10 of 72).[9]

The French PUP Study with rFVIII

A French study with rFVIII was conducted from 1993 to 1996; 52 severely affected PUPs (< 1% FVIII) were enrolled, and evaluated. Fifty received Kogenate®, exclusively, while 2 received Recombinate® exclusively. 15/52 (28.8%) developed inhibitors, 5 of which were low titer (< 10 BU) and 10 high titer (≥ 10 BU). Five of the inhibitors were transient. Eleven of 44 Caucasian children developed inhibitors, while 2 of 6 Arabic and 2 of 3 Black children did. Nine of 20 (45%) children who had the inversion mutation developed inhibitors, while only 18% of those who were negative for the inversion mutation did so.[10]

Prospective clinical trials in PUPs with "second generation" rFVIII concentrates

The PUP Trial with B-Domain Deleted rFVIII (ReFacto®)

A multicenter, multinational prospective trial with rFVIII SQ (ReFacto®) in PUPs began in 1994; severe hemophilia A PUPs were enrolled between October, 1994 and 1996. In this trial, 101 PUPs were enrolled, treated and evaluable.[11] This product differs from Kogenate® and Recombinate® not only in that the rFVIII lacks the B domain, but also in the fact that no human serum albumin is added as a stabilizer (additionally, while chromogenic and two-stage FVIII assays give expected recovery values in recipients, one-stage assays give roughly half the expected values).

After 4 study years, the percentage of patients having developed inhibitors is 30% (30 of 101 severely affected PUPs). In 11 of the inhibitors patients, the peak inhibitor titer was ≥ 10 BU, while in 5 it was 5-10 BU, and in 14 it was < 5 BU.[12] Fifteen of 17 patients who were started on an ITI regimen with rFVIII SQ had a reduction in inhibitor titer, with 9 of the 15 achieving a negative Bethesda assay by the time of the interim data analysis.[12] In 6 additional patients treated "on demand", the inhibitor disappeared spontaneously. Fifteen PUPs still have an inhibitor (9 of the 15 were originally > 10 BU, while the other 6 were < 5 BU). Thus, the current prevalence of inhibitors in the cohort is 15% (15/101), with or without immune tolerance.[12]

The median number of exposure days to inhibitor detection is 12 (range 3-49 ED) in the entire group of 30 inhibitor patients. It was 9

(range 1-34) for patients with high titer (≥ 5 BU) inhibitors. In all patients, the median number of ED at the time of interim data analysis was 55.[12]

The PUP (and, Minimally Treated Patient) Trial with a Second Generation rFVIII Concentrate Formulated with Sucrose Rather than Albumin (Kogenate®FS)

From October, 1997 until April 30, 1999, 62 infants and young children with severe hemophilia A (< 2% FVIII) were enrolled in a prospective, multicenter, multinational safety and efficacy study of a second generation rFVIII concentrate which is formulated with sucrose rather than human serum albumin (rFVIII-FS; Kogenate®FS). The 62 study subjects included 38 PUPs and 24 minimally treated patients (MTPs) with < 4 ED to rFVIII (one MTP received a plasma-derived FVIII concentrate, while the other 23 MTPs had received only rFVIII). All had negative inhibitor assays (Nijmegen modification[13] of the Bethesda assay) at study entry. Once on study, all patients received only rFVIII-FS, for treatment of bleeding or for prophylaxis. Inhibitor assays were performed every 3-4 ED for the first 20 ED or every 3 months (whichever came first); every 10 ED from 21-50 ED or every 3 months (whichever came first); and every 3 months thereafter. As of August, 1999, 61 of 62 study subjects had been treated with rFVIII-FS, and 8 had developed an inhibitor after 2-15 ED (median 7 ED). Among 5 American patients who developed inhibitors, 2 were African-American children (of 5 African-Americans in the study). Of the 8 inhibitor patients, 5 (all American patients) were high titer (16, 23, 109, 249, and 271 BU) while 3 (all European) were low titer (1.25, 1.9, and 4.0 BU). However, many of the subjects in this cohort are still at risk for inhibitor development, having had relatively few ED (29 still had < 10 ED).[14]

Prospective clinical trials with rFVIII preparations in previously treated patients

Prospective clinical trials have been conducted in previously treated patients (PTPs) with the two full-length rFVIII preparations (Kogenate® and Recombinate®), and with the B-domain deleted rFVIII (ReFacto®).[15-18]

In the PTP trial with Kogenate®, 58 patients received Kogenate® exclusively in an international, multicenter, prospective study of more than 5 years duration. Fifty-four of the 58 subjects had severe hemophilia A (< 2% FVIII) and 4 moderate hemophilia A (baseline FVIII 2-5%). Patients were monitored for a median of 4.7 years and received 17,922 infusions of Kogenate®. No *de novo* formation of inhibitors to FVIII was noted.[16]

In the Recombinate® PTP trial, 69 patients with severe (67) or moderately severe (2) hemophilia A received Recombinate® exclusively for 1-5.7 years (median 3.7 years). A total of 17,700 infusions were given. No subject developed an inhibitor. With the B-domain deleted rFVIII (r-VIII SQ; ReFacto®), only one of 113 PTPs developed an inhibitor, after 93 ED and 36 months on study. The peak inhibitor level was 12.6 BU. In this four year study, a total of 32,826 infusions of r-VIII SQ were given over 30,740 ED (median 226 ED).[18]

Studies done in recent years have documented the importance of certain hereditary factors in inhibitor development (certain major defects in the FVIII gene which interfere with FVIII biosynthesis), race, and familial propensity to inhibitor development),[19-23] and most who are genetically destined to develop an inhibitor do so early in life, after relatively few exposures to FVIII. Thus, the FVIII/FIX Subcommittee of the International Society on Thrombosis and Hemostasis (ISTH) has recommended that heavily treated PTPs be used (rather than PUPS) to determine whether or not a new product is particularly immunogenic.[24]

In PTPs (like in PUPs), none of the rFVIII preparations studied to date has appeared to be more immunogenic than other products.

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DISCUSSION 1.   Factor VIII inhibitors with recombinant products
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ALEDORT: Dr.Lusher could you tell us about the last slide you showed us in which eight of sixty-one inhibitors of the population resulted from PUPS and minimally treated patients. What were the differences in the inhibitor and prevalence of those two groups ?

LUSHER: None really and in fact the minimally treated for the most part had received Kogenate as their minimal treatment. The ones who were minimally treated with one exception had received one or two doses of Kogenate before entering the Kogenate-Sucrose formulated PUP study.

ALEDORT: But what was the difference in the inhibitor prevalence in the two groups? You said that there were eight inhibitors. How many developed in the PUPs and how many in the minimally treated group?

LUSHER: I can't remember the exact numbers but I've checked this and there really isn't a statistically significant difference.

LAURIAN: May I ask you something Dr. Lusher? We know that depending on the gene defect the incidence of inhibitors is not the same. Today, we don't know anything about gene defect in the Kogenate stories and so why compare something which is not comparable?

LUSHER: Well, hopefully we will ultimately and I thought we would have by now the complete gene information from each of these studies. We certainly don't have that now. I remember last year I said that by this year we probably would have this piece of information but from the various data bases it's just not there yet. Mainly, because people have not always sent in the samples. Even though it's in each of the trials it was offered as a free service so that you get the patient's gene defect; but many people have not done it yet and so there are big holes in the genetic information. Despite this I'm still hopeful that people can be urged to go back and get that information. But you're right I think what has become definitely clear is an influence of the FV III gene defects on the immune mechanisms.

HOYER: Are there other questions?

ALEDORT: I'd just like to reiterate what Dr. Laurien was saying. I think that as we become more sophisticated in understanding the risk factors for inhibitor development we are going to have to be very careful in designing new prospective studies when we're looking at the prevalence of inhibitor induction. The same is probably true for immune tolerance. For all we know there may very well be predisposing factors beyond titer, age, etc. that make one treatment successful or not successful. And so I think that we are really still in the evolutionary stage of understanding some of this.

LUSHER: And that will probably take on even greater emphasis and importance with gene therapy in terms of who gets inhibitors and so forth with gene therapy.

Risk factors for Inhibitor development in hemophilia A

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Abstract

The development of anti-factor-VIII-antibodies represents the most serious complication of factor VIII concentrate therapy, affecting about 30% of patients with severe hemophilia A. The genetics of the patients, comprising the factor VIII gene mutation and the immune response genes (MHC), have been shown to influence the risk of inhibitor formation. Severe molecular defects within the factor VIII gene, such as intron 22 inversions, nonsense mutations and large deletions, are associated with a 7 to 10 times higher inhibitor prevalence than less severe molecular gene defects such as missense mutations. Unexpectedly, frame shift deletions within runs of adenine nucleotides showed a low inhibitor risk, although most of them result in a stop codon. The explanation of this phenomenon is the restoration of the reading frame in some factor VIII molecules by slippage errors of the polymerase enzymes. Within most mutation types subgroups with significantly different inhibitor risks can be defined, e.g. multi domain vs. single domain large deletions, yielding a total of 10 groups with different risks of inhibitor formation. Alleles of MHC class II genes showed a less impressive influence on inhibitor development. In two studies of patients with a homogenous factor VIII gene mutation, the alleles DQA0102, DQB0602 and DR15 occurred more frequently in patients with inhibitor than without inhibitor (relative risks ranging from 1.9 to 4.0) and therefore could be assigned as risk alleles. Inflammatory processes in early childhood are under discussion as being an environmental influence factor that may modify the immune response to a foreign antigen. Fixing the genetic predisposition by gene analysis will be one important tool in the future to assess further parameters that might influence inhibitor formation.
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Key words: hemophilia A, factor VIII, inhibitor, gene mutation, HLA system

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Despite the significant improvement in hemophilia care, the development of anti-factor VIII antibodies nowadays still represents the most serious complication in hemophilia treatment. Factor VIII(FVIII) antibodies affect about 30% of patients and usually occur in childhood at therapy onset within the first 10-20 days of exposure.[1,2]

The pathogenesis of inhibitor formation is only partly understood. Several parameters are suggested to influence inhibitor formation. These parameters include the genetics of the patient, the FVIII treatment regimen, the factors that challenge the patient's immunologic integrity during early childhood, prenatal experiences and formal aspects such as study design and inhibitor assays used.

The genetics of a patient represents the easiest parameter to investigate because it does not change over time, is independent from the documentation of data concerning clinical course and treatment of the patient and can be investigated from a single blood sample. The observations that the risk of inhibitor is influenced by the degree of severity, the history of inhibitors in family relatives and the ethnic race point to the genetics of a patient having an important role. Candidates for producing a genetic predisposition to inhibitor development are mutations within the FVIII gene[3] and genes that are involved in the immune response, e.g. genes of the MHC class I and II loci.[4,5] The role of the immune response genes is suggested to become more decisive in the presence of an inflammatory process.[6]

This review will report on the current knowledge of the genetic background of inhibitor formation and will also address the role of inflammatory processes.

Mutation type and risk of inhibitor development

The first extensive study on this field was published by Schwaab *et al.* in 1995.[3] Including the patients of the hemophilia center in Bonn, Germany, and the mutation database published by

Tuddenham et al.,[7] a clear correlation between type of FVIII gene mutation and inhibitor prevalence could be demonstrated (Table 1). Two groups of mutations, the missense mutations and the small deletions, showed a low inhibitor prevalence of about 5%. In contrast, the other mutation types, the prevalent intron 22 inversions, the large deletions and the nonsense mutations, exhibited a 7-10 fold higher inhibitor prevalence of about 35%. The data of the Bonn patients were in very good agreement with those of the patients listed in the mutation database. The observation that in non-severe hemophiliacs with a low inhibitor prevalence almost all of the patients had missense mutations corresponded well with these findings.

Concerning the pathomechanism it is conceivable that patients with missense mutations have some – however non-functional – endogenous FVIII protein, which is sufficient to induce immune tolerance to substituted FVIII. In contrast, no endogenous FVIII is synthesized in patients with the more severe molecular defects such as intron 22 inversions, large deletions and nonsense mutations. Thus, substituted FVIII represents a foreign protein, leading to the immune response of FVIII antibodies.

A mutation type profile for patients with severe hemophilia A was published by Becker et al. in 1996[8] (Table 2). Intron 22 inversions account for 37.4% of mutations in severe hemophilia A, nonsense mutations for 13.6%, missense mutations for 18.4%, small deletions for 9.5%, large deletions for 5.4% and insertions for 1.4%. In 11.6% of the patients the mutation could not been identified. These data show that in severe hemophilia A about two third of the patients have high risk mutations and one third of the patients low risk mutations for inhibitor formation.

Since 1995 more information about the various mutation types and the risk of inhibitor formation has been gathered resulting in at least ten groups of mutation types with different characteristics concerning the risk of inhibitor formation. An overview of these groups of mutations is shown in Figure 1 and described in more detail in the following sections of text.

Different inhibitor risks associated with the various types of null mutations

Interestingly, the risk of inhibitor formation is not homogenous within the types of nonsense mutations and large deletions. Data taken from the HAMSTeRS mutation register (http://europium.csc.mrc.ac.uk)[9] showed that (i) nonsense mutations affecting the light chain of the FVIII molecule were more frequently associated with inhibitors (29 inhibitors at nine different codons) than nonsense mutations affecting the heavy chain (3 inhibitors at 3 different codons) and that (ii) patients with large deletions affecting more than one domain of the FVIII molecule have a higher risk of developing an inhibitor than those in whom a single domain is affected (74% inhibitors in multi domain deletions vs. 21% inhibitors in single domain deletions). The inhibitor risk associated with the intron 22 inversion may have been overestimated in the first studies[3] because patients with an inhibitor may have been tested for the causative mutation with higher priority. Antonarakis et al.[10] reported a lower inhibitor prevalence in patients with an intron 22 inversion. Nevertheless, this mutation type is the most prevalent in severe hemophilia A and especially in the subgroup of patients who developed an inhibitor.

The reasons for the different inhibitor risks associated with the various types of null mutations are still not understood, however they raise two important questions i) Why do not all patients with a single type of null mutation develop an inhibitor? and ii) Why do patients with different types of null mutations have different risks for inhibitor development? The first question likely addresses other factors than the nature of the mutation while the second question must be related to the nature of the genetic defect in the FVIII gene.

Inhibitor prevalence in patients with small deletion/insertion mutations

The inhibitor prevalence found in patients with small deletions was unexpectedly low, as most of the deletions led to a frame shift with a stop codon. However, in contrast to that associated with nonsense mutations, the inhibitor prevalence was much lower. The explanation of this phenomenon was given recently in a paper by Young et al.[11] and a paper by our group.[12]

Young et al.[11] described a mildly affected hemophiliac with a T-deletion within an A8TA2-sequence at codons 1439-1441 of exon 14, that led to a run of 10 adenines (A10). Investigating the mRNA transcripts of this patient he found not only the expected transcript of 10 adenines but also small proportions of transcripts of 7, 8, 9 and 11 adenines. The few mRNA transcripts of 8 and 11 adenines restored the reading frame and led to the synthesis of some residual endogenous FVIII protein. This FVIII protein was fully active, probably because the mutation was located in a functionally non-decisive region of the B-domain. The pathomechanism behind the variable length of the mRNA transcripts is a slippage of the DNA/RNA-polymerases within the adenine run during DNA replication and RNA transcription.

Encouraged by the study of Young et al.[11] we

Table 1. Mutation type and inhibitor prevalence in severe hemophilia A according to Schwaab _et al._[3] The proportion of inhibitor patients is given in absolute numbers in brackets and as relative percentage number (data from the hemophilia center Bonn and the hemophilia A mutation databases.[9]

| | Point mutations | | Deletions | | Intron 22 |
	Stop	Missense	Large	Small	inversions
Pts. from Bonn	20 (7)	30 (2)	13 (3)	11 (1)	59 (25)
	35.0 %	6.7%	23.1%	9.1%	42.4%
IMD	53 (21)	39 (1)	57 (22)	16 (1)	66 (18)
	39.6 %	2.6%	38.6%	6.3%	27.3%
Total	73 (28)	69 (3)	70 (25)	27 (2)	125 (43)
	38.4 %	4.3%	35.7%	7.4%	34.4%

IMD: International mutation database.

Table 2. Mutation type in patients with severe hemophilia A according to Becker _et al._[2]

Type of mutation	Patients n=147
Intron 22 inversion	55 (37.4%)
Point mutation	47 (32.0%)
Stop codon	20 (13.6%)
Missense	27 (18.4%)
Large deletion	8 (5.4%)
Small deletion	14 (9.5%)
Insertion	2 (1.4%)
Mutation not characterized	4 (2.7%)
Mutation not identified	17 (11.6%)
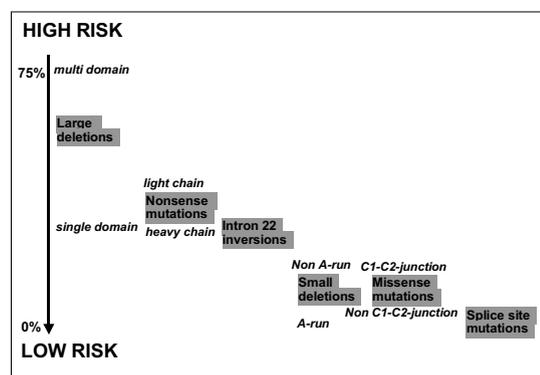
Figure 1. Mutation types and risks of inhibitor development. Main types of mutations are indicated by a grey background. Most of them show subgroups of mutations with different risks of inhibitor formation.

Table 3. Coagulation parameters of patients with small deletion/insertion mutations in exon 14 (upper part) and age-matched patients with standard (stop codon, intron 22 inversion) mutations according to Oldenburg _et al._[12]

Patients & mutation	FVIII:C (U/mL)	Thrombelastogram r (min)	r+k (min)
Group A			
HD25, Exon 14 DelACAC, Codon 1187/88	< 0.01	29	36
HD26, Exon 14, DelA, Codon 1192	< 0.01	26	39
HD26, Exon 14, DelA, Codon 1192	< 0.01	48	71
HD26, Exon 14, DelA, Codon 1192	< 0.01	26	35
Group B			
HP141, Exon 14, CGC(Arg)1689TGC(Stop)	< 0.01	158	199
HP131, Exon 13, TAC(Tyr)636TAG(Stop)	< 0.01	134	202
HP127, Exon 12, CGA(Arg)583TAG(Stop)	< 0.01	175	224
HI36, Proximal Intron 22 inversion	< 0.01	178	259
HI40, Distal Intron 22 inversion	< 0.01	159	219

investigated the phenotype of 5 hemophilia A patients in whom we had found a small deletion or insertion within or near another run of 9 adenines of the B-domain (codons 1191-1194).[12] In all five patients the mutations were associated with a frame shift resulting in a nonsense codon shortly after the mutation. However, although FVIII activity was found to be below 1% – thus fulfilling the criteria of severe hemophilia A – the thrombelastogram parameters r- and r+k-time revealed significant residual clotting activity compared to that in patients with a nonsense mutation or intron 22 inversion (Table 3). These findings clearly point to the synthesis of some functional residual FVIII protein that mitigates the severe hemophilia A phenotype and may cause an almost complete protection against inhibitor development.

Considering that the run of adenines at codons 1191-1194 represents a mutation hotspot responsible for one third of all deletion/insertion mutations within the FVIII gene,[8] our findings might explain the low risk of inhibitor formation found in association with the mutation type of small deletions/insertions. However, it should be noted that those small deletions/insertions that do not affect a series of adenines may produce a significantly higher risk for the development of inhibitors.

Inhibitors in mild hemophilia A

Recently, some very interesting results from studies of inhibitor patients with non-severe hemophilia A have been published. Fijnvandraat _et al._[13] described a mild hemophilia A patient in whom FVIII antibodies were directed against sub-

Table 4. Mutation type and relative risk of inhibitor formation.

Mutation type	Patients with inhibitor n=28	Patients without inhibitor n=67	Relative Risk
Intron 22 inverson	18 (64.3%)	34 (50.7%)	1.7
Nonsense mutation	6 (21.4%)	7 (10.4%)	2.3*
Missense mutation	1 (3.6%)	18 (26.9%)	0.1°
Splice-mutation	0 (0.0%)	3 (4.5%)	n.d.
Small deletion	1 (3.6%)	4 (6.0%)	0.6
Large deletion	2 (7.1%)	1 (1.5%)	5.1
Not detected	5 (15.2%)	7 (9.5%)	1.7

*$p < 0.05$, ° $p < 0.01$, n.d. = not determined.

Table 5. Common (upper part) and rare (lower part) MHC class I and II alleles in severe hemophilia A patients with intron 22 inversion and inhibitor formation according to Oldenburg et al.[4] For the MHC class I loci A, B, C the number of individuals and for the MHC class II loci DR, DQA, DQB the number of chromosomes is given.

Allele	Patients with inhibitor		Patients without inhibitor		Relative risk
	No.	%	No.	%	
Common MHC-alleles in patients with inhibitor					
A3	11	37.9	9	21.4	2.2
B7	14	48.3	8	19.1	4.0
C7	17	58.6	16	38.1	2.3
DQA0102	20	34.5	16	19.1	2.2
DQB0602	18	31.0	12	14.3	2.7
DR15	19	32.8	17	20.2	1.9
Rare MHC-alleles in patients with inhibitor					
C2	1	3.4	6	14.3	0.2
DQA0102	1	1.7	12	14.3	0.1
DQB0602	0	0	6	7.1	0.1
DR15	1	1.7	9	10.7	0.1

stituted FVIII but not against the mutated endogenous FVIII protein. This led to the interesting phenomenon that DDAVP was able to induce a considerable increase of the FVIII activity while substituted FVIII did not. Investigating 26 patients with non-severe hemophilia A and inhibitor development, Hay et al.[14] found that 41% of the patients had hemophilic relatives with inhibitors. In 9 of 11 families in which the FVIII gene mutation was known, the mutation affects the C1/C2 junction (amino acids 2009 to 2229). Moreover, five patients out of three families with an Arg2229Cys mutation developed anti-FVIII antibodies. Suzuki et al.[15] found that a mutation in this region at amino acid 2153 modifies the antigenicity of the C2 domain. Jacquemin et al.[16] and Peerlinck et al.[17] described that the mutations Ile2098Ser, Asn2129Ser, Trp2229Cys, Gln2246Arg[16] and Arg2150His[17] are associated with normal function of the FVIII molecule except for reduced FVIII binding to vWF that causes the decrease of FVIII:C. These findings point to a critical role of this region in the pathogenesis of inhibitor formation. The recently discovered crystal structure of the C-domains of the FVIII molecule[18,19] will form the base for efficient evaluation of this important aspect.

Mutation type and relative risk of inhibitor development

At present only retrospective data and data from the hemophilia A mutation register (HAMSTeRS)[9] are available to correlate the inhibitor risk to the mutation type. These data may be biased to some extent. In the mutation register no information about the cumulative exposure days is given, thus some of these patients might still be at risk of inhibitor development. Moreover the mutation register provides no information about the characteristics of the inhibitor (permanent vs. transient, high titer vs low titer). Another problem is that in the mutation regis-

ter the same mutation is associated with different phenotypes ranging from severe to mild hemophilia A. It is also possible that inhibitor patients have been analyzed with higher priority and therefore may be overrepresented in the mutation register.

For these reasons we initiated a further study in a well defined and characterized cohort of patients. The criteria for entering the study were that the patient had severe hemophilia A, was registered and treated at the Bonn hemophilia center and had been born after the 1.1.1978 (because from this time onwards, computer-assisted patient documentation allowed for complete registration of all patients). Genetic analysis has been completed in 107 patients. The mutation within the FVIII gene could be identified in 95 patients, comprising 28 patients with an inhibitor and 67 patients without an inhibitor. In 12 patients we were not able to find the mutation. Interestingly, 5 of these 12 patients had developed an inhibitor, thus pointing to the fact that mutations not detected by our screening strategy may represent genetic defects associated with a higher risk of inhibitor formation.

The strong selection criteria of the patients allowed the relative risks of inhibitor formation for certain mutation types to be calculated for the first time (Table 4). Intron 22 inversions showed a relative risk of 1.7 in favor of inhibitor development, nonsense mutations of 2.3 and

large deletions of 5.6, respectively. Missense mutations and small deletions were associated with a low relative risk of 0.1 and 0.6, respectively. Notably, genetic defects that were not detected in our study showed a high relative risk of 1.7. The results confirmed the earlier findings that inhibitor formation mainly occurred in intron 22 inversions, nonsense mutations and large deletions. However, more data are needed, especially for the less frequent mutation types.

MHC class I/II genes and risk of inhibitor development

The immune response genes e.g. the genes of the MHC (major histocompatibility complex) classes represent another parameter that may be responsible for a genetic predisposition to inhibitor development. The genes of the MHC classes are located within a small region of the short arm of chromosome 6 and therefore are inherited as a haplotype. The MHC class II genes DQ, DR and DP are in a focus of interest because their function is to present extracellular antigens – such as substitute FVIII – to the patients' immune system. In the past, several studies addressed the influence of these immune response genes on inhibitor formation.[20-23] However, the results were inconclusive and sometimes even contradictory. One main problem of these former studies was that they were not able to consider the patients' FVIII mutation type and therefore the influence of specific immune response genes on inhibitor formation might have been masked by the strong influence of the FVIII gene defect. Our group, therefore, conducted a study in which the influence of the MHC class I/II genotype on inhibitor formation was exclusively investigated in patients with the homogenous intron 22 inversion.[4] A distillate of the results is shown in Table 5. The MHC class I/II alleles A3, B7, C7, DQA0102, DQB0602 and DR15 could be assigned as risk alleles (relative risk 1.9 to 4.0), because they occurred more often in inhibitor than in non-inhibitor patients. In contrast the MHC class I/II alleles C2, DQA0103, DQB0603 and DR13 could be assigned as protective alleles (relative risk 0.1 to 0.2) because they occurred less often in inhibitor than in non-inhibitor patients. These MHC class I/II alleles belonged to extended haplotypes (A3-B7-C7-DQA0102-DQB0602-DR15 and C2-DQA0103-DQB0603-DR13) that were also frequent and less frequent, respectively, in the normal population. Therefore our number of patients was too small to reach a clear statistical significance. Moreover, inheritance as haplotypes might mask those MHC class I/II alleles that are decisive for the risk of or protection from inhibitor formation. From the immuno-

logic point of view the MHC class I alleles should be less important than the MHC class II alleles, which are known to be necessary for the presentation of extracellular antigens. In this context Chics et al.[24] made an interesting finding of a 16 amino residue peptide from the FVIII light chain (amino acids 1706-1721) that could be eluted from a DR15 cell line. The peptide was located on the surface of the FVIII molecule and bound by two functional cleavage sites.

Notably, Hay et al.[5] found the same MHC class II alleles to be associated at similar frequencies with inhibitor formation in patients with intron 22 inversions, thus supporting the concept that the MHC class II alleles are of some significance for the risk of inhibitor formation.

Two further studies provide indirect evidence that genes belonging to the immune response system may influence the risk of inhibitor development. Scharrer et al.[25] made a meta-analysis of three USA studies (Kogenate,[26] Recombinate[27] and US retrospective study[28]) that clearly demonstrated the influence of race on inhibitor formation. In the ethnic group of Africo-Americans the incidence of inhibitors in severe hemophiliacs was double (51.9%, 14 of 27) that of in Caucasians (25.8%, 51 of 191). Cox-Gill[29] compared the incidence of inhibitor formation in hemophilic siblings to that in more extended hemophilic relatives and found a much higher incidence in sibs (50%) than in extended hemophilic relatives (9%). Since the genetic defects of the FVIII gene should have been similar in both studies, the observed difference in inhibitor incidence should be caused by genetic variations of the immune system.

Inflammatory modulation of the immune response

In a recent review Kaufman et al.[6] pointed to the role of inflammatory processes in inhibitor formation, if the inflammatory reaction coincides with exposure to FVIII antigen. He gave two examples from gene therapy studies on animal models. In one study two Auburn dogs tolerated canine FIX infusions but develop FIX antibodies when the protein was synthesized after FIX gene delivery by Lentivirus or AAV (both to liver). A similar observation was made in Rhesus monkeys which tolerated repeated infusions of human FIX, but developed antibodies after delivery of human FIX by an adenovirus to liver. It was suggested that the liver inflammation induced by the infection with the virus vector triggered the onset of inhibitors. In a further study only one of seven dogs in which canine FIX was delivered by AAV to skeletal muscle developed an inhibitor. Notably, this dog had a skin infection at the time of gene delivery. From the experiences of these animal models it may be speculated that inflammatory

reactions activate lymphokine production that triggers an immune response. Consequently the individual experiences of the patient's immune system, with respect to infections, vaccinations and bleedings, during the first exposure to FVIII may influence the risk of inhibitor onset.

Conclusions

In conclusion there is now sufficient evidence that the genetics of a patient – FVIII gene mutation to a greater and immune response genes to a lesser extent – represents an important predisposing factor to inhibitor formation. However, the finding of different inhibitor status in monozygotic twins[30] points to the presence of other non-genetic factors e.g. inflammatory processes in early childhood. Fixing the genetic predisposition by gene analysis will be one important tool to aid assessment of further parameters of the complex system of inhibitor formation in the future.

Funding

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DISCUSSION 2. Risk factors for inhibitor development
Oldenburg, J. Schwaab, R (Bonn)

ALEDORT: Thank you for that very extensive discussion and provocative commentary Dr. Hoyer. I would like to call on you to comment on HLAs. This is an issue you have brought to the literature before and been very interested in, and then I'd like to ask Dr. Di Michele to comment on the inflammatory issue because it's something she has been very interested in and concerned about. Dr. Hoyer could you start?

HOYER: I'm afraid I can't add very much to that very nice discussion of HLA. I think that the bottom line is that it seems that there is some relation, but it is certainly not the dominant factor and this is certainly consistent with all of the earlier studies concerning smaller groups of patients; certainly the Intron-22 Inversion Population is the one to study. It's been studied and the answer was maybe.

ALEDORT: Dr. Di Michele do you want to comment on your interest and concern? I would just like to add another factor in looking at surrounding environmental differences in people developing inhibitors and those not. It is not uncommon for the surgical setting in which it's either the surgery itself or large quantities of antigen but I would add that there is certainly some evidence that a patient having surgery is more likely to develop an inhibitor than one not having surgery. Dr. Di Michele could we have a comment from you?

DI MICHELE: I don't think I can say it any better than Dr. Oldenburg and you when you made your comment. I think it's something that we have all observed clinically, particularly in pediatric patients in whom we've watched inhibitor-titers wax and wane in association with any inflammatory stimulus, usually illness. I just want to add that we are going to look at this issue and Dr. Hay will comment on this a little more when he talks about the International Immune Tolerance Study Protocol. We are also interested in the question of inflammatory responses on success of immune tolerance for exactly those reasons and all of those inflammatory stimuli that you mentioned will be carefully recorded in the International Immunetolerance Study Protocol to try to assess their impact on outcome.

RYPERT: I think the problem with the assessment is the risk of these HLA alleles is that we don't know anything about T-cell epitopes. I think there were three haplotypes in MC class II. Have you ever looked at any data base with these particular haplotypes which might be particularly likely to bind certain peptides within the FVIII molecule that could be T-cell epitopes?

OLDENBURG: No, we have not done this and it's a problem that the haplotypes that are common in the inhibitor patients are also common in the normal population and so it's difficult to differentiate from the background.

RYPERT: What about patients that form auto-antibodies against FVIII; would you find the same HLA types?

OLDENBURG: This a very rare occurrence and we are at present gathering samples of this group. We have about twenty-five and we are trying to increase the number in order to look at this point. These were two subsets of groups with a control group of forty patients and inhibitor was intron 22 inversion of thirty patients and I think we need at least this size to get an answer to this question.

LILLICRAP: I wanted you to comment on this ten per cent of patients in which you looked for mutations and didn't find them. First of all you put this down to a lack of sensitivity in the methodology which it may well be, but do you have a sense of whether these might be cryptic splice sites within introns? Is it possible that some of these mutations may be elsewhere and not at factor VIII? And then whether you've got enough numbers to say whether that ten percent of individuals have a higher or lower incidence of inhibitor formation?

OLDENBURG: This is a very interesting question. We have looked at about 107 patients at the Haemophilia Centre in Bonn and we found that the fact that we don't find the mutation is connected with the risk factor of about 1.7 for developing an inhibitor which was in the same range as for intron 22 inversion. We are using the DGGE method which looks at the melting behavior of DNA and for the B-domain done by

Dr. Schwaab who uses chemical-mismatch cleavage. We think that most of the sensitivity is due to the method we have been using; this is for two reasons: we have looked at a subset of patients in which we were unable to find the mutation with DGGE by another method based on HPLC and we were able to find some mutation in two thirds of these patients. I think this number can be increased.

LUSHER: Dr. Oldenburg this intriguing question of the inflammatory reaction and how much that adds to inhibitor development when we look at small children with severe hemophilia they all have bleeding episodes, childhood diseases and most of them have vaccinations and so we need to ask if it is a matter of degree and severity of these things or how do you envisage this is affecting the development of an inhibitor?

OLDENBURG: I think this is really difficult to answer and we can speculate that it is when such immune system challenge occurs in relation with the administration of factor VIII: when it is administered very close to an immune system challenge of another type it may be of higher significance in terms of inhibitor formation but this is only speculation. I think the answer will come from the immune tolerance studies to induce new tolerance status because all the factors I pointed out, as Dr. Aledort already mentioned, are of the same importance for the outcome and duration of immune tolerance therapy.

SAINT REMY: My comment regards your comments about inflammation and its role in the generation of inhibitors. It's probably no coincidence that the majority of inhibitors pertain to the IgG_4 subtype because the subclass of IgG_4 is driven by interferon and the main cytokine which is produced during inflammation is indeed interferon.

HOYER: That is a very important point and I think that one of the things that is coming from the sequential clinical studies is increased gathering of information that may be able to clarify these studies. We just don't have enough data on the incidence of bleeding, size of bleeding and treatment. I think your point that maybe not all bleeding episodes are the same in that a large bleed may be different from a smaller one in terms of its risk for inhibitor development is a very good point. One thing I'd like to add to your comments about gene therapy and treatment regards the fact that your data were for factor IX. We have a very small amount of information that is consistent with the opposite effect; that is, in the hemophiliac mouse you can very easily induce with plasma factor VIII or with recombinant factor VIII an immune response by intravenous injection but on the other hand in the adenovirus derived gene therapy studies inhibitor formation was not been seen. In this case it would appear to be the opposite but it's a different factor and a different model and so I don't know if they can be generalized.

Strategies for the Treatment of Inhibitor Patients

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Abstract

Symptomatic treatment of patients with hemophilia A and hemophilia B has now reached high levels of safety and efficacy. In consequence, at present the most prominent clinical complication is the development of inhibitors, which are alloantibodies directed against the coagulation factor demanded for substitution therapy in the management and prevention of bleeds. When *de novo* inhibitors are detected for the first time in a patient, several questions are raised. Since the clinical picture with inhibitors is dominated by an excessive tendency to bleed and reduced or lost efficacy of the usual factor concentrate, acute bleeding will often be a problem. Major questions from the patient and his next of kin are whether the inhibitors will disappear, and whether there is a therapy available that can cure this complication. Over the last 20-25 years, treatment programs have been established that seem to offer a reasonably good chance of suppressing inhibitors in hemophilia. Some protocols suggest the use of very high daily doses of factor VIII, whereas others propose much lower doses of factor VIII, seemingly with quite comparable rates of success. Therefore, controlled prospective clinical studies, focusing on the dosage aspect, are urgently required.

Control of bleeding is another issue of great importance. In inhibitor patients with a low responder state, substitution with increased doses of factor VIII or IX may successfully arrest bleeding. In some hemophilia A patients with a high responder state, but with actual inhibitor titers in the lower range, a porcine factor VIII concentrate could be useful. In these cases the patient's anti-factor VIII antibody should display no major cross-reactivity towards the porcine factor VIII molecule. In patients with high-responder inhibitors, so-called bypassing agents may be used to control bleeding. There are two major classes of bypassing agents. Concentrates have been produced for more than two decades derived from plasma and characterized by a high content of vitamin K-dependent coagulation factors. Examples are the prothrombin complex concentrates (PCC), and the activated prothrombin complex concentrates (aPCC). A newly introduced recombinant activated factor VII molecule (rFVIIa) has gained approval in numerous countries based primarily on results from emergency use in a substantial number of individuals with inhibitors. Other, still experimental, products have been proposed, but no human clinical studies are available as of yet. Inhibitors remain a challenge to patients and their physicians.
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Key words: Hemophilia, inhibitors

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The development of alloantibodies that inhibit the function of the substitution factor required to normalize hemostasis represents the most serious long-term complication of hemophilia today. When inhibitors are detected for the first time in a patient, two distinct issues require careful consideration: i) is there a likelihood that the inhibitors will disappear, spontaneously or following intervention? and ii): can bleeds be adequately managed?

The purpose of this review is to address some of the major critical issues related to clinical management of patients with inhibitors.

Inhibitors, risk factors and levels

Predicting inhibitor risk

While publications have reported observations on inhibitors in patients with hemophilia of all degrees of severity,[1] the most prominent clinical problems are seen in patients with severe hemophilia (residual factor level < 0.01 IU/mL). Cumulated data from recent long-term trials conducted in previously untreated patients following exposure to recombinant or plasma-derived factor concentrates have revealed that inhibitors occur early in life in the groups of patients frequently exposed to factor concentrates. A median number of 8-10 exposure days has most often been reported, but a wide range has been observed. While the frequency of inhibitor prevalence in three major clinical trials on recombinant factor VIII was close to 30% in previously untreated patients with severe hemophilia A, the risk of inhibitor formation in hemophilia B still needs to

be established more accurately, although the prevalence appears to be less than 5%.

Recent research has signaled a close relationship between the nature of the causative mutation and the risk of inhibitor development in hemophilia A (J. Oldenburg , data presented in this issue), since the intron 22 inversion, and larger deletions, appear to be responsible for the majority of cases. Other contributing factors are still less well understood. Certain histocompatibility locus class II profiles may be associated with the risk of inhibitors.[2] Most likely, pre-existing immunologic features may also influence the risk of inhibitor formation in an individual. Among pairs of brothers with hemophilia and inhibitors registered in a Swedish study, only one of the two brothers was affected by the inhibitor complication in almost 50% of the pairs.[3] High responder inhibitors, i.e. more than 5 Bethesda Units per mL (BU/mL) of plasma at any time, are the major clinical challenge. Antibody levels that remain persistently below 5 BU/mL (low responders), are more easily managed, and some even disappear on continued treatment with ordinary doses of substitution concentrate. A particularly dangerous complication which can occur in hemophilia B inhibitor patients is anaphylactic reactions to factor IX replacement. This frightening complication is most often seen in patients with complete deletion of the factor IX gene.[4]

Clinical risk problems

Patients with inhibitors are at increased risk of sudden death from major uncontrolable bleeding such as cerebral hemorrhage. Furthermore, "trivial" hemophilic bleeds that may be quite easily managed in non-inhibitor patients are often more difficult to manage in the inhibitor patient, and disabling long-term complications, such as long-standing hematoma or persistent hemarthrosis, are common. The alternative treatment measures available may be less efficient, and more costly, and monitoring of their efficacy may not be feasible.

The new case with inhibitors

A new case of inhibitor development may be detected during patients' three monthly routine check-ups, which are nowadays common practice in many institutions. More often, inhibitors are found because of increased bleeding or insufficient control of bleeds using ordinary doses of concentrate.

Ideally, a strategy aimed at removing or suppressing the inhibitors should be used.

Plasmapheresis may be helpful in emergencies as a very short-lasting remedy in patients with relatively low inhibitor levels. When no acute prob-

lem calls for immediate action, the patient should be re-tested after 2-4 weeks to see whether there is a spontaneous change in inhibitor titer. If inhibitors are of high-responder nature, an attempt to induce immune tolerance may be indicated, but the outcome may be influenced by the actual titer of the inhibitors when this treatment is commenced.

Immune tolerance induction

The aforementioned highlights that management of inhibitors in hemophilia A differs in several ways from that in hemophilia B. While continued treatment with factor VIII apparently poses no immediate physical harm to the patient, there is a highly significant risk of allergic reactions in patients with hemophilia B inhibitors following renewed exposure to factor IX.

Since the first report in 1977 of successful suppression of a high-responder hemophilia A inhibitor using high doses of factor VIII,[5] immune tolerance programs have been increasingly adopted in the several countries. Until now, the tolerizing effect of factor VIII concentrates using daily doses greater than 100 IU/kg b.w. has been documented only in single-case or single-case series reports, whereas controlled clinical studies are lacking. Numerous treatment regimens have been published, some using very high doses of factor VIII according to the so-called Bonn protocol or modifications of this regimen,[6-9] others employing considerably lower doses of factor VIII.[10,11] Three major data collections have been compiled in the *International Immune Tolerance Registry*,[12] the *North American Immune Tolerance Registry* (NAITR),[13] and the German National Immune Tolerance Registry.[14] With fairly small differences, the first two Registries recorded an overall success-rate close to 70%. The pre-induction inhibitor titer was a significant predictor of the outcome of the attempt to make patients tolerant. If high-dose treatment was instituted more than five years after first detection of the inhibitors, the treatment outcome was less successful. The magnitude of the dose of factor VIII adopted seemed more important for the outcome in the European registry than in its North American counterpart. Based on most recent data, the German registry patients displayed an overall success-rate of 81%. The three registries consistently observed that the peak inhibitor titer level predicted the outcome of immune tolerance treatment. In the low responder patient group, all three registries reported an efficacy rate of between 80% and 100%, supporting the early finding by Rizza *et al.* that some of these inhibitors disappear with continued "on demand" treatment.[15]

These registries provide us with only few

details on pre-existing morbidity in patients. Further, submission bias may hinder objectivity. Comparing the success rates of low-dose programs with those utilizing high doses of factor VIII, vastly different doses seem to be equally effective when used in patients with lower level inhibitor titers (< 40 BU/mL). Therefore, comparative clinical studies evaluating different dosing programs are urgently needed. A randomized, controlled study comparing two dosage regimens is expected to be launched very soon (Steering Committee members Drs. C.R. Hay, D.M. DiMichele, and E.P. Mauser-Bunschoten).

In brief, immune tolerance induction therapy seems to be an important, and widely used method of managing a child with a newly detected inhibitor. However, since all registries have shown that the pre-induction level of inhibitors is a significant predictor of outcome, it may be worthwhile observing a new inhibitor case for some weeks or months before starting a costly treatment program, taking advantage of any spontaneous tendency of the inhibitor titer to decline.

Venous access

Maintaining venous access is a major challenge in immune tolerance induction. Twice daily infusions of factor concentrate can damage veins of a patient who most likely has already received a number of venipunctures for diagnostic and therapeutic purposes. In the very young patient, veins may thus be in a bad condition at the start of immune tolerance treatment. Indwelling catheters can be helpful, but infective problems may complicate their prolonged use.

Alternative tolerization methods

As an alternative to continuous treatment with increased doses of factor VIII or factor IX, immune adsorption combined with immunosuppressive agents has been advocated. This is the so-called Malmö protocol. Readers are advised that this model has two slightly different procedures. The original model was immune adsorption of inhibitory immunoglobulins, together with non-specific gammaglobulins, onto columns of protein A sepharose along with administration of high doses of gammaglobulin, cyclophosphamide and prednisolone. A version of the protocol, published later, did not include immunoadsorption, but high-dose intravenous gammaglobulin, cyclophosphamide and prednisolone together with factor VIII or factor IX. Repeated courses of treatment were sometimes necessary with both protocols.

An overview of data registered in the Malmö studies has recently been published[16] showing an overall success rate ot 63% in hemophilia A inhibitor patients and 86% in hemophilia B patients with inhibitors. An advantage of this modality is that, in successful cases, patients reach a tolerant state within one month. Treatment is most efficient in patients with a low residual inhibitor titer at the time of starting of treatment. A Malmoe protocol update can be read in this issue.

Management of bleeds

The use of modern treatment programs, including prophylactic factor administration and early "on-demand" treatment of bleeding at home, has undoubtedly revolutionized the general perspective in hemophilia and the quality of life of the hemophilia patient. Importantly, the presence of inhibitors inevitably prevents patient's use of prophylactic therapy, and home treatment has not been generally available to patients with inhibitors.

Recent advances in clinical study practices, the use of controlled prospective studies in particular, have only rarely been adopted in studies on the general management of hemophilia, and management of bleeds in hemophilia patients with inhibitor complications is no exception. This obvious lack of quality control has some simple explanations. In hemophilia, effective symptomatic treatment compensates for the deficiency by simply infusing adequate amounts of the coagulation factor lacking, thus compensating the phenotypic abnormality. Insufficiently treated bleeding increases the risk of long-term disability. Hence, administration of placebo appears hazardous and ethically disputable. In consequence, placebo-controlled studies have been extremely rare, and even studies comparing various dose-levels for management of identical bleeds have been quite few. Clinical experience of the management of bleeds in patients with inhibitors has primarily been acquired from a limited number of open-label treatment case-series studies.

In selecting the most effective treatment program for patients with inhibitors, the titer and the historical behavior of the inhibitors are of major importance. Options available consist of substitution with supra-normal doses of factor VIII or factor IX, use of a porcine factor VIII molecule, and substitution with a hemostatic agent that improves hemostasis independently of the presence of factor VIII or factor IX (i.e. bypassing agent).

Factor VIII and factor IX substitution

Administration of high doses of factor VIII or factor IX may be useful in patients who present with a low inhibitor titer, and in whom post-infu-

sion monitoring demonstrates an acceptable level of circulating coagulation factor following supra-normal amounts of factor concentrate. In the true low-responder patient, a challenge with increased amounts of factor concentrate will not produce an anamnestic response, and the hemostatic efficacy of increased doses of concentrate will remain constant, or may even improve in the short-term. In the high-responder patient with a low inhibitor titer, an anamnestic response is predicted to occur following 4-8 days of treatment, when the inhibitor rise renders any further use of ordinary substitution concentrate useless for a long time. This knowledge is mostly based on personal experience of individual physicians, and systematic data have not been collected in regular, reported studies.

Porcine factor VIII

Porcine factor VIII molecule displays some homology with human factor VIII. In a proportion of inhibitor patients, the antibody does not inhibit, or only mildly inhibits the porcine factor VIII molecule, in particular if the inhibitor titer against human factor VIII is well below 50 BU/mL. In some inhibitor patients, factor VIII activity in patient's plasma may reach effective treatment levels and clinical hemostasis will be achieved by porcine factor VIII infusion. In other high-responder patients, however, porcine factor VIII may produce an anamnesic response to the human factor VIII as well as the factor VIII of porcine origin. Inhibitor cross-reactivity should be tested before and during use of porcine factor VIII in the inhibitor patient. Although a series of multicenter, open label treatment outcome protocols have been published, systematic, controlled studies with the current version of porcine factor VIII have not been published. With the existing formulation of porcine factor VIII, von Willebrand factor may cause thrombocytopenia in susceptible patients.

Bypassing agents

The early accidental observation of a clinical benefit of a relatively impure factor IX concentrate infused into a bleeding hemophilia A patient with inhibitors raised hope that such kinds of products might provide hemostasis in patients with inhibitors in general. Subsequently, the term factor VIII/IX bypassing agent was adopted. The hemostatic function(s) of factor IX (or prothrombin complex) concentrates of low purity has not been clearly established. Theoretically, factor VIII/IX bypassing agents could improve hemostasis by compensating for the lack of factor VIII or IX function, which is critically important for maintaining the function of the autocatalytic loop of coagulation. Coagulation

factors engaged in this loop include thrombin, thrombin activatable factor VIII, activated factor IX, activated factor X, activated factor V and activatable prothrombin. When factor VIII or IX is absent and substitution with these factors fails because of inhibitors, compensation for the loss of the autocatalytic loop function may be offered by adding one or several coagulation factors.

There are two major classes of bypassing agents: i) products that contain a mixture of some or all of the vitamin K-dependent procoagulant factors of human plasma, and ii): an activated recombinant factor VII molecule. While the effectiveness of factor VIII/IX or porcine factor VIII substitution is limited to patients with inhibitor titers within certain limits, the bypassing agents exert their hemostatic effect independently of the actual level of the inhibitors.

Prothrombin complex containing products

The first reported members of this class of concentrates were historical factor IX concentrates and prothrombin complex concentrates (PCC). These concentrates display some similarity due to their content of several extrinsic pathway coagulation factors, but variations are seen amongst products of different manufacture. By manipulation, or by accident, PCC coagulation factors may become activated and turned into so-called activated prothrombin concentrates (aPCC). These contain varying degrees of mixtures of native and activated vitamin K dependent coagulation factors. There is no formal common standardization available to ensure consistency in production and uniformity in labeling of products.

While the hemostasis promoting function of PCC and of aPPC in the hemophilia patient with inhibitors has not been fully explained, it is reasonable to assume that native as well as activated extrinsic system coagulation factors contribute to hemostasis by strengthening the functional capacity of each of the factors present in the concentrate administered. The individual *in vivo* half-life of each of these factors will determine the duration of enhanced coagulation, and the ability of the individual product to produce adverse complications of thrombotic nature. For instance, factor X has a quite long (days) natural half-life. The role and stochiometry of antithrombin dependent interaction with the activated factors is unknown. Concomitant antithrombin administration has been advocated in some cases, in particular in patients receiving long-term treatment and in patients with compromised liver function.

Major clinical studies, including the few controlled studies available, on the hemostatic effi-

cacy of PCC and aPCC have been published[17-19] Despite approximately 20 years of clinical use, a method well suited for monitoring these products in recipients has still not been devised. Since published works have illustrated the presence of a thrombotic potential of prothrombin complex concentrates, use of a monitoring system might increase safety in this respect.

Recombinant factor VIIa

The rationale for using recombinant factor VIIa (rFVIIa) concentrate in clinical management of bleeds in patients with inhibitors has been the appreciated interaction of rFVIIa with tissue factor (TF) where this thromboplastin is exposed as a result of pertubation of monocytes, released from platelets or presented as a result of local cell damage. Whereas it is generally known that the FVIIa-TF complex activates factor X, whereby thrombin generation can be promoted, it has further been established that FVI-Ia itself may activate factor X. Independently of tissue factor, rFVIIa may directly support coagulation activation on the platelet surface, as recently demonstrated.[20] At present, we have no clear picture of the relative contribution of enhanced plasma coagulation as compared to the platelet and cell surface promotion of coagulation induced by rFVIIa. However, there seems no doubt that cell surface phospholipids are essential in this process.

Open label format, clinical studies on rFVIIa have demonstrated that this drug is efficacious in arresting bleeding in patients with inhibitors, based on data collection of numerous bleeding episodes of various kinds[21] in a large series of cases. Further, data from two studies have illustrated the efficacy of rFVIIa in early "on-demand" treatment of "trivial" bleeds in the home setting,[22,23] and in management of surgical episodes.[24] Recently, a randomized controlled study of elective surgical episodes was reported, comparing two different dose levels of rFVIIa[25] for intraoperative and immediate postoperative hemostasis. This study demonstrated that the higher dose (of 90 µg/kg b.w.) was more effective than the lower dose (of 35 µg/kg b.w.). However, a study comparing rFVIIa against aPCC has not been conducted so far. It will be difficult to make such a study blind since the volume and protein concentration of the two kinds of concentrate as well as the dosage frequency are very different. Thrombotic episodes have been observed in very few recipients of rFVIIa. Most of these episodes occurred in elderly patients with acquired factor VIII inhibitors and pre-existing vascular risk factors (U. Hedner, personal communication). The

rFVIIa concentrate offers the advantage PCC and aPCC in its equal efficacy in hemophilia A and hemophilia B inhibitor cases.

Other modalities

A "de-lipidated" tissue factor and an activated factor X have reportedly been developed and tested in the laboratory setting. However, as of today, no human clinical results have been presented. Hybrid factor VIII molecules, such as a hybrid human factor V-human factor VIII molecules or a hybrid of human factor VIII with porcine factor VIII, are being studied *in vitro*, but have not apparently been tested clinically in humans.

The use of fibrin glue has been advocated by some center as an adjuvant to substitution therapy in inhibitor patients requiring surgical intervention. Tranexamic acid (AMCA) has quite often been adopted concomitant to rFVIIa treatment in surgical episodes.

Selecting the hemostatic agent

Selecting the most appropriate modality for treating critical bleeds requires serious consideration. Regional and national treatment recommendations have been produced by various boards of hemophilia treaters proposing management algorithms for bleeds in patients with inhibitors. However, local experience with the modalities available seem to be a major determinant for the regimen selected. In this, issues such as efficacy, cost of treatment, and the risk of adverse events of infectious and thrombotic nature may be considered. Likewise, other clinical complications, and the nature of the bleeding episode should be taken into account.

Continued surveillance of patients and continued work improving the safety and efficacy of products for the treatment of hemophilia patients with inhibitors are warranted.

Conclusions

Significant improvements have been made over the last few decades in understanding the nature and causes of inhibitors in hemophilia. Further, the introduction and dissemination of immune tolerance regimens has improved the outlook for the patient with an inhibitor, and a host of modalities has been developed for safe, quite effective treatment of bleeds. Recent data have even demonstrated that emergency and elective surgery can quite safely be accomplished in patients with inhibitors. However, continued work is required, supported by controlled clinical studies, to assess the efficacy of various programs for the management of the inhibitor itself as well as its bleeding complications.

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Porcine factor VIII: past, present and future

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Abstract

Porcine factor VIII concentrate is effective in up to 90% of bleeds. Its use is, however, sometimes associated with reactions and a fall in platelet count. Furthermore, it is not virally attenuated, although no viral transmission from this product to humans has ever been demonstrated. A third-generation porcine factor VIII is currently being developed. This is purified from parvovirus-free porcine plasma using immunoaffinity and ion-exchange chromatography. It is virally attenuated using the solvent detergent method. It should have a very high specific activity and be virtually free of porcine von Willebrand factor. These product characteristics should confer greater product safety and a greatly reduced risk of treatment side effects.
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High-purity porcine factor VIII (Hyate:C, Ipsen, UK) has been used for the treatment of bleeding in patients with factor VIII inhibitors for 20 years.[1-5] The advantage of this product over human factor VIII is that, in congenital hemophilia, these inhibitor antibodies have a median of only 15% cross-reactivity to porcine factor VIII.[4] Patients with acquired hemophilia tend to have even lower anti-porcine inhibitor titers, and usually respond well to Hyate:C.[5]

When selecting patients for treatment with porcine factor VIII the anti-porcine inhibitor titer, the clinical circumstances, and the previous anamnestic response to the inhibitor should all be considered. Although the Hyate:C product data sheet suggests that patients with anti-porcine factor VIII inhibitor titers of <50 BU/mL may be treated with this product, patients with inhibitor titers <20 BU/mL have a better response-rate than those with higher titers. Porcine factor VIII is usually used for serious bleeding in patients with acquired or congenital hemophilia and low or intermediate level anti-porcine inhibitor titers.[2-5] An important sub-group of patients with congenital hemophilia have been identified who have very low anti-porcine inhibitor titers and whose inhibitors remain low despite regular treatment with porcine factor VIII. These patients may be treated routinely, using small doses of porcine factor VIII over a period of many years.[4] The clinical response relates to the current inhibitor titer and the post-infusion factor VIII increment achieved, although responses have been reported even in individuals with very high inhibitor titers. It has been suggested that these responses, which are not associated with a measurable factor VIII increment, may be attributable to the platelet agglutinating effect of low levels of porcine von Willebrand factor (vWF) remaining in the concentrate.[7,8] Patients have been reported to obtain a good response to treatment following more than 90% of bleeds.[4]

An anamnestic rise in the anti-human inhibitor titer has been reported to follow between 25 and 35% of treatments with porcine factor VIII.[2-4] This is thought to represent a lower risk of an anamnestic response than that which follows treatment with human factor VIII. The risk of anamnesis is particularly low amongst patients with acquired hemophilia.[2,3,5] In a multicenter study, only 4% of patients in one series of patients with acquired hemophilia were reported to have an increase in inhibitor titer following treatment with Hyate:C.[5] The use of porcine factor VIII can be associated with the emergence of specific anti-porcine inhibitors.[4] In about a third of these situations there may be a brisk anti-porcine anamnestic response which, depending on the titer reached, may limit regular treatment with this product. In a further third the anamnestic response is moderate in degree and may be dose-related. Most of the patients in this group may be treated on a number of occasions for more serious bleeding without becoming resistant to porcine factor VIII. The remaining patients have no anamnestic response to porcine factor VIII and may be treated regularly with this product for *routine* bleeding.[4,6]

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Side effects of Hyate:C

The main side effects of Hyate:C are transfusion reactions and a post-infusion fall in platelet count. Reactions have been reported to occur following 3-7% of infusions[2-4] although those using Hyate:C regularly have a far lower incidence.[5] Most reactions are dose-related, occurring after the administration of large doses only, although a minority of patients react every time they are given porcine factor VIII, however low the dose.[4] These patients cannot be treated with porcine factor VIII regularly. The risk of reactions may be increased by the rapid infusion of Hyate:C and may be reduced by administration by continuous infusion. In a recent series of over 100 continuous infusions of porcine factor VIII the only transfusion reactions observed were those associated with the pre-infusion bolus dose of Hyate:C.[9]

A post-infusion fall in platelet count is common and is also dose-related with an r-value of 0.64.[4] However, the fall in platelet count is usually relatively slight and of no clinical significance; the count fell by a median of only 15% in one series.[4] The fall in platelet count is also usually transient, representing reversible agglutination by porcine VWF.[4,7,8] In this series the platelet count reached its lowest point 30 minutes post-infusion generally recovering within an hour to pre-infusion levels. The fall in platelet count is rarely of any clinical importance but intensive porcine factor VIII replacement using large doses administered several times a day may be accompanied by a progressive fall in platelet count over a period of several days. For this reason, the platelet count should be monitored during intensive replacement therapy with Hyate:C. This progressive fall in platelet count may be reduced by administering the factor VIII by continuous infusion.[9] Although continuous infusion of very large doses of Hyate:C may be associated with a progressive fall in platelet count, this fall seems to be less marked than the change in platelet count associated with the administration of similar doses of porcine factor VIII by bolus injection.

Product characteristics

Hyate:C is a second generation porcine factor VIII concentrate fractionated using polyelectrolyte.[1] This yields a high-purity concentrate, with a specific activity of >100 units/mg of protein, which is relatively depleted of porcine vWF.[1,11,13] The VIIIC:VWF ratio is approximately 10:1. The product is stored at −15 to −20°C, a hangover from the earlier, intermediate-purity, 1st-generation product. Although these storage conditions are specified in the product licence, they are probably not necessary and it is likely that the product storage recommendations will be changed in the future.

As the manufacturing process involves no specific viral attenuation step, the final product is extensively screened for porcine viruses using a 4-cell line general screen and the source plasma is also screened for porcine parvovirus. Porcine viruses known to be zoonotic to man include porcine influenza virus and encephalomyocarditis virus.[11] Hyate:C has never been shown to transmit any viral disease. The adoption of more sensitive cell culture and polymerase chain reaction (PCR) techniques for the detection of porcine parvovirus in 1996 led to the identification of this virus in some batches of product. Subsequent shortages of Hyate:C were caused by the steps taken to eliminate this virus from the end-product, even though this virus is not known to be pathogenic to man.[10,11]

The porcine plasma donors are slaughtered before six months of age, by which time many may have been infected with porcine parvovirus. For agricultural economic reasons, only breeding sows are normally vaccinated against this virus. Pools of porcine plasma are screened for porcine parvovirus using a PCR technique and all pools testing positive are rejected and discarded.[13] Between 50% and 80% of pools can be rejected for this reason. Ipsen are progressing to arrange a second collection facility to increase plasma procurement and improve product supply. Although Hyate:C may have been contaminated with porcine parvovirus for many years before increased test-sensitivity led to its detection in the porcine source-plasma there is no evidence of transmission to man. In a recent pharmaco-vigilance study, 80 patients who had been treated with Hyate:C at some time and 125 controls (abattoir workers, manufacturing personnel, and recipients of porcine heparin or insulin) were tested for porcine parvovirus, infuenza virus and encephalomyocarditis virus. All tested negative, showing that, in this group, despite a variety of types of exposure to pigs or porcine products, often over a period of many years, no transmission of these viruses had taken place.[12]

3rd-generation porcine factor VIII

Even though Hyate:C is used in the clinically high-risk milieu of the bleeding inhibitor patient and though it may be life-saving, and even though the side-effects are usually relatively minor, there is a general desire for a product with an improved side-effect profile. There is, therefore, a demand for a third generation product with these characteristics. Ipsen have been developing such a concentrate, which should soon be subjected to clinical trial.[13]

The new product will be purified from porcine

plasma using immunoaffinity and ion-exchange chromatography. Essentially VWF-free porcine factor VIII is generated.[13]

Viral depletion and attenuation will be achieved in several ways. Firstly the source-plasma will be screened, as before, and porcine parvovirus positive plasma pools rejected. Secondly, chromatographic purification and washing would be expected to be associated with considerable viral reduction. Viral spiking experiments used to estimate the degree of viral reduction associated with similar fractionation methodology of human cryoprecipitate suggests that at least a six-log viral reduction of most viruses might be expected from the fractionation alone. Finally the product will be virally attenuated using the solvent detergent method. This method would not inactivate non-enveloped porcine parvovirus, hence the need for continued plasma screening for this agent. A heat step has not been incorporated due to concern over potential neo-antigen formation.

These processes are anticipated to yield an ultrapure, virally attenuated concentrate with a specific activity of about 5,000 U/mg protein. The final formulation will not include any added human albumin as excipient or stabilizer. The intended storage conditions are 2-8°C.[13]

Possible product characteristics

Since this product has yet to be tested in clinical trials one can only speculate on its likely clinical characteristics. One might anticipate that reactions would be far rarer with the new product. This has been the general experience, as ever-purer human factor VIII concentrates became available. Here, one might expect that the dose-related majority of reactions observed following the administration of Hyate:C would be avoided. Many patients who had reactions with Hyate:C in the past will have reacted not to porcine VIIIC *per se* but to other contaminating porcine proteins which may be present in Hyate:C but which will be absent from the ultrapure concentrate. A very small proportion of patients may react specifically to porcine factor VIII itself.

The third-generation product should have no effect on the post-infusion platelet count since it is not significantly contaminated with porcine VWF. It will be interesting to see if this affects efficacy in any way, since it has been speculated that platelet agglutination may add to the clinical response to Hyate:C. The product should have a very high level of viral safety.

The likelihood of anamnesis should be unaffected by increased product purity just as there is no conclusive evidence that high-purity human factor VIII concentrates are any more antigenic than intermediate-purity products, but this will have to be monitored in prospective clinical trials. Similarly, viral attenuation of human factor VIII using the solvent detergent method has not been shown to cause neo-antigen formation and seems unlikely to cause inhibitor development following the use of third-generation porcine factor VIII.

The future

Even as we prepare to embark upon clinical trials with this product, its successor may be only just over the horizon. An appreciation that porcine factor VIII is less antigenic than human factor VIII in inhibitor patients and that the factor VIII C2 and A2 domains are more antigenic than other sectors of the molecule[14] has led to the exploration of recombinant human-porcine hybrid factor VIII constructs.[15,16] These molecules have variable porcine substitutions of sectors of the human A2 and C2 domains. A range of such constructs is currently being screened against factor VIII inhibitors taken from a number of individuals in order to select the least antigenic. Although such recombinant human-porcine hybrids have major potential as treatments for bleeding in inhibitor patients, they may also prove less antigenic than wild-type recombinant human FVIII in normal use. If this proves to be the case, they may have an application as first-line treatment in PUPS, inducing fewer inhibitors than current standard treatment.

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DISCUSSION 3   Porcine FVIII, Past, Present and Future
C. Hay (Manchester UK)

INGERSLEV: Thank you Dr.Hay for an interesting and very complete overview on the porcine factor VIII molecule and the prospective concerning new products in this field.

HOYER: I have always been fascinated by that group of patients who are repeatedly treated with porcine factor VIII who don't develop inhibitors. Is there anything in the history of these patients that gives you a clue about why this happens. Where these patients treated more frequently or at an earlier age? Are there any other distinguishing characteristics that help you separate this 30% from the other 70%?

HAY: No. There are even patients who have a low response when they are given human factor VIII. Most of them, however, were high responders to Factor VIII and were treated very regularly, in the majority of cases every day or every second day. Three of the patients were treated on demand with an average frequency of only once every two or three weeks. They mostly started treatment in fairly early childhood between the ages of five and ten but the patient who has been treated the longest has now been on treatment with porcine factor VIII for eighteen years. He started in his teens and so did the first patient I managed, so really they are nonresponders. In fact I've noted that myself too.

SAINT-REMY: Dr. Hay, you mentioned that a small proportion of those patients were seemingly allergic to the product. Do you mean that IgE antibodies have been identified?

HAY: No, I don't mean that. It was an entirely clinical observation. All I mean is that however small a dose they are given and however carefully every time they're given porcine factor VIII, they have a reaction. The majority only had reactions with bigger doses and one suspects that this is related to the speed with which you give the product and I think there is an element of truth in this because if you double the dose charac-teristically you don't double the length of time over which someone squeezes the syringe, so it goes in more quickly. There seems to be less of a problem with continuous infusion.

INGERSLEV: You have a long tradition of using high Hyate:C. Do you have patients in who you could show that it is possible to retard the development of arthropathy. Will the bodily problems be less for these patients?

HAY : That hasn't been looked at formally and certainly not in a controlled way but the subgroup in which this product was used as regular treatment appeared to be as effective as human factor VIII would have been. I would expect that in those patients the progression of arthropathy was retarded. Quite frankly it never crossed my mind to examine this aspect. Those patients who use the product intermittently like with all the other patients end up with far worse anthropathy than the average hemophiliac.

INGERSLEV: Is this also your experience when patients have been treated with other modalities and had treatment for every bleed that you can retard?

HAY: I have more patients on home therapy with the progression of the arthropathy on PCCs or FVIIa than I have with porcine factor VIII. I'm sure this matches everyone else's experience and those patients have got very significant arthropathy. Their response to treatment is not as good as that which I'd expect from factor VIII in the absence of an inhibitor.

KOLULEF: In our high responding pediatric patients we usually employ activated PCCs or recombinant factor VIIa. Nevertheless, there are some patients who do not respond. We usually determine their cross-reactivity before using porcine factor VIII and we observe in some patients some cross-reactivity which is slightly above the recommended cut-off. Do you have any experience with the influence of the cross-reactivity which is below the cut-off in terms of clinical response?

HAY: In routine treatment all the patients we treated had very low levels of antibody to porcine factor VIII.

KOLULEF: Which levels were there? It's very important for us as clinicians.

HAY: They were all less than five and amongst that group we found the anti human inhibitor titre was poorly predicted. We had one patient for example with fifty-six Bethesda units against human and 0 against porcine. For routine use, if you were using human factor VIII, you would only use it in an inhibitor patient with a relatively low inhibitor titer. I think the same is true for porcine FVIII; if you want to use it for routine use I would limit it to patients with low inhibitor levels, below five BU who do not have significant anamnesis or side effects and so they end up being selected on an entirely empirical basis, that is, on the basis of their inhibitor titer and their response to porcine initially given in hospital. What your upper cut-off would be for the use of porcine factor VIII for a more severe bleed is controversial. The package inserts suggest a level of twenty Bethesda units against porcine while my suspicion is that it should be lower.

SEIDER: Is there a genotyping programming in place for patients receiving porcine factor VIII?

HAY: There isn't at the moment but it's an interesting question. I think the number of patients in the literature that fall into that category is quite small and I have my doubts as to whether analysis of this small group would yield statistically significant results for that logistic reason alone.

Mapping factor VIII inhibitor epitopes using hybrid human/porcine factor VIII molecules

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Abstract

Four epitopes in the factor VIII (FVIII) molecule have been identified that constitute the targets for antibodies in most inhibitor plasmas. These epitopes are located in the A2, A3 and C2 domains and in the activation peptide (ar3 region) of the FVIII light chain. We have developed a method for mapping FVIII epitopes using recombinant hybrid human/porcine FVIII molecules. Ongoing studies continue to provide higher resolution maps of these epitopes. The manipulation of inhibitor epitopes using recombinant DNA technology may lead to improved forms of FVIII that have lower antigenicity and/or lower immunogenicity.

Factor VIII Inhibitors

Inhibitory antibodies (inhibitors) to FVIII arise in both hemophiliac and non-hemophiliac populations. They develop in response to FVIII infusions in approximately 25% of patients with hemophilia A.[1] In non-hemophiliacs, FVIII autoantibodies develop in a variety of clinical settings, including the post-partum period, systemic lupus erythematosus, and chronic lymphocytic leukemia. FVIII inhibitors are polyclonal populations that are almost always IgG (most often IgG_4) in nature.

Factor VIII Inhibitor Epitopes

FVIII contains a sequence of domains designated A1-ar1-A2-ar2-B-ar3-A3-C1-C2, where ar designates acidic regions that are NH_2-terminal to thrombin cleavage sites between the A1 and A2, A2 and B, and B and A3 domains, respectively. The first inhibitor epitopes identified were mapped to the A2 and C2 domains of FVIII by Western blotting and deletion mapping.[2-4] More recently, inhibitors with specificity for the A3 domain and ar3 region have been identified. The presence of these antibodies was initially suggested by the observation that inhibitor plas-

mas are frequently incompletely neutralized by a combination of recombinant A2 and C2 domains, but are usually completely neutralized by a combination of the A2 domain and the FVIII light chain, which is a fragment containing the ar3-A3-C1-C2 domains.[5]

The A2 Epitope

A2 epitope mapping studies have been done using active recombinant hybrid human/porcine FVIII molecules.[6,7] This strategy is based on the observation that inhibitors usually have limited cross-reactivity with porcine FVIII.[8] Thus, a hybrid human/porcine FVIII molecule that is less reactive with an inhibitor to human FVIII, that is, which is less antigenic, localizes the inhibitor to the region in which the porcine substitution has been made. Because the hybrids are active, reduction in antigenicity is unlikely to be due to improper folding of the protein.

Of several hybrids that contain porcine substitutions in the A2 domain of human FVIII, one, designated HP9, has been most informative. HP9 contains a porcine substitution for residues Arg484-Ile508. The antigenicity of HP9 toward the patient A2-specific inhibitors studied so far is at least ten-fold lower than that of human FVIII.

The A2 epitope has been further characterized by making single alanine substitutions at the nine sites of the human Arg484-Ile508 region where human and porcine fVIII differ: Arg484, Pro485, Tyr487, Ser488, Arg489, Pro492, Val495, Phe501, and Ile508.[9] The inhibition of these mutants by three A2-specific alloimmune and two A2-specific autoimmune human inhibitor plasmas was measured by the Bethesda assay. For all five patient plasmas, the Tyr487Ala mutant displayed the lowest antigenicity, which ranged from 10 to 20 percent of that of human FVIII. The inhibition of the Ser488Ala, Arg489Ala, Pro492Ala, Val495Ala, Phe501Ala, and Ile508Ala mutants by most of the plasmas was also significantly reduced. These results indicate that the side chains recognized by A2 inhibitors are similar, despite the

differing immune settings that give rise to FVIII alloantibodies and autoantibodies.

The C2 epitope

The hybrid human/porcine FVIII approach has been used to map the C2 inhibitor epitope.[10] A hybrid containing a substitution of porcine sequence for Glu2181-Val2243 in the human C2 domain, designated HP24, is less antigenic than human FVIII toward five C2-specific human antibodies and a murine anti-FVIII monoclonal antibody, NMC-VIII/5. Thus, a major FVIII inhibitor epitope determinant is bounded by Glu2181-Val2243 at the NH_2-terminal end of the C2 domain.

This result was unexpected because earlier studies had indicated that the COOH-terminal end of the C2 domain contains the inhibitory antigenic site.[11-14] However, the NH_2-terminal and COOH-terminal sequences of the C2 domain may be close to one another in space and form a single epitope. This may be the case because residues Cys2174-Cys2326, which are at opposite ends of the C2 domain, are disulfide bonded.[15]

Coagulation factor V is homologous to FVIII. Hybrid factor V/FVIII proteins that contain substitutions of FVIII in the factor V C2 domain have been used to map the epitope of an inhibitory anti-factor V antibody that blocks phospholipid binding to the NH_2-terminal third of the C2 domain.[16] This result is consistent with FVIII inhibitor epitope mapping studies and suggests that the NH_2-terminal region of the C2 domain of both factors V and VIII contains an immunodominant epitope that is important for phospholipid binding.

Only one human monoclonal FVIII inhibitor has been characterized, a human IgG4k antibody produced by an immortalized B-cell line.[17] This antibody recognizes the C2 domain of FVIII and inhibits FVIII binding to both vWF and phospholipid. The epitope recognized by this inhibitor has not been mapped. Further analysis of monoclonal inhibitors will be an important way to determine whether there is heterogeneity in the epitopes recognized within given FVIII domains.

The A3 epitope

An anti-A3 inhibitor was recently identified in a patient with hemophilia A by deletion mapping, which placed the epitope within an A3 segment bounded by amino acids 1778-1823.[18] The antibody also inhibited the binding of factor IXa to the FVIII light chain, which is necessary for assembly of the intrinsic factor X activation complex.[19] The FVIII light chain binding site for factor IXa has been localized to amino acids 1811-1818 in the A3 domain. Consistent with these observations, three inhibitor IgGs have been identified that prevented the binding of factor IXa to the FVIII light chain.[20] In that study, the binding of these inhibitors to the FVIII light chain was competed by a synthetic peptide corresponding to A3 amino acids 1804-1819.

The ar3 epitope

We have recently identified an additional epitope in the ar3 region using hybrid human/porcine FVIII molecules.[21] We identified patient plasmas that had activity against the ar3-A3 region and tested their activities against two hybrid human/porcine FVIII molecules designated HP35 and HP41. Both molecules contained porcine substitutions encompassing the A2 and C2 epitopes to eliminate effects of antibodies to these regions. HP35 contains the human ar3 region and porcine A3 domain. Conversely, HP41 contains the human A3 domain and porcine ar3 region. The cross-reactivity of seven patient plasmas against HP35 and HP41 were compared to that of human FVIII. In four of the seven plasmas, HP35 was significantly more cross-reactive than HP32. Conversely, HP41 was less cross-reactive than HP33 in six of the seven plasmas. These results are consistent with the presence of an epitope in the human ar3 region that is recognized by some inhibitor plasmas.

Therapeutic implications

Plasma-derived porcine FVIII concentrates have been used extensively as a low antigenicity product to treat FVIII inhibitor patients.[22,23] A hybrid human/porcine FVIII molecule containing porcine substitutions at known antigenic sites might provide a useful therapeutic alternative. Anti-porcine FVIII antibodies that frequently accompany porcine therapy[24] may be directed toward epitopes that are distinct from the common human FVIII epitopes.[25] Hybrid FVIII might be superior to porcine FVIII because it avoids exposure to porcine neoepitopes. Recombinant hybrid FVIII would also provide a better alternative because of potential infectious problems inherent in the use of plasma-derived material. In addition to reducing the antigenicity of FVIII, it would be desirable to reduce its immunogenicity, that is, to reduce its ability to be recognized by the immune system. The immunogenicity of a molecule depends on the B-cell repertoire, T-cell help and suppression, and the major histocompatibility complex, which together determine the concentration and binding affinity of antibodies for an antigenic site. An important question regarding FVIII B-cell epitopes is whether they are intrinsically immuno-dominant. If so, then mapping and modification of

these epitopes by site-directed mutagenesis could produce a less immunogenic FVIII molecule.

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DISCUSSION 4 **Mapping Factor VIII Inhibitor Epitopes using Hybrid Human/Porcine Factor VIII Molecules**
Lollar, P. (Atlanta)

SCHWARTZ: Did you have the occasion to study the association and the disassociation of HP 41 from human factor?

LOLLAR: No, we didn't but clearly that is a direction in which we would like to go. I think one of the interesting questions about the analysis of inhibitors is how you measure their potency *in vitro* and the Bethesda assay appears to be predictive of the ability to treat someone with porcine factor VIII and yet the binding of an antibody to the activation peptide of factor VIII may

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not be evident in a Bethesda assay and I think it's really a question of how this epitope contributes overall to the average inhibitor plasma. It's possible that in many plasmas some of the inhibitor reactivity isn't seen in the Bethesda titer as clinically insignificant whereas there may be some patients in whom this epitope constitutes a major problem and the Bethesda titer isn't reporting the problem.

RYPERT: You're talking about low antigenicity of your recombinant molecules meaning that you get a reduced binding of antibodies induced against human factor VIII. This, however doesn't give us any reflection on the immunogenicity of the molecule that is the capacity to induce inhibitors which is the real problem of these products.

LOLLAR: I also agree that it's a more generally important issue but the antigenicity is also an important problem in those patients who have inhibitors. It's important to distinguish the difference between antigenicity and immunogenicity. Antigenicity is defined as the ability of something to interact with the product of the immune response, usually an antibody, whereas immunogenicity is defined as an ability to elicit an immune response. One of the very interesting things about factor VIII epitopes which I think is unexplained at this point is that in patients with hemophilia A developing inhibitors and in patients who have acquired inhibitors we have very different immunological backgrounds including the fact that one has been treated with factor VIII exogenously and the other has not. If you look at patient's epitopes in respect to the A2 polypeptide one sees these antibodies are binding to the same A2 molecule and to me this suggests something which is slightly against an immunological dogma and that is there are intrinsically important regions on the factor VIII molecule that drive the immune response whereas the immunological dogma is that the entire surface of a protein is potentially immunogenic. The fact is that the A2 epitope may be an immunodominant epitope; it could be that the intrinsic structures of that epitope are at least in part driving an immune response to the level that high affinity antibodies occur. This indicates that one could potentially modify those epitopes and create a less immunogenic molecule. One of the problems with immunogenicity is that we don't have a nice in vitro test for it, whereas we have a fairly good test for antigenicity with the Bethesda assay. Therefore, it would be nice to have a test-tube or a cell-based assay in which one could predict the immunogenicity of factor VIII molecules, but we don't have it yet. There is a hemophilia A model of immunogenicity which was developed by Dr.Hoyer's group and I think

that right now that would be the standard in which to try and test constructs on a bi- epitope basis evaluating their immunogenicity and we really intend to do that.

LILLICRAP: Do you know whether any of the substitutions are making significant differences to the specific activity of the protein?

LOLLAR: We've made forty-one human porcine hybrids to date, and all of them have either equal activity or greater activity than human factor VIII. As you know porcine factor VIII has more activity than human factor VIII at least in a one stage clotting assay. It's interesting and also a little surprising that some of the constructs did not have decreasing activity. What this means is that one can, not only with the A2 domain but also with the C2 and the A3 domains, make substitutions and create molecules which don't bind to antibodies as they lose a micro-molecular interaction; yet they're still able to interact with regions of the human clotting complex in an in vitro system and get a good specific activity.

SAINT REMY: Have you any idea of the T-cell reactivity of your constructs?

LOLLAR: No, we haven't. T-cell epitopes and B-cell epitopes are usually different. Just because the regions 484 to 508 on factor VIII are a problem area does not mean that's what the T-cells recognize. T-cells recognize small peptides. There is a lot of research in this region: at the ISTH meeting, for example, Bianca Conti presented data on the T-cell epitopes which are present in human factor VIII with respect to infusion of human factor VIII into mice and also humans. She used a T-cell proliferation assay and identified several regions of the factor VIII molecule that indicate that at this point the T-cell epitopes are more frequent and much more complex than the B-cell epitopes and there are a lot more of them.

HOYER: Have you looked at these hybrids with plasmas from patients who have developed inhibitors and then have been treated extensively with porcine factor VIII? The reason I ask is to return to the issue of antigenicity versus immunogenicity. It is an imperfect tool but perhaps the only one we can use without animal studies. One wonders if the patients treated extensively with porcine factor VIII would develop antibodies that would react with your hybrids. On the other hand, if they don't react with the hybrids, this would reinforce the notion that they may be used therapeutically for long periods of time.

LOLLAR: This patient that I discussed who was an auto-antibody patient who had only received porcine factor VIII; he was the only patient we have had who had an anti-human

titer and an anti-porcine titer who we had a good clinical history for. The twenty-three patients that I showed you were patients for whom we did not have a clinical history and were just defined by their high Bethesda titers which ranged from ten to thousands. It would be interesting to accrue that data. I would like to have samples to do that. I think that a panel of porcine- human hybrids would be a potentially useful set of reagents to include in prospective studies to try to find out how the B-cell response varies with exposure to porcine factor VIII.

Antibodies to factor IX

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Antibodies to Factor IX

Alloantibodies (inhibitors) against factor IX (FIX) develop in 1-3% of patients with hemophilia B and the incidence is higher in severe hemophilia B.[1-3]

Alloimmunization occurs only after exposure to FIX containing products. Factor IX antibodies usually occur in severe hemophilia B patients with total absence of FIX antigen, either due to total gene deletion or other major derangements of the FIX gene. The risk of inhibitor development is reported to be 50% for those hemophilia B patients with total gene deletions.[3] Frame shift mutations and stop codon abnormalities are associated with a 20% risk while missense mutations are not associated with any increased risk of inhibitor formation.[4]

Similar to the FVIII inhibitors in hemophilia A, FIX antibodies in hemophilia B patients are detected either by routine surveillance or when the patients do not have an expected response to treatment. But most recently severe allergic reaction/anaphylactoid reaction occurring simultaneously with inhibitor development has identified a subset of patients with FIX inhibitors in hemophilia B.[5] This unique adverse reaction further complicates the treatment of acute bleeding episodes, since the readily available factor IX containing prothrombin complex concentrates can also induce allergic reactions in these patients.[6] Recently licensed recombinant factor VIIa appears to be the only logical treatment for acute bleeding episodes in factor IX inhibitor patients with anaphylaxis. Results with immune tolerance induction (ITI) are poor and this treatment is associated with a unique renal complication of nephrotic syndrome in FIX inhibitor patients with concurrent allergy.

Clinical Experience

Unlike in hemophilia A with FVIII inhibitors, a significant number of patients with FIX inhibitors in hemophilia B present with simul-taneous allergic reactions at the time of inhibitor development. The small number of severely affected subjects and even smaller number with factor IX antibodies limit our clinical experience of this unique complication of hemophilia treatment. Currently, there are over 35 patients worldwide who have had both inhibitors and allergy to FIX products.

In the majority of these patients there was a temporal relationship between inhibitor development and anaphylaxis. Other common features of this group include:
1. High titer inhibitors (> 10 BU);
2. Allergy to alternate FIX products when tested;
3. Genotyping demonstrating either deletions or frameshift mutations.

Most of the reactions reported occurred in early life. The median age reported is 12 months with a range of 7 months–12 years. The median number of exposure days prior to inhibitor development is 11 days with a range of 2-50 days. All ethnic groups are affected, and we have seen this problem more commonly with the use of purer factor IX products.

FIX antibodies

Antibodies to FIX are not as well characterized as FVIII antibodies. From what we know they are predominantly IgG4 subclass. They do not fix complement and have equal affinity to both heavy and light chains of FIX. A recent study from Japan has shown the presence of an IgG 1 subclass of antibody in addition to IgG4 at the time of anaphylactic reaction in some of these FIX inhibitor patients.

Management

1. Treatment of acute bleeding.

Treatment of hemophilia B patients with FIX inhibitors is complicated. The readily available product, factor IX, containing prothrombin complex concentrate (PCC), can only be used in patients without concurrent allergy to FIX. rFVIIa appears to be the most appropriate treatment for bleeding episodes in hemophilia B patients with inhibitors and allergy to FIX. The recommended dose is 90-120 µg/kg every 2

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Table 1. Prevalence of factor IX inhibitors. U.S. survey.

Total no. of patients	Yrs 97-98 (Warrier) 97 centers responded 1990	Yr 95 (Katz) 82 centers responded 1967
Severe	573 (30%)	735 (37%)
Moderate	680 (36%)	644 (33%)
Mild	647 (34%)	588 (30%)
Inhibitors	44 (2.3%)	29 (1.5%)
INH + allergy	20/44 (45%)	NA

hours until bleeding stops. The interval between doses is increased once the bleeding is controlled. Several of the patients in our initial report received rFVIIa with good results.[5]

2. Immune tolerance induction (ITI)

Available data indicate minimal success with ITI in hemophilia B patients with inhibitors. In those with concurrent allergy, the success rate is even lower.[5] In addition, nephrotic syndrome as a complication of ITI has been reported in 9 patients with FIX inhibitors and allergy to FIX (Table 2).

Discussion

Until recently, common serious complications following replacement therapy in hemophilia B patients were thrombosis and inhibitor development. However, with the advent of purer FIX products, thrombosis as an adverse event of treatment with FIX seems to have been overcome. But a new, potentially life-threatening complication, i.e., a severe allergic reaction has occurred in 45% of FIX inhibitor patients who have received FIX-containing products (Table 1).

The simultaneous occurrence of allergy with FIX inhibitor development has limited the treatment options further for FIX inhibitor patients. The only logical treatment for those with allergy to FIX appears to be the most recently licensed rFVIIa.

Although immune tolerance induction (ITI) with permanent eradication of inhibitor has been successful in 70-80% of cases of hemophilia A with inhibitors,[7] this is not the case with hemophilia B inhibitor patients. Moreover, the experience with ITI is limited in hemophilia B with FIX inhibitors. Most commonly cited reasons for the limited experience include low prevalence, thrombogenicity of FIX containing PCC, unavailability of high purity FIX products

Table 2. Nephrotic sydrome as a complication of ITI in hemophilia B.

MD/CTR	Patient's age	Gene defect	INH titer (MAX)	Regimen	Allergic reaction	Clinical symptoms	Treatment	Time to resolution	Refs.
Evenstein/ Boston	2	Deletion	67 BU	Mononine, 100 U/kg qd, 9 mo.	Yes (Prior)	Edema, oliguria, proteinuria	Reduction, Prednisone	8 mo.	1,2
Warrier/ Detroit	2	29, R→stop	30 BU	Mononine, 100 U/kg qd, 8 mo.	Yes (Conco) + INH	Edema, proteinuria	Cessation	7 mo.	1
Ettinger/ UMDNJ	5	NA	40 BU	IVIG, Cf, Bebulin 100 U/kg qd, 8 mo.	Yes (After)	Edema, proteinuria, hypocomplementemia (2X)	Reduction, Hydroxyzine	Mild proteinuria still	1,3
Berntorp/ Sweden	11	6460, C→T; 29, R→stop	100 BU	Mononine 100 U/kg, 18 mo.	Yes	Proteinuria	Cessation	2 mo.	PC
Pollman/ Germany	8	Deletion	160 BU	FIX, 100 U/kg bid, 12 mo.	Yes (After)	Edema, Proteinuria	Steroids	?	4
Lenk/ Leipzig, Germany	6	6460, C→T	32 BU	?, 7 mo.	Yes (Prior)	Edema	Cessation, prednisone + CTX cyclosporine	Mild proteinuria	5
Tengborn/ Sweden	8	Deletion	45 BU	Mononine CTX + IVIG, 3 attempts (15 mo.)	Yes (Conco) + INH	Edema	Steroids + DC FIX		8

PC: personal communication; 1. Ewenstein MB, Takemoto C, Warrier I, et al. 1997. Blood; 89:1115., 2. Dharnidbarka VR, Takemoto C, Ewenstein BM, et al. 1998. Pediatr Nephrol 1998; 12:654-7. 3. Constantinescu AR, Weiss LS, Saidi P, et al. 1997. J Ped Hematolo Oncolo; 19:345. 4. Lenk H, Bierbac U, Schille R. 1996. Haemophilia; 2(Suppl 1): 104. 5. Pollman H. 1996. Haemophilia; 2(Suppl 1): 72. Other cases: Brackmann / Bonn, Germany; Petrini/Sweden.

until recently and lack of any defined standard method for ITI.

The success rate with ITI is poor in hemophilia B with inhibitors and occurrence of nephrotic syndrome during ITI in patients with concurrent allergy has further reduced the success with ITI. Nine cases of nephrotic syndrome have been reported so far (Table 2). All were receiving large doses of high purity factor IX products for ITI when they developed nephrotic syndrome. The median duration of ITI prior to the occurrence of nephrotic syndrome is 9 months (ranged 6-15 months).

Renal biopsy results available in one patient showed membranous glomerulonephritis with no histochemical evidence of deposits containing FIX. The response to immunosuppressive treatment was poor in these patients. However, most of the patients responded to either reduction or discontinuation of FIX infusions. This clinical observation clearly suggests a direct causal relationship between nephrotic syndrome and ITI.

Conclusions and Recommendations

In view of the life-threatening nature of anaphylaxis, the following suggestions may be considered when treating children with severe hemophilia B:

1. identify the children at risk by obtaining molecular diagnosis (gene defect) of severe hemophilia B at the time of initial presentation. Those with large deletions or frameshift mutations can then be monitored closely during the early period of treatment at a medical facility equipped to handle life-threatening emergencies;

2. at the time of initial discussion with the family, mention these complications of treatment and counsel the family accordingly;

3. based on the case reports of nephrotic syndrome during ITI in patients with concurrent allergy, ITI should be considered only for those patients without allergy to FIX.

Further studies are required to determine the characteristics of FIX inhibitors especially in respect to anaphylaxis. An international registry to collect data regarding FIX antibodies, occurrence of anaphylaxis, ITI and complications will help us further our knowledge with regards to prevention of treatment-related complications in hemophilia.

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DISCUSSION 15 **Antibodies to FIX**
Warrier I (Detroit, USA)

DI MICHELE: The issue of product on which this problem develops is an important one to clarify because I know that Dr. Katzs' survey did not mention this but on the other hand we've had a much more heightened awareness which might be a secondary effect in terms of the results you have got in your study. There is a patient in Chicago who developed this problem twenty years ago on PCCs and also in the registry there is a patient who developed this problem of an allergic reaction with an inhibitor developing in the pre-ultrapure product era and I would like to know if, in your registry, you know how many and exactly when these inhibitors were detected

vis à vis the products in use at that time?

WARRIER: Most of the young children on that study are on purer products. There are a few older children and the median age was up to twelve with inhibitor development as early as six months. So, we do see a large variation and those twelve year olds are the ones who were treated prior to the development of newer products or ultrapure products. Nevertheless the ITI information is rather new and I don't think very many people put them on ITI because we haven't had a safe product to start them on ITI. I don't think PCC was considered as a choice because of the thrombogenicity and in addition I don't believe that there were many people on ITI.

DI MICHELE: I was referring to your statement

about the fact that most of the allergic antibodies developed with ultrapure products. Part of this may be an artefact of the surveying of pediatric centers because I have a feeling that there are young adults who have developed this phenomenon and I believe it would be interesting to survey our colleagues of the care centers for adults as well.

WARRIER: That's a good point as in actual fact we did only study the pedriatic centers.

POLLMAN: One of these three patients from Germany was one of the first described with protienuria after immune tolerance. We continued the immune tolerance program with hemophilia A with a hundred units per kg body weight twice a day and now he is free of inhibitor and free of proteinuria for more than one year. Hemophilia B anaphylactic reaction is against high purity concentrates and I don't know what to do to start an immune tolerance program with the same scheme with this young patient because his situation is very poor even with VII; he's crippling and I'm quite sure that at the end of the year he will start the immune tolerance program and I do know that there is a risk of proteinuria. I believe he will go on to develop proteinuria but we have a chance to continue the treatment and to have success in this one patient.

POLLMAN: The first patient was treated with plasma-derived factor IX before recombinant factor IX. The second is one of the Benefix study. I think we must discuss what we can do for these patients; treat them with the immunetolerance program or not?

WARRIER: I would suggest you to look for proteinuria as routine screening because currently that is all the information that we have. From what we know about the patients they all responded with reduction or discontinuation of the factor IX infusions. They didn't respond to immunosuppressive therapy as far as we know. The option is to do routine screening and stop immune tolerance as they develop proteinuria.

INGERSLEV: Are you still accepting new submissions?

WARRIER: Yes, we are, at the suggestion of Dr. Mariani and with his help we have a registry to enroll these patients. Recently we have been able to get an announcement in Thrombosis Haemostasis and on their website you will be able to download the form as well as Dr.Mannucci's study which is subsidized by the Genet-

ics Institute. Dr.Mannucci is looking at complement activation at the time of inhibitor development. That protocol is also going to be available at the same site.

ALEDORT: One should not forget when the first prothrombin complexes were put on the market and anaphylaxis was reported in the New England Journal of Medicine on numerous occasions, going into whether they were kininogen production, etc. It's not new that we have seen anaphylaxis with factor IX materials without having an inhibitor. Possibly, we should look for other issues than just this being an inhibitor-related phenomena.

WARRIER: I agree with you.

BERNTORP: Your cases with hemophilia B and allergy have become anergic by giving them small repeated doses of subcutaneous factor IX. Do you have any such reports in your study that could perhaps provide a way for Dr.Pollman to proceed in the treatment of his patient?

WARRIER: There were other patients who had this desensitization done. I think that these were the only three patients in our registry who had desensitization done subcutaneously. A very small number, and it was not done subcutaneously but intravenously. We gave very small increasing doses of factor IX and desensitized them.

EWENSTEIN: I have two comments to make. Firstly, if you are going to consider ITI, we have to desensitize the patient in the old fashioned allergic sense of the term by giving very small, in our case intravenous, doses over several days before daring to give larger doses because of the allergic manifestations. We need to build up to it very slowly. We treated our patient in an ICU setting because the allergic manifestations were quite severe. Secondly, the complexity of all this is that in the case of our patient we are pretty certain that we had an IgE-mediated response. We did RAST testing and feel confident in those results but other scientists have been unable to document IgE in other patients and think that it may be an IgG-mediated phenomenon. So I think there are still a lot of unknown things here and I would be very cautious in the early phases of ITI. I would also recommend to treaters of these patients to consider skin testing because that appears to be a relatively sensitive way of detecting early allergies.

Inhibition of CD40 ligand (CD154) in the treatment of factor VIII inhibitors

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Abstract

The development of persistent, high titer inhibitors represents a serious complication of the treatment of patients with severe hemophilia A. Elimination of these inhibitory antibodies is usually attempted through repeated administration of high doses of factor VIII. Such regimens are costly, time-consuming and often fail when the inhibitor is of very high titer or of longstanding duration. A potential alternative approach to inhibit the production of anti-factor VIII antibodies is blockade of the T-cell/B-cell collaboration that is required to generate humoral responses. One cognate receptor pair that is required for T-cell-dependent B-cell activation consists of CD40, which is expressed on B-lympho-cytes and other antigen presenting cells, and CD40 ligand (CD40L, CD154), which is transiently expressed on activated T-cells. To determine whether blockade of the CD40-CD40L pathway can inhibit the production of anti-factor VIII antibodies, a clinical study has been designed in which patients with hemophilia A and a high titer inhibitor (> 10 BU) receive monthly exposures to factor VIII in the presence of a humanized mouse monoclonal antibody to human CD40L (hu5c8*). Subjects must be between the ages of 5 and 60 years old and be HIV seronegative. To date, three subjects have received at least three doses of hu5c8 at the initial protocol dose of 10 mg/kg. Preliminary results suggest that anti-CD40L inhibition may be effective in blocking anamnestic responses to factor VIII in some patients. It remains to be determined whether this effect will persist and whether patients may eventually become tolerant to factor VIII in the absence of hu5c8 co-administration.
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Key words: hemophilia, factor VIII, inhibitors, CD40 ligand, immune tolerance, CD154

*Hu5c8 is being developed under the trademark name ANTOVA at Biogen, Inc., Cambridge, Massachusetts.

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Replacement of factor VIII with high purity or recombinant factor VIII concentrates represents the mainstay of the treatment of hemophilia. This treatment is associated with the development of inhibitory IgG antibodies in up to 40% of patients with severe hemophilia A (factor VIII <1%).[1] Approximately one half of these inhibitors are both persistent and of high titer (> 5-10 Bethesda units [BU]), rendering the further use of factor VIII replacement impractical. Bleeding episodes in patients who develop inhibitors must be treated with other agents that "bypass" the need for factor VIII in the generation of thrombin.[2] Because these agents are both expensive and generally less effective than factor VIII concentrates, the development of a high titer inhibitor is associated with an inevitable increase in morbidity and resource utilization.[3] It is thus highly desirable to characterize the immune response to factor VIII more fully and to devise therapeutic strategies capable of abrogating this response.

Immune responses to factor VIII

Mutations in the factor VIII gene that result in a major loss of coding sequence (multi-domain deletions, nonsense mutations) are associated with the highest incidence of inhibitor formation.[4] This is not surprising since these mutations are also associated with a lack of detectable factor VIII protein. Natural tolerance, which may be defined as the abrogation of the immune response to non-pathogenic or self-antigens, begins prenatally and must be maintained during adult life. Theory predicts that patients expressing mutations that result in the total absence of factor VIII would be more likely not to become tolerized to the protein and therefore be at greatest risk for the development of factor VIII inhibitors. However, not all patients who lack detectable factor VIII develop inhibitors. Other determinants, such as the HLA class II phenotype[1,5,6] and the type of factor VIII concentrate to which the patient is

exposed,[7-9] have also been shown to be determinants of inhibitor development.

There is a growing body of evidence in support of the concept that the humoral response to factor VIII is T-cell-dependent, as with most protein antigens. Findings to date include the serendipitous discovery of an association of a factor VIII-derived peptide with purified human DR molecules[10] and the demonstration of T-lymphocyte proliferative responses to rFVIII in patients with hemophilia A and inhibitors.[11] More recently, results obtained in a murine model of hemophilia A demonstrated that blockade of the B7/CD28 co-stimulatory pathway can prevent or diminish the development of anti-factor VIII antibodies.[12] The results of these experiments are also consistent with the clinical observation that longstanding factor VIII inhibitors often disappear in HIV-infected patients concurrent with an overall decline of T-cell immune function.[13] The inhibitory antibodies to factor VIII are predominantly of the IgG4 and, to a lesser extent, IgG1 subclasses,[14,15] further suggesting the involvement of both Th1 and Th2 subsets of CD4+ T-cells.

Induction of immune tolerance

The elimination of inhibitory antibodies to factor VIII has often been successfully accomplished through a variety of treatment protocols collectively termed induction of immune tolerance (ITI), which typically involves the daily infusion of large doses of factor VIII for periods as long as 18-24 months. In some instances, immune modulating agents and procedures are also employed. The cellular mechanism(s) responsible for the success of ITI using these largely empiric approaches have not been elucidated, but may involve the induction of anergy or active suppression as has been reported in other tolerance regimes.[16] The majority of protocols used to date demonstrate similar efficacy (63-83%) but differ appreciably with respect to dose and the time required for success.[17] Reports from three large registries with pooled data from multiple centers and a variety of ITI regimens have identified high historical peak titers, longstanding inhibitors and inhibitor titers greater than 10 BU at the initiation of ITI therapy as significant negative predictors of eventual success.[17,18] Thus, current ITI protocols are particularly likely to fail in the subset of patients with very high titer inhibitors that remain elevated (≥ 10 BU) despite prolonged intervals without exposure to factor VIII.

Role of CD40/CD40L in the humoral response

The interaction of activated T-cells with B-cells is a required component in the humoral response to most protein antigens. This B-cell/T-cell interaction is mediated through several receptor-ligand binding signal events. Prominent among these are CD40, which is expressed on B-lymphocytes and other antigen presenting cells, and CD40 ligand (CD40L, CD154), which is transiently expressed on activated T-cells. CD40 is a 50 kD protein that belongs to the tumor necrosis factor (TNF)-α receptor family of cell surface receptors that have been shown to mediate both cell activation and programmed cell death. CD40L is a 32 kD type II membrane protein with significant sequence homology to TNF.[19] In addition to lymphoid cells, CD40 has also been identified on a variety of non-lymphoid cell types, including fibroblasts, endothelial cells and renal tubular epithelial cells, while CD40L has been identified on both macrophages and the cell surface of activated platelets.

Interactions between CD40 and CD40L have been shown to be essential for the full development of humoral responses.[20-23] B-lymphocytes and other antigen presenting cells (APC) display antigen in the form of MHC-peptide complexes to T-lymphocytes which are thus stimulated to upregulate CD40L on their cell surface (Figure 1). Engagement of CD40L by CD40 then stimulates the B-cell in a variety of ways, including proliferation and differentiation, immunoglobulin class switch, and upregulation of CD80 (B7-1) and CD86 (B7-2). Engagement of CD80 and CD86 by CD28 induced on the T-cell further amplifies the T-cell response. T-cells lacking CD40L are unable to activate antigen presenting cells resulting in the failure to produce IgG, as well as IgA and IgE, responses.[24,25] Importantly, in the absence of co-stimulation, antigen-specific CD4+ T-cells may be anergized or selectively deleted by apoptosis, resulting in a durable state of tolerance.[26]

Study Design

Methodology

The study is being conducted as an open-label, multi-center, multiple-dose trial to evaluate the safety and efficacy of hu5c8 when given approximately two days prior to an infusion of factor VIII to stable hemophilia A subjects with high-titer factor VIII inhibitors. Fifteen to twenty hemodynamically stable subjects, aged 5-60 years old, with inhibitor titers of greater than 10 BU will be enrolled at five or more treatment centers. On day 1 of the 29-day treatment cycle, hu5c8 is administered at a dose of 10 mg/kg. Forty to sixty hours later, recombinant factor VIII (Recombinate™ [Hyland Immuno, Baxter Healthcare Corp], 100 U/kg) is infused. The
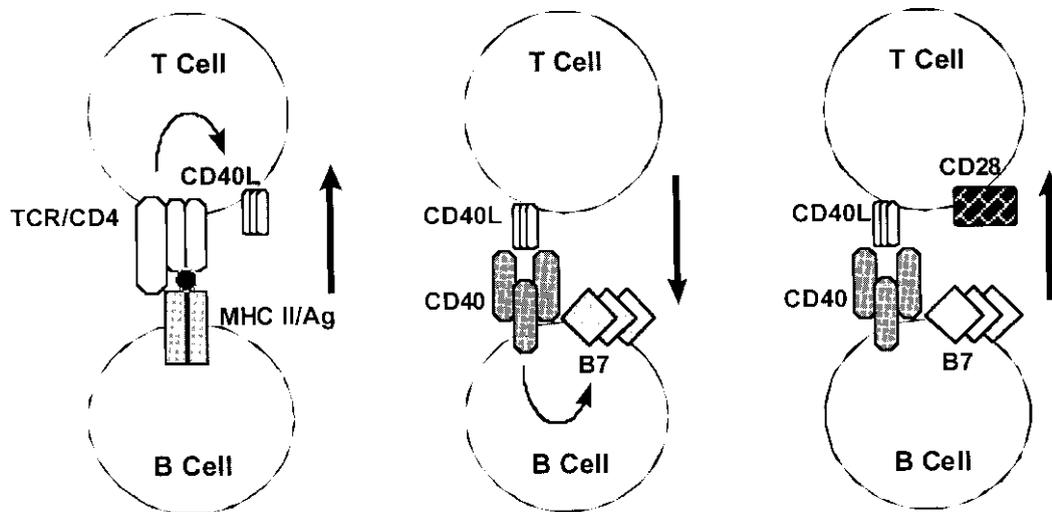
Figure 1. Pathways of B-Cell/T-Cell co-stimulation. Interaction of the T-cell receptor (TCR) with the antigen-MHC class II complex on the antigen presenting cell (APC) stimulates the expression of CD40L on the responding T-cell. Engagement of CD40 on the cognate B-cell causes the upregulation of B7-1 (CD80) and B7-2 (CD86). B-cells reciprocally trigger the T-cells via B7/CD28 signaling. In peripheral blood, both activated B-cells and monocytes can act as APCs. *Adapted from Foy et al., 1996.*

occurrence of an anamnestic response is ascertained at day 12 of each cycle and is defined as ≥ 25% increase in the inhibitor level from the pre-dose titer, measured at the initiation of the study or during that treatment cycle, whichever is lower. Subjects who show evidence of an anamnestic response on day 12 of the first, second or third cycles will receive hu5c8 at 20 mg/kg for the remainder of the study. All subjects not experiencing a serious adverse event will receive at least four treatment cycles of study drug and factor VIII concentrate. Intercurrent bleeding episodes may be treated with any of several prothrombin complex concentrates (PCC), activated PCC or recombinant factor VIIa, at the investigator's discretion.

Objectives

The primary objective of the study is to determine whether, in hemophilia A subjects with inhibitors levels >10 BU, administration of hu5c8 will decrease the inhibitor level by 50% from baseline. The safety and pharmacokinetics of hu5c8 in this patient population will also be determined. Additional secondary objectives, applicable to the subset of subjects whose baseline inhibitor levels are <50% of the historical peak, are to determine whether the contemporaneous administration of hu5c8 prevents an anamnestic response. Anamnestic responses are to be alternatively defined as a greater than 25% increase from study baseline or less than 10% of

the difference between the historical maximum and baseline inhibitor titers.

Inclusion and exclusion criteria

To be eligible for the study, subjects must have congenital severe hemophilia A with a current factor VIII inhibitor titer of >10 BU, must be between the ages of 5 and 60 years old and must be seronegative for HIV. Patients over the age of 12 who are seropositive for hepatitis C virus are eligible so long as they are clinically well and serum ALT and AST levels are less than three times the upper limit of normal. Patients who have previously failed ITI regimens are eligible. Principle exclusion criteria include: any clinically significant abnormality not related directly to hemophilia A and its complications, renal insufficiency (creatinine > 2 mg/dL), a CD4+ lymphocyte count of < 250/mm³, and a serious infection or treatment with any immunosuppressive drug within four weeks prior to the first dose of hu5c8.

Study results

To date, four patients have been enrolled in the study, three of whom have completed at least two treatment cycles (Table 1). All treatments were well tolerated. Patient #1 is a 53-year old man with a factor VIII inhibitor of more than 12 years' duration and no prior attempts at ITI. He had an inhibitor titer of 108 BU on day 1 of the study, which was somewhat higher than

Table 1. Hu5c8 factor VIII inhibitor study subjects.

Pt.	Age, sex (yrs)	Historical max. titer	Previous duration (yrs)	Baseline titer	Cycle
1	53, M	80 BU	>12	91 BU	4
2	12, M	188 BU	10	116 BU	3
3	13, M	206 BU	6, ITI (2)	18 BU	3
4	46, M	82 BU	<1	33 BU	1

Baseline titer defined as the mean of the factor VIII inhibitor titers determined on day −14 (± 7), on day −3 (±2) and immediately prior to the first dose of hu5c8.

his previously recorded historical maximum titer of 80 BU. The titer level remained essentially unchanged throughout the first two cycles of treatment, then declined to a minimum of 50 BU on day 1 of cycle 4. On day 12 of cycle 4, the titer rose modestly to 69 BU. Patient #2 is a 12-year old boy with an inhibitor of 10 years' duration and no prior attempt at ITI who has completed two cycles of hu5c8 treatment. He had an inhibitor titer of 139 BU on day 1 of the study compared to a historical maximum of 188 BU. On day 1 of cycle 3, his inhibitor titer was 200 BU; the dose of hu5c8 will be increased to 20 mg/kg for each of the next two cycles. Patient #3 is a 13-year old boy with an inhibitor of 6 years' duration who had failed two previous attempts of ITI. The historical maximum factor VIII inhibitor titer was 206 BU. He had a titer of 8 BU on day 3 of cycle 1, which remained essentially unchanged throughout three treatment cycles with hu5c8 at a dose of 10 mg/kg. Patient #4 is a 46-year old man with a high titer inhibitor of short duration who has just begun cycle 1. None of the patients has experienced any serious adverse events while on study.

Summary and conclusions

Inhibition of the CD40/CD40L co-stimulatory pathway provides a tempting novel approach to the management of hemophilia A patients with high titer inhibitors to factor VIII. Preliminary results in three subjects suggest that hu5c8 at a dose of 10 mg/kg may be effective in blocking anamnestic responses to factor VIII in some patients. Hu5c8 is currently also being investigated in several clinical trials of autoimmune disorders, including chronic immune thrombocytopenia purpura, lupus nephritis and multiple sclerosis. It remains to be determined whether higher doses of hu5c8 yield improved results in the present study of patients with a well-established, alloimmune humoral response. It also remains to be determined whether the early effects observed to date will persist and whether patients may eventually become tolerant to factor VIII in the absence of hu5c8 co-administration. Further studies on the use of hu5c8 in hemophilia B patients with inhibitors and in patients with acquired hemophilia A are also warranted.

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The Maintenence of Tolerance after Successful Immune Tolerance Induction in Hemophilia A and B: The North American Registry

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Abstract

The North American Immune Tolerance Registry (NAITR) was initiated with the goal of determining, by questionnaire, immune tolerance (ITT) practices in hemophilia treatment centers in Canada and the United States. Sixty-eight centers (40%) responded. Of the 130 registry subjects with hemophilia A who completed ITT, 93 (72%) achieved tolerance. Of the 11 completed ITT courses in patients with hemophilia B, 4 (36%) were successful. Maintenance therapy was defined as any clotting factor regimen administered subsequent to the patient achieving the treating physician's criteria for successful immune tolerance. Seventy-five (81%) of 93 individuals in the hemophilia A cohort who successfully achieved tolerance were maintained on a regular (prophylactic factor VIII (FVIII) regimen for a variable time period post-ITT. The median dose used was 150 units/kg/week (range: 17-700). Forty-eight (64%) subjects remained tolerant while receiving regular doses of FVIII for a median observation period of 13 months (range 0-129 months). Of 27 patients whose maintenance therapy had been stopped, 17 (68%) remained tolerant over a median period of 19 months (range 1-54 months) and 9 relapsed. Among the relapses, 3 occurred after maintenance therapy was stopped; 6 were noted on prophylactic FVIII at a median time of 11 months (range 2-61 months). The definition of tolerance was reviewed for the 9 subjects who relapsed and was defined by a normal recovery and survival in only 1/9 patients. Among the 11 hemophilia B subjects in the cohort who completed tolerance, 4 had a successful outcome. Four individuals were placed on maintenance regimens of 25-100 units FIX/kg/day and all remained tolerant.
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Key Words: hemophilia A, hemophilia B, immune tolerance, inhibitors

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The North American Immune Tolerance Registry (NAITR) was a project of the ISTH Factor VIII/IX Subcommittee conceived in 1992 by Dr. Carol Kasper. The NAITR was initiated with the goal of further determining immune tolerance (ITT) practices in Canada and the United States with respect to the identification of 1) therapeutic regimens in use; 2) therapeutic outcomes; 3) predictors of successful outcome, 4) complications of therapy and 5) the maintenance of successful tolerance over time. We now report the data accumulated from the NAITR between March 1993 and August 1997 with respect to maintenance of tolerance after successful induction in hemophilia A and B inhibitor patients.

Design and Methods

Data collection

Data collection forms were initially sent in March 1993 to 168 hemophilia treatment centers (HTCs) in the United States (US) and Canada. Data updates were requested from participating HTCs in March 1994 and April 1996. The last update on the data set was performed in August 1997 to include several additional participating centers. Data forms were completed by the HTC medical directors or hemophilia nursing staff. The collection tool was intended to study the following parameters: 1) the frequency of past and current use of ITT among hemophilia A and B patients with inhibitors; 2) the therapeutic regimens in use including treatment product purity, dosing and duration of therapy; 3) the clinical outcome (success or failure) relative to the following aspects of treatment: a) duration of ITT; b) interval between inhibitor diagnosis and initiation of ITT; c) age at ITT induction; d) HIV status; e) race; f) and inhibitor titers including i) historical pre-ITT peak; ii) titer immediately prior to ITT induction (pre-induction titer) and iii) peak titer on ITT. Data were also collected on dosing and product regimens

used for maintenance of tolerance following successful ITT, as well as the access and therapeutic complications incurred while on ITT.

Definition of variables

ITT outcome was categorized as a success, a failure, or indeterminate because of ongoing therapy. The NAITR permitted the criteria for success and failure to be determined by the individual respondents, but these criteria were all recorded to document the current standards of ITT practices in North America. The ITT was categorized as ongoing if the enrollee had not yet achieved the criteria of success established by the individual center and was continuing to receive ITT doses of clotting factor. Maintenance therapy was defined as any clotting factor regimen administered subsequent to the patient achieving the treating physician's criteria for successful immune tolerance.

Results

Inhibitor demographics/frequency of ITT

A total of 68 centers, 40% of those polled, submitted data to the NAITR. The data represented 5,000 (43%) of the known North American individuals with hemophilia A and 1,325 (39%) with hemophilia B followed at the recognized HTCs. Included were 2,489 (43%) of the estimated severe hemophilia A patients and 473 (35%) of the estimated severe hemophilia B population.[1]

Outcome data

Of the 130 registry subjects with hemophilia A who completed ITT, 93 (72%) successfully achieved tolerance. Of the 11 completed ITT courses in individuals with hemophilia B, only 4 (36%) were successful.

Success was defined by criteria established at each participating center. The definition of success included a negative inhibitor titer (≤ 1 BU) in 90/97 (93%) of hemophilia A and B cases. Success was further defined by a normal recovery of $\geq 70\%$ of predicted in 59/97 (61%) of individuals. Only 29/97 (30%) of the registry subjects had a successful immune tolerance determination that included a negative inhibitor titer as well as both a normal recovery, and factor survival (≥ 12 hours). The criterion for successful ITT in four patients was conversion from high to low responding inhibitor status.

Maintenance of tolerance

Hemophilia A

Seventy-five (81%) of the 93 individuals in the hemophilia A cohort who successfully achieved immune tolerance were maintained on a regular (prophylactic) regimen of factor VIII (FVIII) infu-

sion for a variable period of time post-IT induction. The median dose used for maintenance therapy was 150 units/kg/week (range: 17-700) administered in variable dosing regimens. At the time of the last data analysis, 48 (64%) subjects remained tolerant while continuing to receive regular doses of FVIII for a median observation period of 13 months (range 0-129 months). Of the 27 patients whose maintenance therapy had been stopped, the status of 1 patient was unknown, 17 (68%) remained tolerant over a median observation period of 19 months (range 1-54 months), and 9 had relapsed. Among the relapses, 3 occurred at between 1 and 8 months after maintenance therapy was stopped, and 6 were noted during maintenance therapy at a median time of 11 months (range 2-61 months). Due to the questionnaire design, no outcome data were obtained on the 18/93 patients who were never maintained on a FVIII regimen after successful tolerance was induced.

The definition of tolerance was reviewed for those 9 subjects who relapsed on maintenance treatment. Tolerance had been defined by a normal recovery and survival in only 1/9 patients and by a normal recovery alone in 4/9 patients. Three relapses had a negative Bethesda titer as the sole determinant of successful tolerance, and one success had been initially defined as a conversion from high to low responder status (Table 1).

Hemophilia B

Among the 11 hemophilia B subjects in the cohort who completed the tolerance induction protocol, 4 had a successful outcome using factor IX therapeutic dosing regimens of between 43 and 200 units/kg/day. This included one patient with an inhibitor – associated allergic phenotype. Once tolerance was induced, all 4 individuals were placed on maintenance regimens of 25-100 units/kg/day of factor IX. At the time of the last data analysis in 1997, maintenance was ongoing for all subjects and 4/4 remained tolerant.

Table 1. NAITR maintenance of tolerance. Comparison of total/relapse cohorts.

Definition	HA/HB ITT Successes* (n = 94)	HA/Relapse (n = 9)
≤ 1 BU alone	31 (33%)	3 (33%)
≤ 1 BU / Rec $\geq 70\%$ expected	30 (32%)	4 (44%)
≤ 1 BU / Normal Rec / $T^{1/2}$	29 (31%)	1 (11%)
Conversion to LR	4 (4%)	1 (11%)

*Information not available for 3/97 patients.

Discussion

The North American Immune Tolerance Registry represents the second retrospective characterization of a large cohort of hemophilia A inhibitor patients who underwent immune tolerance through August 1997.[2] It was performed in an effort to further understand both the outcome and outcome predictors of ITT as practised in North America. The study differs from its predecessor, the International Immune Tolerance Registry previously reported by Mariani et al. in 1994[3] in that 1) hemophilia B inhibitor patients were included; 2) potential outcome predictors such as race and peak inhibitor titer on ITT were also studied; 3) inhibitor patients still undergoing tolerance were also characterized in an effort to study trends in ITT over time; 4) complications related to clotting factor administration and frequent venous access were recorded and 5) the maintenance of tolerance after successful therapy was documented.

In this paper, the maintenance of tolerance in successfully induced hemophilia A and B patients was reviewed. The results suggest that over a significant period of observation, tolerance, once achieved, was maintained in the majority of both hemophilia A and B patients studied. However, the weekly doses of clotting factor received by successfully tolerized patients with hemophilia A and B varied widely. In the small hemophilia B cohort, all four patients were maintained on regular (prophylactic) factor IX infusions following tolerance achievement. This was not the case, however, in the hemophilia A cohort. In these patients, it was therefore possible to study the maintenance of tolerance both on and off regular FVIII infusions. Interestingly, tolerance was maintained equally well over similar median observation periods, whether or not a prophylactic FVIII regimen was continued after its initiation. However, the important question of whether or not tolerance is maintained when patients are immediately switched to an "on demand" factor regimen after successful induction, could not be answered by this study.

The small number of hemophilia A patients with inhibitor recurrence upon either the reduction of regular FVIII dosing or complete cessation of the prophylactic regimen, presented an opportunity to examine the impact of the definition of successful ITT on long-term tolerance. The most stringent definition of success (normal recovery and survival) was underrepresented (11% vs. 31%) and conversion to low responder status was overrepresented (11 vs. 4%) in the relapse cohort when compared to the entire group of hemophilia A and B patients undergoing ITT (Table 1). However, given the small number of patients who relapsed, no significant inverse correlation between the definition of success and the maintenance of successful tolerance could be definitively ascertained.

Although useful, the retrospective study of maintenance of tolerance in cohorts of immune tolerance patients is confounded by the limited ability to ensure adequate reporting and conduct ongoing long-term follow-up. However, this important question will be further addressed prospectively in the hemophilia A inhibitor population undergoing ITT through the International Immune Tolerance Study due to begin early in the year 2000.

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DISCUSSION 5   **The maintenance of tolerance after successful ITT induction in hemophilia A and B: the North American Registry**
D. Di Michele, B. Kroner and the Factor VIII/IX Subcommittee of the International Society for Thrombosis and Haemostasis (New York, NY and Rockville, MD, USA)

ALEDORT: Dr. Di Michele my first comment is with respect to your definition of success. Does it really compare to the data shown by Dr. Mariani or the German registry because you used a very different definition, in that success in the other two registries was defined unequivocally as a normal recovery and a normal half life? My second comment relates to your showing that although you didn't reach significance in the difference between the recombinant and mono-

clonal you implied that the differences would go away rather than the fact that they might expand. I think that since you showed us that all the characteristics that the other registries showed as benefits to success were all in the opposite direction specifically if it is more likely that the monoclonals will be better than the recombinant rather than the reverse. Therefore, I wasn't sure that I understood your final comment that more people will use recombinant for immune tolerance if the data don't support that. I think it would be folly because it's a lot more expensive. I'd like to understand better your logic in that particular situation.

DI MICHELE: In terms of the definition issues that has always been an important difference between the registries, iIt's interesting that when we look at the same predictors of success and non-success, despite the fact that definitions of success were quite variable in the North American Registry, I think it is amazing that a lot of the same success parameters and outcome parameters continued to hold up regardless of that definition. I can't comment any further on that because the definitions are the definitions. With respect to your comments on the possible differences between recombinant and monoclonal concentrates I think that this difference might go away as the data are few and the P Value is 0.04. This was significant but now it has only borderline significance. Therefore it is possible that as we get more data and the numbers become real, this difference may disappear. So, I don't know which way it is going to go and I think the important thing is that you can't over-interpret these preliminary data. Consequently, I don't think people should be making choices of product based on the preliminary data.

HULMAN. You found a poor difference between the recombinant and the monoclonal products. Are you quite sure that the dosage was the same in these two groups? The German Registry is very old. We started therapy following the Bonn protocol twenty five years ago and that is an era without any recombinant products. We started this with intermediate products and the success at that time was about eighty percent as Dr.Lenk has shown. Are you sure we could do better with monoclonal products?

DI MICHELE: No, I'm not sure. I'm raising this as an important issue because in the past we've discussed product with respect to purity. We have not looked at products of similar purity and overall success rates. I think it is very important that we continue looking at this issue in order to become more sure about issues such as outcome prediction which might be connected to treatment with recombinant products. My opinion is that the biggest factor which

affected the recombinant success rate was the fact that most patients started at titers that were greater than ten. If I had to predict that there was going to be one variable that affected that group of patients I would have to say that such a parameter probably did. It's important to look at this. We have the opportunity to look at this in the prospective studies that are going on in Germany. It is also important to look at all the PUP data and to look at the true outcome of immune tolerance with recombinant products. If we look at some of the data that were presented by Dr.Lusher and the Refacto data and the Kogenate data the successes on recombinant were 45% and 60% which is not very different from the 55% that I reported. I don't think we can get a clear idea about monoclonal unless we have those data coming from somewhere else. I just raised it as an interesting issue and I didn't wish to make any definitive statements.

MARIANI: I have a comment and a question for you. Why did you try to analyze the difference between monoclonal and recombinant? I'd like to know what was behind this decision because they are very similar in terms of unitage per milliliter and purity. I don't think that this could lead to anything in terms of likelihood of success. In addition, I would like to point out that many of the cells in the tables with regard to your statistical analyses contain are very small numbers. Therefore I would be very cautious in drawing any conclusions. People might get confused. It is probably better not to create many groups but rather to understand the major differences unless numbers allow a complete and detailed statistical analysis.

DI MICHELE: I think your points on the analysis are well founded. Nevertheless, the reason we looked at this was because the registry gave us the opportunity to look at this. The data are there and to my knowledge they haven't been previously examined. You wanted to know if we will get the answer to that question. As a result of small overall numbers in the North American Registry I can't be sure yet. If you look at it, the population is small but its sizeable because there are forty patients in each group and they are numerically well matched. Therefore, I think that even though a more detailed analysis is required I don't see any reason not to see this as a variable.

LENK: I think in the case of our patients it would be very difficult to answer this question because most patients are treated with the same product. I think it's quite difficult with relatively small numbers to give an answer as to whether a product is better than another product.

CIAVARELLA: I think in America they only use monoclonal and recombinant products while in

Italy and in Europe we use intermediate products or other chromatographic concentrates. I would like to know your opinion about the possibility that other plasma derived products could be more successful than the recombinant ones; for example, some people said that the presence of VWF containing products correlated with factor VIII might be more successful than the other products. I think this is a very important point.

DI MICHELE: Americans are very "state-of-the-art", but indeed the higher purity products have become the standard of care in the United States. This is certainly true after 1990 and you have to remember that in the North American Registry 75% of the tolerance induction procedures were done after 1990: those were the products that were available and that was the standard of care at that time. In terms of the product purity issue I can only really comment on the data from the registry with regard to the high predisposition of high purity product usage in the registry versus what Dr.Mariani presented in his registry which is just the opposite. We can't answer that question through the registries and maybe others who are looking at this issue such as Dr. Kreutz might have more to comment. I'm afraid that I can't comment from the data that I have.

ALEDORT: I disagree with Prof.Mariani in saying that it isn't so important to know whether or not monoclonals differ from recombinant. Firstly, we don't have enough data to tell us if you aren't induced to have an inhibitor with one kind of product and whether you're better of getting tolerized with a different product. We don't have enough data yet. On the other hand to assume that a recombinant product is identical because of its purity to a human derived product is not correct. They are different molecules and so they are not identical. There may very well be a difference whether it's high purity, intermediate or low. We don't know the answer to that as Dr.Di Michele has already said. Understanding the possible differences between human versus recombinant is, in my opinion, very important. This is also important in terms of cost. Immune tolerance is extremely expensive. The costs would vary substantially if you use a human-derived intermediate cheap product or an expen-

sive recombinant.

MARIANI: I didn't say that they are the same thing. I said that the contaminants and the other components are very little. The difference in terms of purity is negligible because both of them have a purity of more than 1000 units per milliliter and consequently in these terms they are very similar. I do hope that the molecules are not so different.

BERNTORP: Dr. Lenk presented success rate data from different large centers. Have you also done that Dr.Di Michele for your registry? It could perhaps explain the difference in concentrate policy.

DI MICHELE: No, I haven't.

BERNTORP: Did you switch to recombinant products all over the US for immune tolerance at the same time or did small centers keep using monoclonal products for a long time thus leading to a lower success rate for other reasons?

DI MICHELE: That's a good question but I don't have the answer. I didn't analyze it *vis à vis* large centers versus small centers. It's going to be a very difficult analysis because of the fact that hemophilia care in the US is very decentralized when you compare it to many of the countries in Europe. When I sent out this questionnaire to the US and Canada, I sent out a hundred and sixty questionnaires to centers that were certified by the Canadian and American National Haemophilia Foundations as hemophilia treatment centers.

BERNTORP: And so, perhaps you should reorganize your hemophilia care.

DI MICHELE: That's beyond the scope of this conference.

SCANDELLA: Could you comment on the definition of an interruption in therapy. It was mentioned by Dr.Lenk that it was something that was not longer than one to two days. Do you have information about that?

LENK: We had some patients who started therapy with very low dosage and the therapy was unsuccessful. After a certain time a high dosage therapy was given. In other patients they stopped for months because of compliance problems.

The German Registry of immune tolerance treatment in Hemophilia – 1999 update

H. Lenk* and the ITT Study Group°

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Abstract

As of 1999, the German registry of immune tolerance treatment in hemophilia has received reports on 146 patients who have undergone this therapy from 25 hemophilia centers. In 16 of the reported patients treatment is ongoing. Therapy has been completed in 126 patients of all groups with hemophilia A; most of them are children. In 78.6% of hemophilia A patients full success was achieved, 8.7% finished with partial success, and in 12.7% ITT failed. Statistical analysis demonstrates that interruptions of therapy have a negative influence on success. The inhibitor titer has the highest predictive value for success or failure of therapy. A high maximum titer as well as a high titer at start of treatment were related to a low success rate. Other variables such as exposure days and time interval between inhibitor detection and start of ITT were not statistically significant. Four patients with hemophilia B have also completed therapy, only one of them with success.
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Key words: factor VIII and IX inhibitors; ITT registries; ITT treatment

The German immune tolerance registry was established in 1993. As a result of the participation of 25 treatment centers in the immune tolerance treatment (ITT) study group the number of patients is increasing continuously. In the last two years alone, 28 new cases were reported. The registry includes all inhibitor patients who have been or are being treated in Germany since the introduction of this method 25 years ago. The registry should give the opportunity to overview the rate of eradicating inhibitors as well as the factors influencing success or failure.

Patients

As of October 1999, 146 patients with hemophilia and inhibitors to factor VIII or IX have

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been reported to the registry. All have undergone immune tolerance treatment. In most patients ITT was completed. There are only 16 patients, 14 of them with hemophilia A and two with hemophilia B, in whom treatment is still ongoing. The registry will receive reports from the centers about the further course of ITT in these patients. Out of the 140 patients with hemophilia A, 23 were low and 117 were high responders. The distribution of the patients according to their maximum titers is shown in Figure 1. The mean age of the patients at diagnosis of the inhibitor was 11 years and at start of ITT it was 12.9 years. In our survey two years ago, in 1997, the mean age at diagnosis of the inhibitor had been 11.9 years and that for starting treatment 14.1 years. These mean ages are decreasing continuously because most inhibitors nowadays are discovered and treated in young children.

Results

One hundred and twenty-six patients with hemophilia A and 4 with hemophilia B have completed ITT. ITT was successful in only one of the 4 hemophilia B patients. As shown in Table 1, hemophilia A patients who complete treatment can be divided into three groups according to treatment results. Out of 126 patients 99 (78.6%) belong to group 1, in which full treatment success was achieved, and 11 patients (8.7%) belong to group 2. From the clinical point of view patients in group 2 demonstrate a satisfactory improvement but they have a borderline inhibitor and/or recovery and a half life that is not completely normal. Since completion of ITT all these patients have received continuous prophylactic FVIII treatment, their inhibitor remains permanently below 2 BU and bleeding episodes are very rare. Sixteen patients (12.7%) belong to group 3, in which ITT failed completely. In 8 of these patients therapy was stopped because of lack of success, but in two
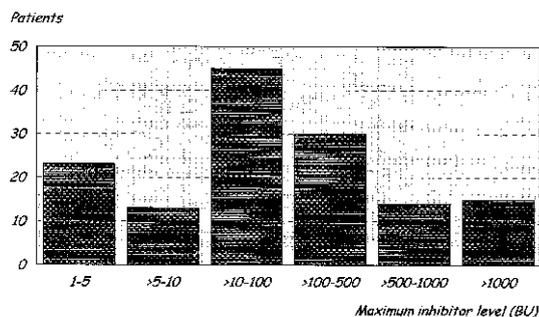
Figure 1. Maximum inhibitor level in hemophilia A (n=140).

Table 1. Outcome of ITT in 126 patients with hemophilia A.

Results	All pts (n=126)	low responders (≤5 BU, n=22)	high responders (>5 BU, n=104)
Therapy completed, normal recovery and FVIII half-life	99 (78.6%)	20 (91%)	79 (76%)
Therapy completed, inhibitor titer permanently < 2 BU and/or low recovery and shortened FVIII half-life	11 (8.7%)	1 (4.5%)	10 (9.6%)
Therapy without success (interrupted due to lack of compliance or death during therapy included)	16 (12.7%)	1 (4.5%)	15 (14.4%)

therapy was stopped because of insufficient compliance and in two others because of additional diseases. Four patients died during therapy. The mean duration of ITT was one year and 3 months in group 1, it was 5 2/12 years in group 2 and 2 4/12 years in group 3. The influence of different conditions and variables was checked by statistical evaluation. The parameters were: the maximum inhibitor level in general and during ITT, the influence of the time interval and of exposure days between detection of the inhibitor and start of treatment, dosage of FVIII, APCC administration, the age of patients and interruptions of therapy. We also evaluated the few patients with inhibitor eradication in moderate and mild hemophilia to see whether the outcome of ITT was different in this subset of patients (Table 2). The results of the evaluation of 126 patients do not differ very much

Figure 2. Variables that affect the outcome of immune tolerance therapy (classification 1->2+3). Logistic regression analysis, backward variable selection (n=121).

Table 2. Outcome of ITT in mild, moderate and severe hemophilia A.

| Classification of success | HEMOPHILIA | | |
	severe n=109	moderate n=13	mild n=3
Full success (1)	87 (80%)	10 (77%)	1
Partial success (2)	7 (6%)	2 (15%)	2
Failure (3)	15 (14%)	1 (8%)	

Table 3. Outcome of ITT at different inhibitor levels.

Maximum inhibitor level (BU)	n	Full success (1)	Partial success (2)	failure (3)
<5	22	20	1	1
5-10	12	12		
>10-100	37	31	4	2
>100-500	27	20	4	3
>500-1000	13	8	2	3
>1000	15	8		7
0.6-100	71	63	5	3
>100	55	36	6	13

from those reported two years ago concerning 100 patients. The inhibitor titers had an overwhelming importance for success (Table 3). The other variables included in the regression analysis with backward variable selection had only low significance or did not reach statistical significance (Figure 2). Figure 3 shows the success rate in patients starting ITT at different intervals after detection of the inhibitor. The 27 patients who started immediately had nearly the same outcome as the group starting after more than
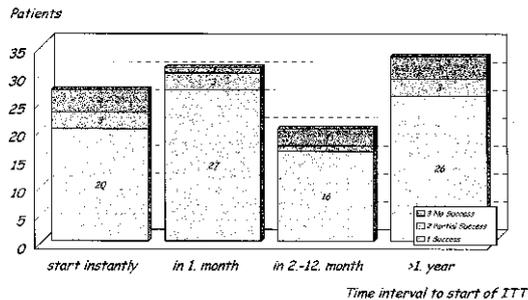
Fig. 3: Time interval between detection of the inhibitor and start of ITT in Haemophilia A and success rate (n–111)

one year. Table 2 shows the comparison of group 1 with full success with groups 2 and 3 without full success. The significance of the parameters does not change when groups 1 and 2 with full and partial success are evaluated composed against group 3 together.

Discussion

ITT is now the most accepted method of eliminating inhibitors, especially high titer, in hemophilia A. In Germany immune tolerance induction generally follows the Bonn protocol.[1] This means that patients are mostly treated with a high dose regimen. Statistical evaluation of high against low dosage therapy therefore is not possible in the patients included here. The registry contains all patients of the participating centers from the start of this method of treatment. Consequently, the age distribution of the patients treated in the '70s and '80s is shifted in the direction of older patients compared to the mostly young children which tend to dominate nowadays.

Inhibitor titer

The importance of the inhibitor titer as a predictor of the success was noted from when the registry was established. Other registries also show the strong influence of titer on outcome,[2,3] although it is not easy to compare outcome results of different studies[4,5] because inclusion criteria are not similar. The ITT outcome was 79% of complete success in our patients. If only patients with inhibitor titers below 100 BU are included the success rate of ITT is 89%. The success rate also depends on the criteria chosen to

define success. The patients in our group 2 became *mild low responders* with inhibitor values constantly below 2 BU without an increase at FVIII challenge. Treatment with FVIII is possible again. If patients with full and partial success are counted together the overall success rate is 87%.

Time from diagnosis to start of ITT

There has always been discussion about the importance of the interval between diagnosis of the inhibitor and start of ITT. The advantages of an early eradication of the inhibitor after its detection, especially in children, is obvious. The danger of bleeding is diminished, joints can be protected by prophylactic treatment again and the costs of ITT are lower because of the low body weight. However, the time interval between inhibitor detection and start of ITT or the number of exposure days in this interval does not significantly influence success in our registry. In the international[3] and North American immune tolerance registry[2,4] only patients who started ITT more than 5 years after inhibitor detection had a significantly worse outcome; starting earlier did not influence success rate. A high inhibitor titer at the start of ITT is significantly related to a negative outcome. It may, therefore, be better to wait in such cases until the inhibitor titer is reduced so that treatment can be commenced under more favorable conditions.

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Malmö protocol update

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Abstract

The Malmö protocol for immune tolerance induction includes high doses of Factor VIII/IX, intravenous IgG and cyclophosphamide. If the inhibitor titer exceeds 10 Bethesda units at start, extracorporeal adsorption of IgG is performed using protein A. The protocol sometimes has to be repeated. A successful response may occur within a few weeks. In hemophilia A the success rate so far is 10/17 patients (15 high-responders, 2 low-responders) or 59%, whereas in hemophilia B 6/9 patients (8 high-responders, 1 low-responder), or 67%, have become tolerant. In one hemophilia B patient, a relapse occurred after 6 months. During a second treatment episode he developed an acute myocardial infarction, probably caused by replacement with prothrombin complex concentrate. We conclude that the Malmö protocol is efficient for induction of immune tolerance but the patients must be selected particularly with regard to inhibitor duration and time of last booster.
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Key words: hemophilia A, hemophilia B, immune tolerance, protein A adsorption, factor VIII, factor IX

Professor Inga Marie Nilsson developed the Malmö protocol for immune tolerance induction during the 1980s after the clinical observation of a patient with hemophilia B and a high responding inhibitor.[1] The protocol has since then been used both in hemophilia A and hemophilia B and the purpose of this report is to give an update of the results.

The Malmö treatment model for induction of immune tolerance

The protocol

If the inhibitor titer is above 10 Bethesda inhibitor units (BU) immune tolerance induction (IT) is preceded by extracorporeal adsorption of inhibitor to protein A (CITEM 10 Excorim KB, Lund, Sweden) in order to reduce the inhibitor to 3 BU or below.[2-4] Cyclophosphamide is given from the first day of treatment, first intravenously at daily doses of 12-15 mg/kg bodyweight (bw) for 2 days and then orally at daily doses of 2-3 mg/kg bw for 8-10 days, the time period depending on the development of low leukocyte counts. The initial dose of factor VIII (FVIII) for hemophilia A or factor IX (FIX) for hemophilia B varies, as it is necessary to increase the VIII:C/IX:C levels to about 40-100 units/dL. FVIII/IX is then given at intervals of 8-12 hours to maintain the VIII:C/IX:C concentration at a level of 30-80 units/dL. When the VIII:C/IX:C levels decrease, usually after one week, shortening of the interval between doses, usually 6 hours, increases the total daily dosage of FVIII/IX. Beginning on day 4 of treatment, IgG is given intravenously at daily doses of 0.4 g/kg bw for 5 days. After disappearance of the inhibitor, regular FVIII/IX treatment (around 30 units/kg bw) is given 2-3 times a week according to the usual prophylaxis protocol (Nilsson, 1992).

At the start of immune tolerance induction hydrocortisone (100 mg) is usually given i.v. daily for the first two days in order to prevent side-effects such as allergic reactions caused by the drugs. In children Uromitexan® is given in order to counteract toxicity to the urinary bladder by cyclophosphamide.

Strategy for the use of the Malmö model for inhibitor treatment

In the most recent comprehensive follow-up of the Malmö protocol, Freiburghaus *et al.*[4] analyzed the prognostic factors for success. The most important factors seem to be the following: low current inhibitor titer, low historical peak and long interval since previous replacement therapy. It also seems to be important that the patient at the start of immune tolerance induction should be in a steady state without any ongoing reactive process. Based on these factors we have developed a strategy for the Malmö inhibitor treatment, which is shown in Figure 1. Thus, IT is started when the inhibitor has not been boostered in at least 6 months. If still above 10 BU, protein A adsorption is included in the protocol. If the treatment is successful, the patient continues with ordinary prophylaxis; if not successful the treatment is repeated after 6-12 months. In the meantime recombinant FVI-
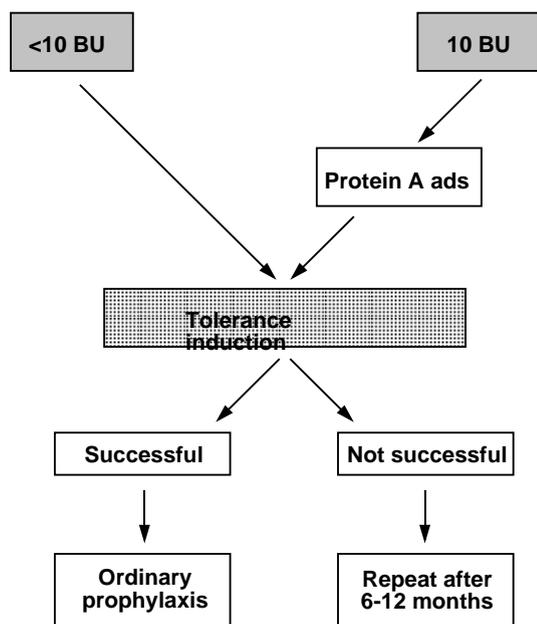
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Figure 1. The Malmö protocol for inhibitor treatment.

Ia (NovoSeven) is used as needed to cover for acute bleeds.

Definition of response

Successful tolerance is achieved when the recovery and half-life of infused FVIII/IX are within the normal range, and the clinical hemostatic response is normal.

Results and comments

In the last report on the treatment result[4] 15 hemophilia A patients (13 high responders) had been treated with 21 courses of treatment. Ten (67%) became tolerant. A median of 162,000 (range 20,000-862,000) units of FVIII had been given over a mean of 20 days (range 9-37). The mean daily dosage per kg bw was 207 units (range 83-441). Seven patients with hemophilia B had been treated, all of whom were high responders. Six (86%) became tolerant. The mean dose was 219,000 units of FIX (range 14,000-616,000) over a mean of 23 days (range 8-53). The mean daily dose per kg bw was 304 units (range 36-953).

A brief update of the results for the Malmö protocol, regarding both hemophilia A and hemophilia B, was made in October 1999. The results for hemophilia A are presented in Table 1. All of the 10 patients who had become tolerant are still tolerant and thus no relapse has been seen in patients with hemophilia A. No major side-effects occurred. It can be seen that since the report in 1997, 2 patients have been treated, both unsuccessfully. In fact we have seen several failures during the 1990s, when those treated have been primarily small children, and recombinant or monoclonal antibody purified concentrates have been used. Because of this we have looked back at a number of hemophilia A patients and found that several of those who were successfully treated, were treated repeatedly during the years when they had a long

Table 1. Malmö protocol. Results in hemophilia A, as of October 1999.

Patients	No. of episodes	Historical peak inhibitor titer (BU) median and range	Age first ITT median and range, yrs	Success	Follow-up time median and range, yrs
n=17 (16 severe, 1 moderate)	24	96 (6-45,000)	14 (2-64)	10/17 (59%)	12 (6-14)

Table 2. Malmö protocol. Results in severe hemophilia B , as of October 1999.

Patients	No. of episodes	Historical peak inhibitor titer(BU)	Age first ITT	Tolerance	Follow-up time, yrs
1	1	>300	5	Yes	7.7
2	2	150	12	Yes, relapse	Relapse after 6 months
3	1	>300	14	After 6 months	16
4	1	>600	10	Yes	13.6
5	1	120	46	Yes	Not known
6	1	100	22	Yes	9
7	3	>300	5	No	Not tolerant
8	2	429	1.6	No	Not tolerant
9	1	3	29	No	Not tolerant

standing inhibitor, prior to immune tolerance induction. In several patients it is evident that frequent treatment with FVIII alone or in combination with cyclophosphamide attenuated the anamnestic response. Thus the historical peak is probably not really relevant to the type of immune response in certain patients entering the Malmö protocol. This might explain the apparent decrease in the success rate during recent years. An alternative explanation may be that a switch from intermediate purity products to ultrapure or recombinant products has occurred, especially during the last decade. Whether product purity has an impact on IT tolerance outcome remains an open question, however. Our IT regimen for hemophilia A has changed somewhat, because of the observation that important factors for a successful outcome are, among others, low current inhibitor titer and long intervals since previous replacement therapy. This means that in a small child who develops an inhibitor, the optimal use of the Malmö protocol would require a long waiting period before IT is implemented. This could jeopardize the child´s health, due to recurrent hemorrhages. Therefore, in children with a newly detected inhibitor we prefer to start immediately with a Bonn-like regimen, giving at least 200 units of FVIII per kg bw daily. The Malmö protocol is mainly reserved for patients with longstanding inhibitors prior to IT.

For hemophilia B, we have treated 9 patients as of October 1999. The study material and the results are shown in Table 2, from which it can be seen that one relapse occurred after 6 months. This patient received another course of treatment, which was terminated when the patient developed signs of an acute myocardial infarction. He later developed cardiac failure and expired from heart failure several years later. The treatment given was the full protocol including protein A adsorption. The FIX concentrate used was a prothrombin complex concentrate. Thus, of the 9 patients treated of whom one had a low-responding inhibitor, tolerance was achieved in 6 (67%). Relapse occurred in one patient so the final success rate was 5/9 (56%). The conclusions drawn from treatment of patients with hemophilia B are that the Malmö model gives a comparatively high response rate in the treatment of high-responding FIX inhibitors, and that only purified FIX concentrate should be used, in order to minimize the risk of thromboembolic complications. In addition, the short treatment period used for the Malmö protocol should, from a theoretical point of view, minimize the risk of developing nephrotic syndrome, a known complication of long-term high-dose FIX treatment.[5]

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DISCUSSION 6   The Malmö Protocol Update
Berntorp E (Malmö, Sweden)

DI MICHELE: I would like to make a comment and ask a question with respect to the hemophilia B data. If you look at your success rates and the relapse in six months, I'm not convinced that you shouldn't consider it five successes out of nine patients. What is more important is that if you count up the number of courses of treatment that they were required to achieve those successes, what you really have are seven successful courses out of a total of thirteen. Most of us were presenting success in terms of immune tolerance perceived success in terms of a single course of therapy. You have multiple courses here.

BERNTORP: In a few patients we gave multiple courses. It is very important to look at the economic aspects here. We have always stressed that these courses sometimes have to be repeated. There is a big difference between a median of twelve months immune tolerance induction

with 200 units per kg bw versus two or three courses with the Malmö protocol but it is true that sometimes that courses have to be repeated.

DI MICHELE: I'm just looking at the overall success rates and not quibbling over the cost effectiveness. My second comment refers to the nephrotic syndrome. You didn't mention whether any of the patients in your cohort were allergic phenotype patients because those are the ones in whom the nephrotic syndrome with immune tolerance has been reported. In the American registry five out of the seven failures were patients who had this allergic phenotype and failed immune tolerance whereas only one of the four successes had this phenotype. There were a total of six out of the eleven patients who were tolerized as part of the North American Registry that had this phenotype and so we may be looking at somewhat different populations. I'm not sure that we can compare the tolerance rates in the North American Registry with the data you have presented here unless there were allergic phenotype patients.

BERNTORP: We had one patient who failed who had the allergic phenotype. He developed this allergy and the nephrotic syndrome when we continued with the long-term high dose regimen. There is also the case of another patient who has been subjected to the Malmö protocol who also developed this allergic phenotype in the long term treatment. He was a failure with the Malmö protocol and then he continued.

MARIANI: Your data concerning the fact that you start with the lowest possible inhibitor titer either by letting the titer go down because you don't treat the patient or by using the sepharose column fits very well with the statistical analysis showing that the pre-titer is a very important predictor of outcome.

MARTINOWITZ: We had a few patients who failed with the Malmö protocol. We hired a team and a machine which is a very complicated military operation to bring togheter. In two of the five patients we found that there was a change in the pattern of the inhibitor. I believe one had a remaining titer of 20 and the other of 10. They couldn't have been treated with continuous infusion of factor VIII because the inhibitor became more like a type two inhibitor with much lower avidity and affidity. I don't know if you have had such experience or if you tested the patients. In all the protocols we should check the inhibitor pattern at the end because if you can treat the patient with factor VIII successfully even for surgical procedures by

continuous infusion, then the definition should be changed.

BERNTORP: That is not exactly our experience but we have patients on high dose regimens. At the present time we have one small child who has been on 200 units per kg bw for a long time and indeed his bleedings have entirely disappeared. Therefore I agree with you that factor VIII may have a hemostatic effect despite the fact that you measure a rather high inhibitor titer *in vitro*.

HUGHWORTH: As for nephrotic syndrome I'm not absolutely certain that it is always associated with the Ig. We were referred a patient to go on the FVII a surgical trial for renal biopsy who had developed nephrotic syndrome, who had no prior history of anaphylactoid reactions whatsoever and had been on it for about six and a half months. On laproscopic biopsy of the kidney we found focal-sclerosis and glomerulonephritis. Thus I'm not entirely convinced that you have to have a prior Ig event to get nephritic syndrome. It may well be related to the stoichiomotry of nine versus eight in that you're giving fifty fold greater amounts of protein to treat therapeutically the same number of units.

HYBERG: How many absorptions do you perform and how fast does the inhibitor titer rise after the absorption?

BERNTORP: Usually, we perform two; one absorption each during two consecutive days and two to three plasma volumes are processed each day. The typical pattern is that you take down the inhibitor within the first absorption to, for example, ten Bethesda units and the next morning it will have risen and therefore you perform a new absorption and then you can come close to zero in the average patient. If you then give the patient factor VIII for treatment of a bleed as we have done sometimes, and you do not start with an immune tolerance induction dose then the inhibitor comes back within approximately seven days.

MAUSER: We have one hemophilia B patient who was tolerized with the low dose Dutch regimen with 50 I.U of factor IX every other day and he had no anaphylaxis in his history, but after about five years of tolerance induction regime he developed nephritic syndrome while he was on a thousand units every other day of factor IX monoclonally purified. He had a very low dose of factor IX if you consider that he was a patient of 80 kg.

BERNTORP: Had he been an inhibitor patient five years before?

MAUSER: Yes.

Immune tolerance induction: prospective clinical trials

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ABSTRACT

Successful immune tolerance induction (ITI) relates significantly to the starting inhibitor titer but not the peak historical inhibitor titer. Below a starting titer of 10 BU/mL success appears to be unrelated to the dose of factor VIII (FVIII) used for ITI. Above this starting titer, larger doses of FVIII may be associated with a better outcome from ITI. Opinion on the importance of the dose of FVIII used for ITI is polarized and there is no agreement on the optimal regimen for ITI. A prospective randomized clinical trial is proposed in which patients will initially be treated on demand with bypass therapy until the inhibitor titer has fallen below 10 BU/mL. Patients will then be randomized to receive a high or low-dose regime. Efficacy, cost-effectiveness and morbidity will be compared.
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Our current knowledge of immune tolerance induction is not derived from prospective comparative clinical trials but from several series of patients treated with various single regimens, reported partly retrospectively and partly prospectively,[1-4] and from three retrospective surveys or registries.[5-9] The former studies report the single center experience of individual regimes. Although all used a similar pharmacokinetic definition of success (restoration of normal half-life and recovery), patient-selection varied from study to study and in some cases the regime evolved during the course of the study.[1-4] Although the VanCreveld[1] and Malmö[2] studies are both true cohort studies, the Bonn series excluded low level inhibitors and the precise inclusion criteria are unclear.[3] These differences render a valid direct comparison of the regimes difficult, even though the reported success-rate from the three centers is very similar.

The registries were set up to determine predictors of success or failure of ITI and to provide some comparison between regimes. The registries differ in their method of data collection and even their end points and this limits the extent to which their data may be compared. All are retrospective surveys in which the data are relatively soft and reporting bias cannot be excluded. Although all three registries use a standardized questionnaire, this is not standardized between the registries. The North American Registry (NAITR)[7,8] appears to have a softer definition of success than the other registries. The German Registry[9] collected no data on the use of low-dose regimens and so can provide no insight into their efficacy.

Despite these limitations the registries have established most of the predictors of successful ITI, although a number of questions remain unanswered or a source of controversy. ITI is successful in up to 90% of patients although it is not agreed how these patients should be selected or which regime should be used. Both the International Immune Tolerance Registry (IITR) and the NAITR showed that the most important predictor of successful ITI was the inhibitor titer at the start of ITI, which affected both the likelihood of success and the time taken to achieve tolerance.[5-8] An inhibitor titer < 10 BU at the outset of ITI significantly correlated with successful outcome in both the IITR and the NAITR (p= 0.001 and 0.004, respectively).[5-8] The success-rate and time to success for patients starting ITI with an inhibitor titer <10 BU/mL was 85% and 11 months compared with 43% and 15 months in patients with inhibitors > 10 BU/mL. Most other studies show a similar relationship between the starting inhibitor titer, outcome and the time taken to achieve tolerance.[1,3,4]

In contrast, the peak historical inhibitor titer was not statistically related to outcome even though a low peak historical titer is commonly said to be predictive of successful ITI and a very high peak inhibitor titer > 500 BU/mL to predict a poor outcome. Since the starting inhibitor titer is far more predictive of outcome than the peak inhibitor titer, the chance of successful ITI may be enhanced by deliberately deferring the initiation of ITI until the inhibitor titer has declined below 10BU/mL. During this interval,

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exposure to factor VIII should be avoided or minimized by treating bleeding, on-demand, using bypass-therapy, preferably recombinant VIIa (Novoseven, Novo, Denmark). If ITI is started at the first opportunity, as is common current practice, ITI will often start shortly after some earlier treatment with factor VIII, during a secondary immune response to factor VIII and when the inhibitor titer may be rising rapidly. This may reduce the chance of achieving successful ITI. Treatment in which ITI was deferred until the inhibitor titer was <10 BU/mL, either through circumstance[1] or by design[11,12] has been notably very successful. These groups report very impressive success rates of 88% to 100%, despite using widely varying factor VIII dose–rates.

The major area of contention between the Registries is the importance of the dose of factor VIII administered. The International Immune Tolerance Registry (IITR) suggests that this is the principal determinant of success (p <0.0001), particularly in patients whose starting inhibitor titer was >10 BU/mL. The NAITR found that success was unrelated to the dose of factor VIII used although the time to success was statistically related to dose.[5-7] A meta-analysis of the registries conducted by Kroner suggests that, in patients with an inhibitor titer <10 BU/ml at the start of ITI, success is unrelated to the dose of factor VIII used.[10] This lends further support to the proposal that ITI should be deferred until the inhibitor titer has fallen below 10B U/mL.

Not only is it unclear which regimen is most effective, there is no published comparison of the morbidity associated with high or low-dose regimes or their relative cost-effectiveness. Low-dose regimes may be administered without the use of a central venous catheter and, although perhaps slower, the three times a week regimen is likely to be more acceptable to patients and parents than a twice daily regimen using a central line. Line infections commonly cause a nonspecific rise in inhibitor titer and may cause failure of ITI. One might also speculate that, although line infections may be more common among patients using high-dose regimes, intercurrent bleeding may be commoner during low-dose ITI. These and other questions would best be answered by a randomized controlled trial in which a comparison of the efficacy of a high and low-dose regime is accompanied by an analysis of morbidity and cost-effectiveness.

Randomized controlled trial of high and low-dose ITI

We propose a randomized, controlled comparison of high and low-dose ITI. The hypothesis of this study is that efficacy of the two regimes is similar (given a starting inhibitor titer of <10 BU/mL) but that the high-dose regime may achieve success more rapidly. If this is correct, the low-dose regime should prove more cost-effective but it is difficult to predict how the morbidity of the two regimes may differ.

Patients will be recruited who have severe hemophilia A, a newly detected inhibitor > 5 BU/mL and aged < 7 years. Their bleeds will initially be treated on-demand using bypassing agents (preferably Novoseven) to allow the inhibitor titer to decline. When the inhibitor has declined below 10 BU/mL, they will be randomized to receive either 200 IU/kg/day as a single bolus or 50 IU/kg three times a week, these being pragmatic approximations to the Bonn and van Creveld regimens.

Central lines will be used at the discretion of the managing clinician and data on line infections carefully collected. The once daily factor VIII administration for the high dose regime was chosen partly to minimize the risk of line infection.

ITI will continue until tolerance is achieved or the induction is deemed to have failed. Successful ITI is defined by the restoration of normal factor VIII recovery and half-life. Failure is defined as a failure of the inhibitor to decline by at least 20% during any six-month period or patient withdrawal for any other reason. It is anticipated that most patients will be tolerant within six to twelve months but a few resistant patients will take significantly longer to become so. Details of all intercurrent bleeding, all line infections, all hospital admissions and all treatments will be kept. This will enable a direct comparison of both the morbidity and cost-effectiveness of the two regimes. It is important that all concomitant therapy be included in such a comparison.

Two interim analyses will be conducted and the results reported to a committee of non-participating senior investigators (Data Safety Committee). If a large difference between the two arms appears or if an unacceptable number of adverse events is encountered then the Data Safety Committee may terminate the study prematurely.

Data will be collected partly using a traditional paper CRF but also using a very user-friendly electronic CRF. This may be called up on the internet on *http://www.itistudy.com*. Once the study has started, this interactive CRF will permit direct entry of screened and anonymous data into the database which is held in a dedicated fileserver in Manchester. Encryption, password protection and a secure socket layer provide security.

Logistic barriers to conducting a randomized trial of ITI

Patient numbers

The literature is disparate, which makes an accurate estimation of the power of the study difficult. However, using a crude meta-analysis of the available studies suggests that about seventy patients may be required in each treatment arm. A further three patients are required per arm to compensate for the loss of statistical power introduced by the two interim safety analyses. This gives a total sample size of about 75 per treatment-arm. To recruit such a large number is clearly challenging. The size of the problem is well illustrated by considering the potential for recruitment from a single country, the United States of America. In the USA about 200 PUPS a year are born with severe hemophilia. At least 20 % will develop persistent inhibitors i.e. 40 patients per year. About 70% of these are estimated to be eligible for the study giving a potential total of 56 patients over two years. Clearly, multinational collaboration is required for such a study to be completed within a reasonable time. It is therefore, proposed to conduct this study throughout North America and Canada, throughout Western, Northern and Southern Europe, Israel, Australia, New Zealand and possibly Japan.

Factor VIII shortages

The study permits the use of any brand of factor VIII for ITI, from intermediate-purity plasma-derived concentrates to second-generation B-domain deleted recombinants. The choice of product is left to the managing clinician, the only stipulation in the protocol being that we would not wish a change of product during ITI. [The clinician is not obliged to tolerize using the same product that the patient was using when the inhibitor was first detected, although they commonly would.] Despite reports suggesting that intermediate-purity products are more effective for ITI,[13] there is no convincing evidence that product type affects the outcome of ITI and there are several reports of good results using high-purity or recombinant factor VIII for ITI.[9,10,14]

We anticipate that most clinicians will choose to induce tolerance using recombinant factor VIII because most children in the participating countries are treated using these products. Demand for recombinant factor VIII has outstripped supply, and increasing production capacity is a very slow process with a very long lead-in time. Some manufacturers are rationing their products on a world-wide basis and have refused to supply recombinant factor VIII for immune tolerance because they have already too small an allocation for their existing customers. We estimate that the average patient tolerized using the van Creveld regime will require about 0.25M units compared to 1.0M units required for the average patient tolerized using the Bonn regime.

We have deliberately deferred starting this study for over a year now because the supply of recombinant factor VIII was insufficient to sustain the study. It would appear that the supply of recombinant will soon ease, permitting the study to start. The recent opening of Baxter's Thousand Oaks Plant, licensing of Refacto (Wyeth/GI B-domain deleted factor VIII) and imminent licensing of Kogenate SF should all lead to an easing in supply from the end of the first quarter of the year 2000. We hope to start our study at about that time.

Further information on this study is available from *haemophilia@man.ac.uk, dmdimich@mail.med.cornell.edu* or *http://www.itistudy.com.*

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DISCUSSION 7 Immune tolerance Induction: Prospective Clinical Trials
Hay C, (Manchester, UK)

LENK: You told us that there is a difference in the duration of the treatment with higher doses but not in the result. I think what I have shown with the German Registry is that in those patients with between 5 and 100 BU we have a success rate of more than 90%, and so I believe there is a higher success rate. Nevertheless, in our experience there is an extremely high success rate in the between 5 and 100 BU group.

HAY: The Van Kreveld data suggests that they have around an 80% success rate with unselected patients. I find it quite difficult to interpret the success rate of the Bonn Regime because the published results with respect to forty years of the Bonn Regime certainly don't lay claim to a hundred percent success. Some of the data which regard how long some of the patients were going through tolerance are missing and that makes it impossible for me to evaluate that data using the criteria which we have been using because we made a judgement on how long we were prepared to continue with immune tolerance and as you know in most countries people are not prepared to continue forever. Using our criteria for failure the maximum time that you would be tolerized before being considered a failure would be 33 months. If you use those criteria I am not so sure that the Bonn Regime would be seen to have such a high success rate, particularly with those in whom there had been a significant interuption in treatment or who were described in the reports as undergoing prolonged treatment. We need to ask ourselves the question what is prolonged treatment and the answer is that it is a matter of judgement; one person's prolonged treatment is interminable to another.

ESCAURNA: With regard to the cost-effectiveness and the data with which we are familiar with, the low dose regime costs more as it lasts longer than the high dose regime. Do you also include the costs which arise from recombinant factor rVIIa or PPCs during that period because patients who need a longer treatment period bleed more than the other patients. In addition to the issue of cost effectiveness do you also calculate the complications which derive from those bleedings. Will you keep a record of such occurrences?

HAY: I think it's extremely important that we collect that data. We'll collect data on all concomitant hemostatic treatments, on-line infections and intercurrent bleeding because these may differ significantly. Those are the kind of safety details that will be reported to the data safety committee so that if we find that in one regime bleeding is far greater than in another we may be faced with a criterion to stop.

KREUTZ: If we speak about success rates we have to understand that the definitions of success in all the studies are different. Dr.Brackman and my group in Frankfurt have other definitions of success rates. Consequently, I don't think you can say that the success rates are the same.

HAY: With regard to criteria and the data of the Van Kreveld Regime I wish to emphasize the normalization of half life and recovery. I believe that you were using the same criteria and so I think that those two studies are comparable in that respect.

LAURIEN: The Germans seem very determined to continue with what they are doing. Thus it is important to start your protocol and show data to us which compares low dose to 200 once a day. We need this type of trial.

HAY: We're certainly not waiting for Germany although we would welcome their participation. The only reason that we haven't started already is that it seemed an unsuitable time to start because of supply issues which I think are about to improve rather than being resolved. I do understand that they are now following our protocol although without the randomization which I think is very sad because even with the best will in the world I'm not entirely certain what is going to happen to that data. The way our study is designed means we can't incorporate that data in any way because we've decid-

ed to go for a fully randomized design rather than the original compromise of a comprehensive study. The original compromise was to permit German clinicians to participate in the study.

DI MICHELE: My final comment relates to what Dr.Laurien said; I don't think anyone is going to agree with this protocol one hundred percent. However, I do want to mention the fact that this protocol has been several years in development and I believe that I'm speaking on behalf of all the principal members of the steering committee when I say that the input from everyone, including the dissenting views, has been very important because all the critical discussion which has led to this has resulted in an end protocol that we feel very good about and that we can bring to the world with a lot of consensus. There are views that are discrepant from ours but I believe that the dissenting view has been very important in shaping this protocol. Nonetheless, as you say Dr. Laurien we will go ahead.

Acquired factor VIII autoantibody inhibitors: current concepts and potential therapeutic strategies for the future

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The extremely low incidence of acquired autoantibody inhibitors directed against factor VIII procoagulant activity (estimated at 0.2-1 case per one million population per year) belies its importance as a clinical complication and socioeconomic burden. Furthermore, insight into the pathogenesis of this disease entity may provide clues as to how the immune system responds to factor VIII and how the immune system can be manipulated for treatment. This review will examine current therapeutic concepts and will speculate on future approaches to treatment based on emerging information about immune responses to the factor VIII protein.

Clinical presentation

Acquired factor VIII inhibitors occur predominantly in previously non-coagulopathic young adults (peaking 20-30 years of age) and the elderly (peaking at 60-80 years of age), assuming an approximate biphasic age distribution. Whereas almost half of these individuals present with no identifiable underlying medical condition, the majority do have defined or evolving autoimmune or lymphoproliferative disorders. Immunologic based diseases, usually observed in younger patients, have included systemic lupus erythematosys, rheumatoid arthritis, dermatomyositis, myasthenia gravis, multiple sclerosis, and graft-versus-host disease after allogeneic bone marrow transplantation. Asthma, chronic inflammatory bowel disease, and pemphigus also have been reported; several antibiotics, particularly the penicillins and sulfonamides, and BCG vaccination have been implicated. Several cases of autoantibody inhibitors have been described in association with chronic interferon-alpha therapy. In older individuals, malignant lymphoproliferative disorders, such as chronic lymphocytic leukemia and lymphomas, predominate. These underlying diseases occur in the context of a dysregulated immune system and suggest that an oth-

erwise *silent* lymphoid clone has been induced or that a previously suppressed lymphoid clone has emerged from its inhibited state. The end result is the production of a humoral immunoglobulin, which interacts with specific epitope(s) on the factor VIII protein molecule and neutralizes its procoagulant function (see below). Very recently, a number of solid tumor malignancies have been accompanied by autoantibody inhibitors; however, it is unclear whether these are coincidental or causal events. In favor of the latter possibility are: (1) the frequently close temporal relationship between inhibitor and tumor detection, and (2) the absence of other conditions known to be associated with factor VIII autoantibody formation. Inhibitors of coagulation may be an additional example of the numerous autoimmune paraneoplastic phenomena which have previously been observed with solid tumor malignancies, e.g. autoimmune thrombocytopenic purpura, hemolytic anemia, pemphigus. On the other hand, successful treatment of these tumors with surgery, chemotherapy, or radiation therapy usually does not result in inhibitor disappearance.

The appearance of autoantibody inhibitors against factor VIII during the post-partum period is a rare but serious condition, which most usually occurs in primiparas within 3 months after delivery.[1,2] Low titer inhibitors (<5 Bethesda Units, BU) characteristically disappear spontaneously and do not usually recur with subsequent pregnancies. High titer autoantibody inhibitors (>10 BU) may persist for years and may be particularly resistant to the typical immunosuppressive treatment modalities employed in this disease, e.g. corticosteroids, intravenous gammaglobulin, cytotoxic/myelosuppressive agents. Whether the post-partum autoantibody antedates the development of an autoimmune syndrome or represents an epiphenomenon related to the immunosuppressive factors synthesized by the placenta remains unclear.

Acquired autoantibody inhibitors against factor VIII appear spontaneously in otherwise normal non-bleeding patients[3,4] and, similar to the alloantibody situation in individuals with severe

hemophilia A, have been characterized predominantly as polyclonal IgG4 and occasionally as IgG1. In contrast to congenital hemophilia, IgM and IgA monoclonal inhibitors have been reported in acquired hemophilia. Approximately 15-17% of normal, healthy individuals with no evidence of a coagulopathy possess low titers of clinically irrelevant anti-factor VIII antibodies in their plasmas. They are predominantly of the IgG1 and IgG2 variety and may exert detectable inhibitory activity against factor VIII in *in vitro* mixing studies with normal plasma. Kazatchkine *et al.* have hypothesized the presence of anti-idiotypic antibodies in these healthy individuals, which neutralize the inhibitory potential of any circulating anti-factor VIII autoantibodies. Thus, there is no clinical evidence of bleeding but the existence of autoantibodies against this self-antigen suggests the presence of factor VIII-specific CD4+ T-helper lymphocytes in the normal immunologic repertoire.

Autoantibody inhibitors are predominantly directed against single epitopes on the factor VIII molecule, contained in either the amino terminal end of the A2 domain, or much more frequently (around 50%), in the carboxyl end of the C2 domain of the factor VIII protein. In contrast to the alloantibody situation, autoantibodies to factor VIII are not directed to both sites. Alloantibody inhibitors are usually directed against both the A2 and C2 domains and sometimes against the A3 domain. The low titer, clinically irrelevant autoantibodies found in normal individuals are also directed primarily against the A3 domain. As with alloantibodies, autoantibodies can also be directed against the B domain and other areas of the factor VIII protein; however, they convey no inhibitory activity. Recent data suggest that anti-B domain antibodies may influence the circulating half-life of the factor VIII protein, but this remains to be confirmed. Anti-A2 autoantibodies interfere with factor activation by the factor VIIIa-IXa-phospholipid complex; anti-C2 autoantibodies interfere with the binding of von Willebrand factor protein and/or phospholipid to factor VIII; and anti-A3 autoantibodies block interactions between factor IXa and factor VIII. How these autoantibodies determine the pharmacokinetics of factor VIII remains to be elucidated; however, it is apparent that autoantibodies typically produce a *type II* pattern with non-linear inactivation and the presence of residual factor VIII activity despite very high titers of the inhibitor. Rarely, autoantibodies possess a *type I* pattern of inhibition, in which there is total and linear inactivation of factor VIII activity. This is the characteristic pattern for alloantibodies.

Treatment

Individuals with autoantibody inhibitors of coagulation tend to present with spontaneous bruising and/or serious bleeding. Their hemorrhagic manifestations are typically more dramatic and are more likely to be life- and limb-threatening than compared with alloantibody inhibitor associated bleeds. The mortality rate of patients with acquired hemophilia approaches 15%, due primarily to bleeding complications occurring early in the course of the disease. With time, infectious complications, mediated by the myelosuppressive effects of cytotoxic agents intended to repress inhibitor formation, contribute to morbidity and mortality.

The choice of treatment modalities for acquired inhibitors is predicted on the acuity and severity of bleeding and the BU titer. Acute bleeds occurring in association with low titer inhibitors (<5 BU) directed against factor VIII or IX usually respond readily when the neutralizing antibody is overwhelmed by large doses of human factor VIII or IX concentrates. There is no difference in efficacy between the pooled plasma-derived and genetically engineered products. By definition, individuals with low titer inhibitors do not manifest an anamnestic rise in antibody titers after re-exposure to the offending coagulation protein; thus, they are *low responders*. The administration of intravenous desmopressin (DDAVP, Stimate) at 0.3 µg/kg may stimulate the release of enough factor VIII coagulant protein from its physiologic storage sites to overcome autoantibody inhibitor effects and promote adequate hemostasis for a limited time.

In contrast, high titer inhibitor patients (>10 BU) are *high responders* and will mount an anamnestic antibody increase after re-exposure to the specific coagulant antigen. The high titer inhibitor characteristically cannot be overwhelmed by large amounts of high purity human factor concentrates. Rather, acute bleeds associated with this type of inhibitor must be treated with *bypassing* agents, which circumvent the site of antibody neutralization within the intrinsic pathway of the coagulation cascade, or heterologous porcine factor VIII concentrate, which has reduced cross-reactivity with anti-human factor VIII antibodies and is not neutralized by them. None of these products produces universal hemostasis.

By-pass products include prothrombin (factor IX) complex concentrates of the unactivated (PCC) (Konyne-80 {Bayer, Inc.}, Bebulin {Alpha Therapeutics, Inc.}, etc.) or activated (APCC) (FEIBA{Baxter-Immuno, Inc.} or Autoplex{NABI, Inc.}) varieties, all of which are plasma-derived, or the new genetically engineered recombinant factor VIIa (rFVIIa) concentrate. The mecha-

nism(s) of action for the PCCs and APCCs remains unclear, but their content of factor VIIa and/or other activated vitamin K-dependent clotting factors (such as factors IXa and Xa) certainly must be pivotal both to their efficacy and to their thrombogenic potential. rFVIIa concentrate may promote hemostasis by generating thrombin at sites of tissue factor exposure or by interacting with phosphatidylserine exposed on the surface of platelets activated by small amounts of factor VIIa-tissue factor complexes.

Although their efficacies for alloantibody bleeds approach 90% in open label studies, the limited data derived from randomized controlled studies demonstrated hemostatic efficacy of around 40-60% for PCCs and APCCs versus placebo.[5] The few prospectively randomized studies comparing the efficacy of PCCs to APCCs reveal a slight but statistically insignificant advantage to APCCs. No comparative studies have been conducted between porcine factor VIII versus rFVIIa concentrate versus APCCs. Furthermore, no information exists to determine whether one *by-passing* product is superior to another in autoantibody inhibitor associated bleeds; however, porcine FVIII administered to such patients as first-line therapy rather than for salvage may be superior to the PCCs and APCCs in the absence of high titer cross-reactive antibodies.[6] Anamnestic rises in factor VIII inhibitor titers occur less frequently in autoantibody patients (10-15%) after administration of porcine factor VIII than in individuals with alloantibodies. Interestingly, in non-hemophilic patients, the anamnestic antibodies developing to porcine factor VIII have kinetics of inhibition that are second order (type I), even though their anti-human anti-factor VIII antibodies demonstrate type II kinetics.[7] Anamnestic responses have also been reported to occur occasionally after FEIBA[8] and Konyne.[9]

If acute life or limb-threatening bleeding fails to abate despite aggressive use of PCCs, APCCs, r-FVIIa, or porcine FVIII, alone or in combination, extracorporeal plasmapheresis and exchange can be utilized to reduce high titer autoantibody inhibitors rapidly but temporarily. This can be achieved when plasmapheresis is coupled with use of Staphylococcal protein A or sephacryl columns, which *leach out* IgG antibodies. The exchange (typically, at least 3 liters) can be accomplished ideally with large amounts of high purity human or porcine FVIII concentrate to overwhelm residual antibody. Intravenous gamma globulin may be administered to replenish intrinsic IgG; autoantibody formation also may be suppressed as intravenous immuno-globulin IVIG is a potential source of anti-idiotypic antibodies. Anecdotal reports suggest that IVIG

administered in conjunction with porcine FVIII may enhance the factor VIII incremental response. As the amino acid sequences of the pertinent autoantibody epitopes on the A2, C2, and A3 domains on human factor VIII are identified, one may envision the time when synthesized amino sequences could be linked to the solid phase surfaces within extracorporeal plasmapheresis columns and be used to adsorb out only the specific IgG molecules which are directed to those epitopes. This theoretically should reduce or eliminate inhibitor effects.

The acquired hemophilias appear to be somewhat responsive to immunomodulation regimens. The only published prospective study examining high-dose intravenous gammaglobulin administration for the treatment of autoantibody inhibitors revealed a response rate (disappearance or >25% reduction in inhibitor titer) between 25–37%.[10] Complete and rapid disappearance within days to weeks of the autoantibody was observed predominantly in individuals with low titer inhibitors. High titer inhibitor patients usually demonstrated partial responses over weeks to months (except for the rare, dramatic exception), which ultimately were not associated with reduction in bleeding complications or the ability to change from by-passing agents or porcine factor VIII concentrate to human factor VIII for treatment of acute bleeding episodes. Multiple, prolonged courses of IVIG may be necessary to sustain a positive response. These data suggest that IVIG is not useful as a first-line or single agent therapy for most autoantibody inhibitor situations, but may be a useful adjunctive approach with plasmapheresis/exchange or porcine factor VIII concentrate. It is possible that some of the successes observed in this study represented the spontaneous disappearance of the inhibitors.

The use of the biological response modifier, interferon-alpha, has anecdotally been successful in the suppression of autoantibody formation;[11] however, few other successes have been reported and, in fact, the therapeutic administration of interferon-alpha may be just as likely to stimulate the development of autoantibody inhibitors against factor VIII.[12,13]

Additional potential innovative approaches to immunomodulation may include biotherapy with the unconjugated, chimeric anti-CD20 monoclonal antibody, rituximab, which has been recently approved for use in the treatment of follicular center cell lymphomas.[14] Such treatment may be capable of eradicating clone(s) of lymphocytes responsible for production of autoantibody immunoglobulins, including those with the capacity to neutralize coagulant protein activity.[15]

Immune tolerance induction (ITI) is a form of immunomodulation which has been successful

in eradicating alloantibody inhibitors against factor VIII and factor IX.[16] ITI regimens have rarely been used in acquired hemophilia and up to the present have not been very successful, perhaps because immunosuppression has been so successful. The considerable cost of the coagulation factor concentrates necessary to achieve successful ITI in this predominantly adult disease has been another important deterrent. This may explain why porcine factor VIII-based protocols have not been attempted for ITI, even though this low cross-reactivity product has theoretical advantages over human factor VIII concentrates and has induced immune tolerance in 3 hemophilic patients with anti-human factor VIII alloantibodies.[17] Notwithstanding our ignorance about the mechanism(s) by which ITI is achieved, there has been renewed interest in applying ITI to acquired hemophilia. A protocol employing high or ultra-high purity factor VIII concentrates (15-30 U/kg/day for 3 weeks) combined with intravenous cyclophosphamide (200 mg/day to a total dose of 2-3 g) and intravenous methylprednisolone (100 mg/day for 1 week, followed by tapering doses during the next 2 weeks) resulted in eradication of the autoantibody inhibitor against factor VIII in 11/12 consecutive non-hemophilic patients.[18] The mean time to complete disappearance of inhibitor was 5 weeks compared to 28.3 weeks in a historical control group who received conventional corticosteroids ± cyclophosphamide. There were no deaths in the ITI group, and the only 2 relapses over the 35 month observation period responded successfully to a second course of the original regimen.

Equally provocative has been the report of an oral ITI regimen in acquired hemophilia.[19] Although only 1/3 individuals with acquired hemophilia A demonstrated a decrease in the titer of anti-factor VIII antibodies, oral administration of factor VIII concentrate (1,250 U daily for 3 months) inhibited the factor VIII-specific lymphocyte proliferation and cytokine production by peripheral mononuclear cells cultured *in vitro*. Further studies employing ITI in autoantibody inhibitor states are warranted to establish feasibility and cost-to-benefit relationships.

Most of the successful experience in treating autoimmune antibody inhibitors against coagulation factors has been described with the administration of immunosuppressive and cytotoxic medications. Over 50% of autoantibody inhibitors to factor VIII will be suppressed by prednisone administered ± cyclophosphamide or azathioprine. This includes those individuals with underlying autoimmune diseases, lymphoproliferative and solid tumor malignances, and idiopathic etiologies.[20] Approximately 40% of the autoantibodies disappeared after 3-6 weeks of prednisone alone (1 mg/kg/day). Fifty per cent of those who were steroid resistant manifested inhibitor suppression following cyclophosphamide ± prednisone. A combined immunosuppressive therapeutic regimen consisting of cyclophosphamide (500 mg po on day 1 and 200 mg/day on days 2-5), vincristine (2 mg IV on day 1), and prednisone (100 mg/day on days 1-5) administered immediately after a bolus dose of factor VIII concentrate (50-100 U/kg) to treat an acute bleed resulted in a remarkable 90% (11/12) complete disappearance rate of the autoantibody inhibitor.[21] Immunosuppressive therapy may not be as useful in eradication of alloantibody inhibitors since none disappeared in the 5 hemophilic patients studied. Post-partum autoantibody inhibitors also appear to be more resistant to early immunosuppression,2 but many may spontaneously remit and usually do not recur with subsequent pregnancies.

Immunosuppressive strategies in transplantation medicine may provide new and improved medications which can be applied to the treatment of acquired hemophilia. The currently available cytotoxic agents have non-specific myelosuppressive effects and render the autoantibody inhibitor patient at increased risk of life-threatening infections, the second most frequent cause of death in this population behind bleeding complications. Cyclosporine and FK506 (tacrolimus) are potent inhibitors of calcineurin, a pivotal enzyme in T-lymphocyte receptor signaling. Calcineurin inhibition prevents interleukin-2 transcription and production and thus prevents the autocrine-mediated clonal expansion and proliferation of T-cells and their cytokine production. FK506, which has greater affinity for calcineurin, appears to be more potent than cyclosporine.

Antiproliferative agents inhibit the clonal expansion of T and B lymphocytes. Azathioprine is the prototype by its inhibitory actions on *de novo* purine synthesis in the lymphocyte cell cycle. Unfortunately, its myelosuppressive effects limit its clinical usefulness. Mycophenolate mofetil is a new purine anti-metabolite currently being used in clinical studies of transplant rejection and may be a promising agent for autoantibody inhibitor suppression. Its advantages over azathioprine remain to be elucidated. Similarly, sirolimus (rapamycin) is an anti-lymphoproliferating agent by virtue of its ability to inhibit second messenger signals induced by interleukin-2.

Techniques to block lymphocyte-antigen-presenting cell *cross-talk* provide a provocative and innovative approach to immunosuppression by inhibiting only the CD4 T-lymphocytes which are stimulated by the offending antigen. This can be

accomplished by blocking pathways of T-cell co-stimulatory signals, involving CD40-CD154 and CD28-B7 interactions. A clinical study is anticipated, which will employ an anti-CD40 ligand (Biogen) in the treatment of allo and autoantibody factor VIII inhibitors. The schedule of administration, duration and sequence of treatment, and the need for concomitant immunosuppressive agents remain to be established.

Conclusions

Autoantibody inhibitors against factor VIII and factor IX are associated with considerable morbidity and mortality. Treatment of acute bleeds has been enhanced by the availability of recombinant factor VIIa; however, the use of plasma-derived products, such as porcine factor VIII concentrate, may provide at least equal efficacy and safety and may be more cost-effective. In the absence of comparative trials for all of the commercial products, trial and error, physicians' personal preference based on previous experience, and reimbursement considerations often determine the approach to treatment of acute bleeding episodes. Long-term treatment aimed at eradicating the autoantibody inhibitor has been based on immunosuppression. Evolving knowledge gained from transplantation medicine may provide novel drugs without the side effects which have limited the use of currently available immunosuppressive and cytotoxic agents. Finally, there is preliminary indication that ITI regimens may be applied successfully to the acquired hemophilias.

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DISCUSSION 12   Acquired factor VIII auto-antibody inhibitors: concepts and potential therapeutic strategies for the future
Kessler C, (Washington, USA)

HYBERG: You mentioned the different diseases that are associated with the appearance of auto-antibodies and the fact that you looked for epitope specificity in these auto-antibodies. It is very well known that the specificity of all the auto-antibodies differs from the allo-antibodies obvi-

ously. Do you see differences in the epitope repertoire depending on the actual disease the auto- antibody is associated with?

KESSLER: I don't believe that there have been enough antibodies isolated in this group of patients to answer that question but it is a very important question and hopefully with more collaboration worldwide we may be able to answer it.

HYBERG: When you look at tolerance induction in your patients do you see differences which depend on the disease with which it is associated with the appearance of the auto-antibody? Do you see long-lasting effects for instance if the auto-antibodies are associated with other auto immune diseases?

KESSLER: I'm going to let Dr. Nemes describe his own data but I can tell you that other studies in the literature suggest these are all durable responses. There is no differentiation among the various autoimmune diseases as to whether you can achieve a complete remission.

INGERSLEV: I want to emphasize though that we have suffered in many cases an unavoidable tendency of hematuria when using cyclophosphamide. This has been a major problem and so we have been seeking a new standard procedure in which we have pointed to the use of cyclosporine as the most likely. Do you think we can have some help in future studies being set up in the way that you and Dr. David Green and others have done. All centers have very few patients and we need to have these multi-center studies in order to learn from our patients.

KESSLER: I certainly agree with you. Unfortunately, Dr. Green with his own prospective control study which he has been doing for over nine years has gathered a very small number of patients. Ideally, what you are saying is correct and hopefully there will be much more interest in the future so that worldwide participation will be facilitated.

SULTAN: Dr.Kessler, do you think that the group of patients who developed an inhibitor during pregnancy or after delivery are affected by the same mechanism, for instance associated with solid tumors? Do you think that these patients should have special treatment-not giving immunosuppressive agents? I would like to bring together the immunologists who are gathered here to share their ideas on the development of inhibitors in cases of pregnant women. I would like to know if there is a specific immunologic context which might explain the appearance of an inhibitor antibody.

KESSLER: I am not an immunologist, but I am fascinated by the fact that the placenta elaborates so many substances which can thereby activate lymphocyte release of cytokines. Whether or not the cytokines are the same for all of the oth-

er types of auto-antibody inhibitor situations I can't say, but they certainly don't respond in the same way. Perhaps one of the immunologists present could shed some light on this matter?

BAUDO: Just a comment from the Italian Registry of acquired inhibitor. We have collected the data from about 80 patients up to now. The age of occurrence is higher in men than women. The mortality rate is about 15%, but 50% of the deaths were related to bleeding. The data from immunosuppressive therapy with prednisone suggest that sustained remission is only obtained with the association of cyclophosphamide or azathioprine with prednisone. The rate of remission is over 60%.

SULTAN: But you are talking about the whole group of patients with auto-antibodies and not especially about those appearing during pregnancy.

ALEDORT: Dr. Kessler, I think the issue of the potential for recurrence in another pregnancy is a very important one and I've had several patients who have had horrendous clinical problems when they developed the inhibitor and yet they want more children. I think it is very important and I've tried myself to collect local data as there is very little information on this and the likelihood of recurrence and what influences it. There are enormous implications for family planning and we need to focus some attention on that issue in the future. It may not be as simple as it appears. We possibly need to look at epitopes and there could be something to know about the babies'antigens in order to predict whether the patient should abort or possibly avoid becoming pregnant again. There are a lot of unresolved issues that we need to look at.

KESSLER: Certainly, I agree with you.

SULTAN: Dr. Aledort is there a chance that the inhibitor to factor VIII in pregnant women could be an allo-antibody?

ALEDORT: Yes, it's possible. It may well be that it is a response to a factor VIII molecule in pregnancy that is different because it occurs late. It's not particularly early. It frequently occurs at the time of delivery at which time there is a mixture of maternal and fetal blood. It may very well occur during that time. It is very unusual that it happens in the first trimester or for that matter in the second. Frequently, the bleeding occurs right after the delivery and the antibody is present. We know very little about all this.

KESSLER: I think you are right. You have made an astute observation based on the idea that the women who do develop recurrences do often show up in the late first trimester with their inhibitor whereas their original inhibitor did not develop until the post- partum period.

MARTINOWITZ: I would like to draw your

attention to acute bleeds because the kinetic neutralization is so different from allo-antibodies. A patient can be successfully treated by continuous infusion. What we do is to mix the plasma of the patient with concentrate and we test the neutralization after 5, 10, 20 and 30 minute intervals. In most patients you find a plateau at a certain level. From this plateau you can plan the continuous infusion. We were successful in 6 out of 7 patients regardless of the level of their inhibitor. Even in cases of high titer, neutralization is much slower. The seventh patient who didn't respond to continuous infusion was treated with APPCs. She developed a massive myocardial infarction.

CIAVARELLA: Speaking as a physician, I have a patient who is eighteen with an acquired inhibitor but no underlying disease. The patient is now stable after four years of treatment but she needs immunosuppressive therapy. She asked me about having children and would like to cure the disease.

KESSLER: I'm glad she's your patient. On a more serious note I think there are a lot of ways of approaching this and I hope you have success in your treatment.

LUSHER: What about the patients that we all encounter with hemophilia who seem to have the bleeding manifestations and the reaction kinetics of an auto-antibody rather than an allo-antibody; has that been studied? I'd like to know about that group of people with severe hemophilia who start having excessive ecchymoses and the types of things you see in acquired hemophilia and if you do the kinetics they look like auto-antibodies.

KESSLER: I don't recall seeing anything in the literature. I don't believe that anyone has looked at epitope specificity in those individuals even to confirm that they had changed from a type 1 to a type 2 antibody kinetics other than in Dr. David Green's recent paper where he showed that to be possible after getting porcine factor VIII. Perhaps there is someone else in the audience who has some information on that.

SAINT REMY: Professor Sultan why on earth would you consider an anti-factor VIII immune response during gestation or post-partum period to be an allo-immunization? It's obviously an auto-immunization. It might be an allo-factor VIII but we don't know whether the mother's immune system is exposed to the factor VIII of the baby and basically this is an autoimmune response.

SULTAN: For a long time it was believed that women immunized with factor VIII could be immunized from the fetus factor VIII. That was something we believed twenty years ago. Dr.Aledort suggests something which could explain the relapsing of auto-antibody to factor VIII in a second pregnancy.

SAINT REMY: I am not an expert on immunology of pregnancy but there is now ample evidence coming from animal experiments that the repertoire of the fetus as well as the immune repertoire of the mother is shaped towards the other. This is something which has been very well described in animal experiments. My other comment is that solid tumor might generate antibodies cross-reacting against factor VIII.

New protocol for immune tolerance induction in acquired hemophilia

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Abstract

Background and objectives. Immune tolerance induction (ITI) regimens with human factor VIII concentrates are rarely if ever implemented in adult patients with auto-inhibitors, in contrast to allo-antibody suppression, which is used primarily in young children with congenital hemophilia. On the basis of some earlier experience with synchronization of plasma exchange therapy of various autoimmune disorders we have developed a new aggressive protocol for the treatment of patients with acquired factor VIII (FVIII) antibody. We have evaluated the outcome of 14 consecutive nonhemophilic FVIII inhibitor patients treated in a single center with our ITI protocol between 1992 and 1999, comparing them to 6 historical control patients, treated with traditional immunosuppression therapy (steroid ± cyclophosphamide) between 1988 and 1992.

Design and Methods. Our ITI protocol consists of three weeks of treatment with 1) human FVIII concentrates (30 U/kg/day for the 1st week, 20 U/kg/day for the 2nd, and 15 U/kg/day for the 3rd week), plus 2) iv. cyclophosphamide (200mg/day to a total dose of 2-3 grams), plus 3) methylprednisolone (100 mg/day iv. for one week and than tapering down the dose gradually over the next two weeks). The treatment of acute bleeding episodes in the two groups was not different. High purity and ultra-high purity factor VIII concentrates were used for the ITI. We performed aPTT and mixing tests before and after two hours of incubation, Bethesda inhibitor assay, porcine FVIII cross-reactivity, FVIII:C before and after FVIII administration (recovery), three times a week. The sex ratio and mean age (64 years for the ITI group versus 57 years for the controls), the initial and peak inhibitor titers, and residual FVIII: C values at the diagnosis were similar in the two groups.

Results. Eradication of the inhibitor occurred in 13/14 patients in the ITI vs. 4/6 patients in the control group. The main difference between the two groups was in the time needed for the complete disappearance of the inhibitor (4.6 weeks for ITI vs. 28.3 weeks for controls). In the ITI group we have observed only two relapses during the relatively long follow-up period (26 months), and in both cases the same re-induction protocol was successful again. No bleeding-related mortality occurred in this group in contrast to that of 33 % in the controls. Apart from the well-known adverse effects of glucocorticoid therapy, we have observed only one patient with transient cytopenia. We have not seen any adverse event which could be attributed to the use of FVIII concentrates.

Interpretation and Conclusions. We conclude that the ITI protocol described here is highly effective for the treatment of acquired hemophilia, induces quick therapeutic responses and favorably influences the underlying autoimmune disorder. We suggest that our ITI protocol is suitable for the eradication of idiopathic and autoimmune-associated FVIII autoantibodies in patients presenting with severe bleeding.
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Acquired hemophilia is caused by IgG1-IgG4 anti-factor VIII autoantibodies emerging either spontaneously (idiopathic inhibitor in the elderly) or in connection with pregnancy, various autoimmune and malignant diseases and drug therapy. The reported incidence of this rare condition is 0.2-$1.0/10^6$ population/year.[1] The bleeding manifestations are often dramatic with 80-90% major hemorrhage and 10-22% mortality rates directly or indirectly attributable to the inhibitor.[2]

The long-term management of acquired hemophilia is still controversial. It is possible that different treatments should be used for different subgroups of patients (a conservative approach for children, peripartum and drug-induced cases in whom spontaneous remission can be reasonably expected, and combined immunomodulatory therapy for idiopathic and autoimmune-associated cases).[3] However, indi-

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Table 1. Control group.

M/F	Age at dx	Underlying condition	Initial bleeding	res. FVIII:C	Initial BIA	rem. time*	rem. duration**	eradication treatment
F	36	PSS	sc., musc. hematomas	1	3200	–	–	CPH+st.
F	66	Idipathic	sc., musc. hematomas	2	610	8	36	st
F	27	Post-partum	Uterine	2	5.5	3	1	st.
F	85	Idiopathic	Hematuria	1	15	107	12	st.
M	69	Idiopathic	Pharyngeal	5	20	2	3	st.
M	62	Idiopathic	Femoral hematomas	1	437	–	–	CPH+st.
Mean	57.5			2	714	28.25	13	

*rem. time: time needed to achieve remission in weeks; **rem. duration: duration of remission (follow-up); BIA: Bethesda inhibitor assay

Table 2. ITI group.

Pt.	M/F	Age at dx	Underlying condition	Initial bleeding	res. FVIII:C	Initial BIA	rem. time*	rem. duration**
JM	M	70	Idiopathic	sc., musc. hematomas	3.5	21	3	58
JT	F	53	Idiopathic	sc., musc. hematomas	4	20	4	21
PD	F	62	Idiopathic	sc., musc. hematomas	3.5	8	3	53
FR	M	74	Gastric cc.	sc., musc. hematomas	1.4	19	2	20
Gy.Sz	M	55	Renal cc.+IFNα tx	Retroperitoneal	5	320	10	24
PF	M	79	Gastric cc.	Retroperitoneal	4	22	10	36
LL	M	65	Idiopathic	sc., musc. hematomas	2	30	3	35
EB	M	58	Psoriasis	Pharyngeal	1	60	3	43
IJ	F	6ç	Psoriasis	Retroperitoneal	1	1128	–	–
JB	F	49	Idiopathic+MGUS	Retroperitoneal	7	64	12	15
KL	F	75	Idiopathic	Femoral hematoma	14	28	3	25
MK	F	60	PSS	Brachial hematoma	16	10.3	2	18
JA	F	57	Idiopathic	CNS, intra-abdominal	9	6	4	7
AP	M	74	Idiopathic	Femoral hematoma	2	58	5	3
Mean		64			5.2	128.2	4.6	25.6

*rem. time: time needed to achieve remission in weeks; **rem. duration: duration of remission (follow-up); BIA; Bethesda inhibitor assay

viduals presenting with acquired hemophilia and severe hemorrhage need rapid and effective treatment. On the basis of some earlier experience with synchronization of plasma exchange therapy of various autoimmune disorders,[4,5] and some limited experience with the combined administration of factor VIII and immunosuppressive treatment,[6-8] we have developed a new aggressive protocol for the treatment of patients with acquired factor VIII antibody.

Design and Methods

We evaluated the results of 14 consecutive non-hemophiliac patients with factor VIII inhibitor treated in a single center with our ITI protocol between 1992 and 1999, comparing them to 6 historical control patients treated with the traditional immunosuppressive therapy (steroid ± cyclophosphamide) between 1988 and 1992 in the same setting.

The treatment of acute bleeding episodes was not different (PCC, FEIBA, porcine and human

FVIII concentrates, plasmapheresis and transfusions) in the two study groups.

Our ITI protocol consists of 3 weeks of treatment with:
I. human FVIII, 1st week: 30 U/kg/day,
    2nd week: 20 U/kg/day,
    3rd week: 15 U/kg/day, and
II. cyclophosphamide 200 mg/day to a total dose of 2-3 grams, and
III. methylprednisolone 1st week: 100 mg/day intravenously; 2-3rd week: tapering of the dose gradually.

I/II. were immediately discontinued if FVIII:C normalized within the 3 weeks of treatment. After the disappearance of the inhibitor no further maintenance immunosuppression was given.

High purity (Beriate-P, Hemoctin SDH, Koate-HP) and ultra-high purity (Octonativ-M, Hemofil-M) FVIII concentrates were used for the ITI. We performed aPTT and mixing tests before and after

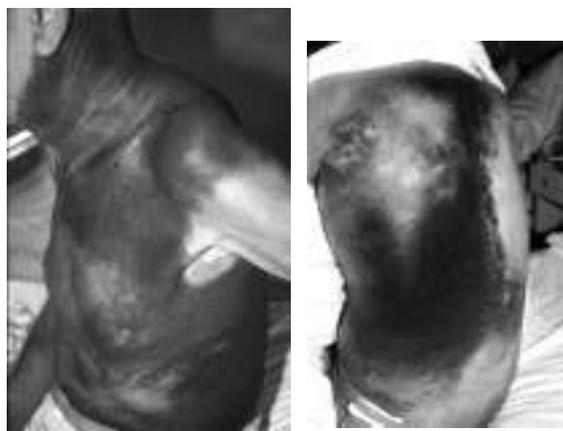**Figure 1. A giant life-threatening dorsal hematoma causing severe anemia in patient #1 of the control group.**

Figure 2 (left). Extensive subcutaneous hematomas in patient #4 of the ITI group.

Figure 5 (right). Gross intra-abdominal and retroperitoneal hematoma causing a life-threatening condition in patient #6 of the ITI group.

Figure 4. Subcutaneous and retroperitoneal hematoma in patient #5 of the ITI group.

two hours of incubation, Bethesda inhibitor assay, porcine FVIII cross-reactivity, FVIII:C before and after FVIII administration (recovery), three

Figure 3. Subcutaneous brachial haematoma in patient #7 of the ITI group.

times a week. The definition of success was the disappearance of the inhibitor in the Bethesda assay system and the persistent normalization of the FVIII:C value (i.e. >70% activity of the normal). The characteristics of the control and the ITI groups are demonstrated in Tables 1 and 2. The sex ratio, mean age at diagnosis, the initial and peak inhibitor titers and residual FVIII:C values were similar in the two groups.

Table 3. A summary of the patients' mean values.

Female/Male	11	9
Age at diagnosis:	62 years	(27-85 years)
Residual FVIII:C:	4.3%	(1-16%)
Initial human inhibitor titer:	304.6 BU	(5.5-3,200 BU)
Peak human inhibitor titer:	356.5 BU	(8-3,200 BU)
(maximal value after FVIII challenge)		
Initial porcine cross-reactivity:	12.4 IU/mL	(0-104 IU/mL)
(measured in 11 patients)		
Peak porcine cross-reactivity:	12.65 IU/mL	(0-104 IU/mL)
(measured in 11 patients)		

Some typical bleeding manifestations are shown in Figures 1 to 5.

Results

Typical courses of the ITI therapy are shown in Figures 6 and 7.

Eradication of the inhibitor occurred in 13/14 patients in the ITI group versus 4/6 patients in the control group. The comparison of the control and ITI groups is shown in Table 4. The main difference between the two groups was in the time needed for complete disappearance of the inhibitor (4.6 weeks for ITI vs. 28.3 weeks for controls). In the ITI group we have observed only two relapses during the relatively long mean follow-up period (25.6 months), and in both cases the same re-induction protocol was successful again. No bleeding-related mortality occurred in the ITI group in contrast to that of 33% in the controls. Apart from the well-known adverse

Table 4. Comparison of the control and ITI groups.

	No. of pts. who achieved rem.	Time needed to achieve rem. (weeks)	Duration of rem. follow-up (months)	MR	Bleeding-related mortality
Control	4/6	28.3 (2-107)	13	3/6	2/6
ITI	13/14	4.6 (2-12)	25.6	4/14	0/14

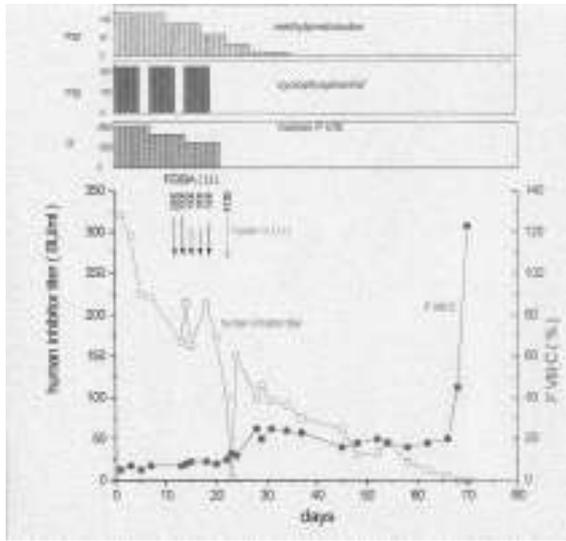*rem. = remission; MR = mortality rate.*

Figure 6. ITI therapy of patient #5 (bw: 92 kg). Example of high antibody titer and late remission.
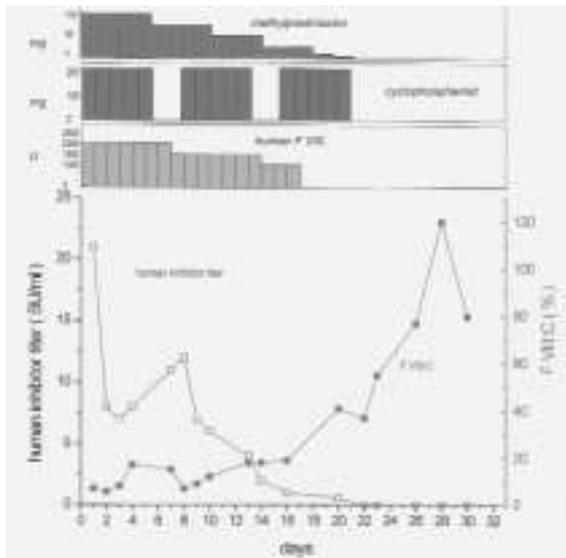

Figure 7. ITI therapy of patient #1 (bw: 90 kg). Example of quick response.

effects of glucocorticoid therapy, we have observed only one patient with transient cytopenia, which resolved spontaneously without any further consequence. We have not seen any adverse event which could be attributed to the use of FVIII concentrates.

Discussion

Immune tolerance induction regimens with human factor VIII concentrates are rarely if ever implemented in adult patients with auto-inhibitors, in contrast to alloantibody suppression, which is used primarily in young children with congenital hemophilia.[9] There has been a claim[9] that sufficient data are not available to determine the efficacy of ITI in patients with autoantibodies.

Our approach is based on the assumption that repeated administration of exogenous FVIII causes additional stimulation of the inhibitor-producing B-cell clones, making them more susceptible to the effects of cytotoxic drugs. The historical background of our hypothesis goes back to the late 80s, when repeated plasma exchanges synchronized with immunosuppressive therapy were tried in patients with progressive autoimmunity, in whom conventionally administered immunomodulatory treatments had failed.[5] This approach used the plasmapheresis-induced proliferation of pathogenic clones for partial clonal deletion by giving large doses of cytotoxic drugs during the assumed period of increased B-cell vulnerability.[4]

It is also known that in acquired hemophilia the endogenously synthesized FVIII is providing constant stimulation to the patient's antibody-producing cells. The administration of exogenous FVIII may have resulted in additional stimulation with a corresponding increase in the susceptibility of the immunocytes to the effect of the cytotoxic drug.[6]

In the original case-report massive doses of FVIII were given simultaneously with 1.5 g intravenous cyclophosphamide to a patient previously unresponsive to combined immunosuppressive therapy containing azathioprine, cyclophosphamide and methotrexate.

In later trials[7,8] patients were also infused with a single dose of FVIII concentrate, 50-100 IU/kg body weight, followed by cyclophosphamide 500 mg on day 1, and 200 mg/day on days 2 to 5; vincristine 2 mg on day 1; and prednisone 100 mg/day on days 1 to 5 (modified CVP protocol). This regimen was repeated every 3 to 4 weeks.

On the basis of these earlier experiences we have developed a new aggressive protocol for the treatment of patients with idiopathic or autoimmune-associated acquired factor VIII antibody and dangerous bleeding manifestations. In con-

trast to the earlier reports mentioned above, in our approach FVIII was administered daily in lower doses, simultaneously with cyclophosphamide and steroid aiming at quick disappearance of the inhibitor. After the conclusion of the three weeks of ITI therapy no further immunosuppression was given and if the FVIII:C normalized within the three weeks of treatment, cyclophosphamide and FVIII administration were immediately discontinued. The only exception was the single patient (#9 of the ITI group) who was not cured by the ITI protocol (and who presented with the highest initial inhibitor titer) but in this case further immunosuppressive attempts have also failed. No maintenance immunomodulatory therapy was administered to the patients treated successfully by ITI. In the two patients who relapsed, re-induction with the same protocol resulted in complete and long-lasting therapeutic success again.

Accepting that the rarity of the condition and the lack of published prospective trials make the prescription of definite therapeutic algorithms extremely difficult,[8] we conclude that the ITI protocol described above is highly effective for the treatment of acquired hemophilia, induces exceptionally rapid therapeutic responses and advantageously influences the underlying autoimmune disorder. We suggest that our ITI protocol is suitable for the eradication of idiopathic and autoimmune associated FVIII autoantibodies in patients presenting with severe bleeding.

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DISCUSSION 13   New protocol for immune tolerance induction in acquired hemophilia
Nemes L, (Budapest, Hungary)

SULTAN: These patients with auto-antibodies have a normal synthesis of factor VIII and so they have a lot of factor VIII in their circulation every day. Do you think 15 units/kg/day will change something in their status *vis à vis* factor VIII?

NEMES: I think this causes additional stimulation. This exogenous factor VIII has an effect and this is one of the factors why we had such rapid responses.

ALEDORT: We have all been struck by the difference in the bleeding areas with people with inhibitors versus hemophiliacs and your slides are classic. And yet we have never had any real understanding as to why people making factor VIII whilst at the same time inhibiting it on a regular basis bleed the way they do.

KESSLER: I would like to follow up Professor Sultan's question. You obviously showed decreased mortality and presumably decreased morbidity but what wasn't clear to me was whether that decreased morbidity begins immediately on initiation of the ITI or did it only begin after you were able to achieve an expanded factor VIII level beyond the baseline?

NEMES: Of course, I have no concrete evidence but I have the feeling that administering factor VIII has a beneficial effect even on the early bleeding complications but I repeat this is only a feeling.

KESSLER: Could there be a benefit even while we aren't seeing a factor VIII level come back. Maybe this is too strong a term but the anti-factor VIII antibody may be a surrogate marker for other autoantibodies that are truly causing the mucocutaneous bleeding. Otherwise it is difficult to explain this degree and this type of bleeding in the presence of circulating levels and that we follow the factor VIII levels and the titers because it's what we know how to measure. Perhaps, it's not the only antibody that's involved in the pathogenesis of this bleeding.

Regimens of factor VIII administration – continuous infusion vs. bolus

ERIK BERNTORP

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Abstract

The Malmö protocol for immune tolerance induction uses intermittent injections of factor VIII/IX together with intravenous IgG and cyclophosphamide. In order to increase the production of antigen-antibody complexes, and also to improve cost-efficacy, we applied continuous infusion instead of intermittent injection in 5 high-responders (3 hemophilia A and 2 hemophilia B). All treatment attempts failed. However, we conclude that continuous infusion may play a role in immune tolerance induction, and the treatment failures in our study could probably be explained by the fact that the patients were partially selected to be resistant cases.
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Key words: continuous infusion, factor VIII, factor IX, hemophilia A, hemophilia B

The Malmö protocol for immune tolerance induction (IT) includes high doses of factor VIII or IX (FVIII/IX), intravenous IgG and cyclophosphamide.[1] When the inhibitor level is at or above 10 Bethesda units (BU) at the start of treatment, the IT protocol is preceded by extracorporeal adsorption to protein A. In accordance with the results of the IT registry[2] we believe that it is important to administer high doses of FVIII/IX concentrates. This is done from the start of treatment, and when the inhibitor returns after about a week the dose should be increased, usually by shortening the interval between infusions. In this way the immune system is exposed to high antigen levels. In order to ensure constant high antigen levels, continuous infusion during IT is an attractive approach. In addition, delivery via a pump would facilitate product administration. In terms of pharmacoeconomics, constant infusion should also be an advantage for several reasons. It is superior to intermittent injections as it saves concentrate, as shown for surgery in hemophilia.[3] In particular, in products with a short half-life, constant infusion saves concentrate for pharmacokinetic reasons and is more cost-effective during regular prophylaxis.[4] In patients with an inhibitor, the half-life of FVIII/IX is very short and thus there is a pharmacoeconomic rationale for using constant infusion for IT. In the present study, the primary goal was to see whether constant infusion could be a way to induce IT in patients who either had been resistant to the traditional Malmö protocol or could be anticipated to be difficult to tolerize.

Design and Methods

The study material comprised 3 patients with severe hemophilia A (FVIII <0.01 units/mL) and 2 patients with severe hemophilia B (FIX <0.01 units/mL). In the 2 hemophilia B patients and one of the hemophilia A patients IT tolerance induction had previously failed, using the traditional Malmö protocol at ages of 4-10 years. In the hemophilia A patient, two additional attempts were made using continuous infusion, at the age of 4 and 8 years. The study protocol was as follows. When the inhibitor titer at start of treatment exceeded 10 BU, extracorporeal protein A adsorption was performed (one hemophilia A and one hemophilia B patient) to reduce the inhibitor levels to near zero. Then a bolus dose of FVIII/IX was given to raise the VIII:C/IX:C level to at least 1 IU/mL. Concomitantly with the bolus, continuous infusion was given, with the aim of keeping a constant level of around 1 IU/mL. Intravenous IgG was given for 5 consecutive days, day 4-8. Cyclophosphamide 12-15 mg per kg bw i.v. was given daily on days 1-2 and then p.o. on days 8-10. In two patients, cyclophosphamide was substituted by prednisone 60 mg per m^2 per day p.o. When the VIII:C/IX:C plasma level decreased and the inhibitor increased, the rate of continuous infusion was increased by 2-3 times. The concentrates used for hemophilia A were, Recombinate© (Baxter), Kogenate© (Bayer) or Emoclot© (AIMA) and for hemophilia B, Mononine© (Centeon).

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Hemophilia A severe 4 years, 15 kg. High-responder >450
BU. Malmö protocol with continuous infusion of FVIII
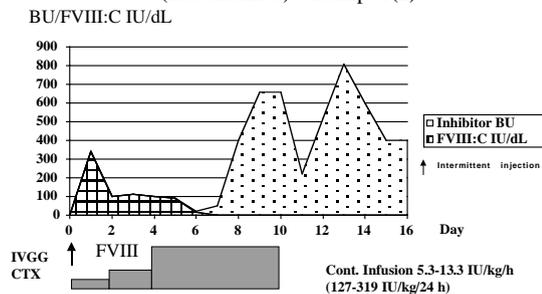(Recombinate). Attempt 1(2)

Figure 1A. Severe hemophilia A, 4 years, 15 kg. High-responder >450 BU. Malmö protocol with continuous infusion of FVIII (Recombinate). Attempt 1 (2).

Hemophilia A severe 8 years, 23 kg. High-responder >450
BU. Modified Malmö protocol (CTX replaced by
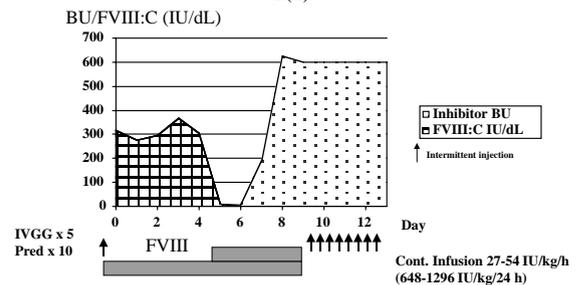prednisone 60mg/m2/day po) (Recombinate). Attempt
2(2)

Figure 1B. Hemophilia A severe 8 years, 23 kg. High-responder >450 BU. Modified Malmö protocol (CTX replaced by prednisone 60 mg/m2/day p.o.) (Recombinate).

Results

Hemophilia A

Three hemophilia A patients were treated. In one patient, who was a high-responder with a historical peak above 450 BU, the first attempt was performed using the original Malmö protocol with cyclophosphamide but with the intermittent injections replaced by continuous infusion (Figure 1A). The inhibitor reappeared after 6 days and had increased to 800 BU after 2 weeks. The number of FVIII units given was 5.3-13.3 units per kg per hour. Four years later, this patient was treated again but now the protocol was modified to include prednisone instead of cyclophosphamide (Figure 1B). The FVIII level during the first few days was kept higher than during the first attempt, approximating 3 IU/mL, which was achieved by giving a continuous infusion of 27 units per kg per hour. However, the VIII:C level dropped after only 4-5 days. The infusion was doubled but a brisk anamnestic response was seen, reaching a peak of around 600 BU, and remaining at this level until day 13 of the observation period. As in the first attempt the treatment was discontinued, as the IT was obviously a failure, according to previous experience with the Malmö protocol.[5]

Of the other 2 hemophilia A patients, one was a high responder with more than 300 BU. He was treated using protein A adsorption followed by continuous infusion of IVgG and cyclophosphamide. The VIII:C level went up to more than 2 IU/mL during the first days but then declined. The inhibitor reappeared after 6 days and peaked at 1,000 BU. The treatment was discontinued after 8 days, as it was an obvious failure in this case also. The number of units of FVIII

Hemophilia B severe. 5 years, 24 kg. High-responder >90 BU. Malmö protocol with
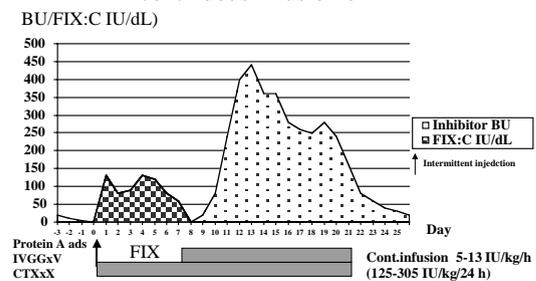continuous infusion of FIX

Figure 2. Hemophilia B severe. 5 years, 24 kg. High-responder >90 BU. Malmö protocol with continuous infusion of FIX.

given was 6-29 per kg per hour.

In the third patient cyclophosphamide was replaced by prednisone. The VIII:C level was kept very high, around 5 units per ml during the first 6 days, but the inhibitor reappeared after a week and peaked at 70 BU on day 14, exceeding the historical peak of 47 BU. The inhibitor declined to a plateau of around 40 BU, and the patient was kept on a Bonn-like regimen. He became tolerant one year after start of treatment.

Haemophilia B

Two patients were treated as reported earlier.[6] Both patients had already been treated unsuccessfully according to the Malmö protocol. Both

were high-responders, with historical peaks of above 300 and 90 BU, respectively. In the first patient (Figure 2), the FIX concentration could be kept at around 1 IU/mL during the first 6 days, with a continuous infusion rate of 8 units per kg per hour. IX:C dropped to zero on day 7, and the continuous infusion was increased to 25 units per kg per hour. The inhibitor reappeared, peaked on day 12 at more than 350 BU, and levelled out at around 250-300 BU. The treatment was considered a failure and stopped on day 23.

The second patient was pre-treated with protein A adsorption, after which FIX:C in plasma could be kept at a high level of around 1 IU/mL. However, the level dropped to zero on day 8 and the inhibitor reappeared, peaking at 450 BU. The treatment was stopped on day 21 because of failure. No side-effects of the treatment were seen.

Discussion

We have thus treated 3 patients with hemophilia A and 2 patients with hemophilia B using the Malmö protocol, but with continuous infusion of FVIII/IX instead of intermittent injection. In 2 episodes, cyclophosphamide was replaced by prednisone in order to avoid potential long-term side-effects of chemotherapy. In one patient with hemophilia A 2 courses of treatment were given. Immune tolerance induction had already been tried unsuccessfully in 3 of the patients. A brisk inhibitor response was seen in all cases, with levels rising to the historical peak or beyond. There are only scarce reports in the literature regarding continuous infusion during IT. Kucharski and co-workers[7] treated 15 high responders (7-1,400 BU) with the modified Malmö protocol, in which continuous infusion was given for 1-6 weeks. For 5 patients the authors report a very good result, meaning that they considered successfully induced tolerance;

in 5 patients a fairly good result was obtained, i.e. partial tolerance and 5 patients did not respond. Thus from currently available data it does not seem that continuous infusion during IT using treatment schedules based on the original Malmö protocol improves the success rate. However, as previously explained, continuous infusion is an attractive mode of delivery for clotting factor concentrates and if technically feasible the role of continuous infusion during IT should be further explored. The study material used was highly selected and probably more resistant to treatment than the average inhibitor patient. Attempts in a more non-selected patient group seem reasonable.

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DISCUSSION 8 **Regimes of factor VIII administration-continuous infusion vs. bolus**
Berntorp E, (Malmö, Sweden)

OLDENBURG: My comment may not be directly related to continuous infusion therapy but yesterday you showed some risk factors for the success of immune tolerance which were that the success rate is correlated with inhibitor titer at the start of therapy and with the dosage. One could speculate that it's free factor VIII antigen that induces the active immune response and one

can also speculate that a bolus of factor VIII and the circulation time of free factor VIII may be higher than when it's given continuously; in fact, factor VIII is bound directly by an antibody and so it could be less effective to induce an immune response than factor VIII that is given by bolus and may circulate longer and hence can produce an active immune response in the sense of anti idiotypic antibody creation.

BERNTORP: On the other hand when we give intermediate injections they are given very frequently and so we never go below 40% of factor

VIII.

MARIANI: It would seem more logical that continuous infusion would be more useful in low responders because we can overcome the antibody and we are more likely to find free factor VIII in the blood stream than in high responders who after a few days we will have a swift anamnesis.

ALEDORT: Isn't it possible that through continuous infusion you are continually absorbing antigen and antibody complexes and there isn't sufficient immune suppression by antigen overload. It's hard to be certain but it would be interesting to have some idea if we are going to interest ourselves in this type of issue. We could possibly look at the kinetics of those antigen-antibody complexes in given patients to see how they disassociate and associate and if you really do get free antigen and then to ask how important free antigen is versus complexing. The answers to these questions are unknown to me but I believe that if we are going to look at this issue we should look at it not only in the final clinical setting but we have to correlate it in some way to laboratory findings.

RYPERT: When you give a bolus injection you can calculate also exactly how much factor VIII you have to give when the inhibitor is less than 10 BU. You also get a factor VIII response when you give a very high dose and if you continue twice daily with factor VIII you see exactly the same anamnesic response as you see in continuous infusion. I don't think it makes much difference whether you give it continuously or twice daily.

BERNTORP: I don't think that in the protocols we have used that it makes very much difference but still you keep the average level of free factor VIII which you can get free during the first week higher. It's a matter of dosage but I think it is more cost effective to give it by continuous infusion.

MARIANI: And then there are of course the organizational aspects to be taken into consideration, such as the need for hospitalization.

Immune tolerance by intermittent factor VIII boluses in two high responder hemophilia A patients

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Abstract

Immune tolerance (IT) in hemophilia A patients with anti-factor VIII antibodies is generally based on daily factor VIII administrations. Here we report the preliminary results of an immune tolerance regimen based on recombinant high-dose (400 U/kg) factor VIII boluses administered at 48-hour intervals. Two high responder hemophilia A patients aged 2 and 3 years received this treatment without the need of permanent venous access. In both cases the IT regimen caused an anamnestic response of less than three weeks' duration and an antibody reduction to less than 5 Bethesda units was achieved in about 8-10 weeks.
In the child with a more prolonged follow-up the inhibitor became undetectable after four months and factor VIII recovery at 6 months was > 85%. This intermittent high-dose regimen seems to be effective in rapidly inducing immune tolerance and seems particularly suitable for very young children in whom it may be useful to avoid the risks and to reduce the psychological burden of permanent venous access.
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The development of anti-factor VIII/IX antibodies, which is relatively common in hemophilia, complicates the treatment and clinical course of this disease. Thus, achieving immune-tolerance (IT) to factor VIII/IX is a major therapeutic goal in hemophiliacs with inhibitors. Infusions of high-dose factor VIII/IX constitutes the common IT strategy while other therapeutic options have a more uncertain role but may be useful in selected patient groups.[1] Most regimens of factor VIII/IX administrations have relied on daily or continuous administrations of factor VIII/IX. In hemophilia A, data collected from different protocols by the International Registry of the Immune-Tolerance Protocols, have made it possible to establish that

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factor VIII dose, young age and early treatment are the main determinants of success rate.[2]

A regimen based on low-dose FVIII boluses administered every other day was shown to be effective, particularly in young patients with a maximum antibody titer of < 40 Bethesda units.[3] Thus, FVIII infusions given every other day may be as effective as daily administrations and provide obvious practical advantages. This strategy may reduce the need for permanent venous access, particularly in very young children. Permanent venous access constitutes a not to be underestimated psychological burden and, in addition, is often complicated by infections. Here we report a pilot experience with a high-dose IT regimen based on 400 U/kg recombinant factor VIII (Recombinate) boluses given at 48-hour intervals. This regimen was administered to two very young children through peripheral veins and appeared to be effective in rapidly lowering the inhibitor titer.

Case Reports and Results

The clinical characteristics of the two patients are reported in Table 1. Both were high responder hemophilia A children referred to the Hemophilia Center of the A. Gemelli Hospital in Rome. In both cases an IT regimen was started within 5 weeks of the recognition of the inhibitor and consisted of high-dose (400 U/kg) FVIII boluses administered every other day. The preliminary results of this regimen are summarized in Table 1. The inhibitor rise after the start of IT lasted 2-3 weeks and an antibody titer < 5 Bethesda units was reached in 8-10 weeks. The clinical course of the two children is reported in Figure 1. An evaluation of FVIII pharmacokinetics in the child with a more prolonged follow-up was performed at six monthly intervals. The evaluations performed at 6 and 12 months showed a good FVIII recovery (>85%) and reduced half-life ($t_{1/2}$ at 6 and 12 months = 4.3 and 4.25 hours, respectively). After the first year the dose of FVI-II was reduced to 200 U/kg/48h.

Table 1. Results of treatment in 2 hemophilia A patients.

		Inhibitor level (BU)		Treatment Response (months)	
Case	Age	Before	Peak	Inhibitor <5BU	Not detectable
1	17 months	20	251	1.5	2.5
2	3 years	45	164	3	n.a.*

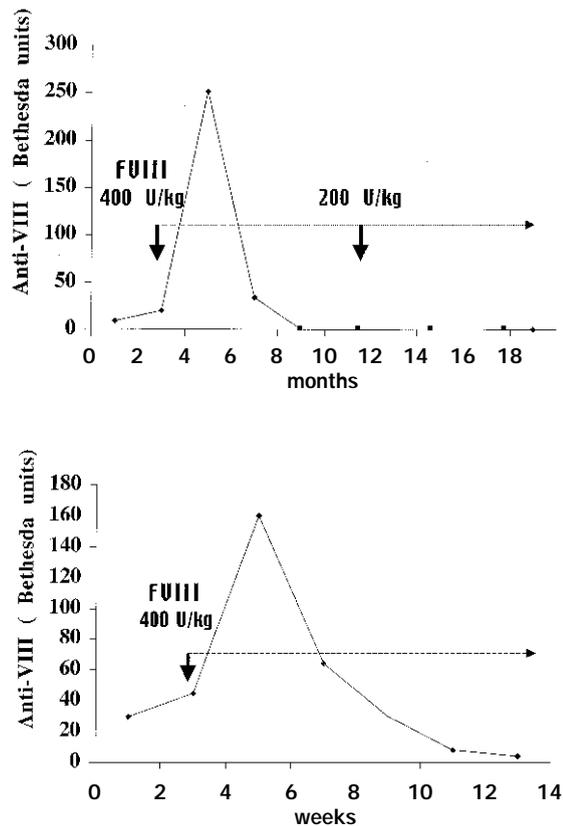*Not achieved after a 4-month follow-up.

Figure 1. Tolerance induction with high-dose (400 U/kg BW every other day) intermittent boluses of FVIII given to a 17-month old (Figure 1A) and a 3-year old (Figure 1B) with hemophilia A.

Discussion

The mechanisms by which factor VIII infusions are able to induce IT in hemophiliacs with anti-FVIII antibodies are still largely elusive. Saturating the inhibitor capacity in order to have circulating free factor VIII is thought to be critical for successful IT induction. Consequently, most IT regimens rely on daily administrations of high-dose FVIII. A low-dose regimen administered every other day has also been successfully tested although, in this case, the time required for treatment success was found to be relatively long.[3] In our patients we used intermittent boluses at a higher dosage in order to induce IT more rapidly. The use of a regimen based on intermittent infusions seemed particularly suitable for our very young patients in order to avoid a permanent venous access.

The results obtained with our high dose intermittent protocol, although preliminary, seem encouraging. The rapid lowering of the inhibitor titer in both children suggests that this treatment regimen is very effective in the induction phase of immune-tolerance. Thus it may be considered for comparative evaluations with other regimens, particularly in young children in whom this approach may help to avoid the risks and psychological burden of permanent venous access.

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DISCUSSION 9	Immune tolerance by intermittent factor VIII boluses in two high responder hemophilia. A patients

Landolfi R, De Cristofaro, Lazzareschi I, Riccardi R (Rome), Mariani G (Palermo)

BEARDSLEY: During the period of time of the anamnestic response did you need to treat these children for bleeding episodes and did you use VIIa?

LANDOLFI: Yes, we did in both children. Both of them had a major bleeding episode before they had treatment and were treated again during the first two weeks of the immune tolerance. The interesting thing was that in both cases the bleeding manifestation completely disappeared after four months' treatment. They had disappeared well before the antibody titer had disappeared.

MARIANI: How did you treat these children?

LANDOLFI: With recombinant VIIa.

Side effects during immune tolerance induction

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The development of inhibitors is currently the most severe complication of hemophilia A. Inhibitors render normal doses of factor VIII for treatment of bleeding episodes inefficient and frequently require other agents to control acute bleeding, which do not have the same therapeutic results as factor VIII in a non-inhibitor hemophilia A patient.

The elimination of the inhibitor by immune tolerance induction (ITI) has, therefore, nowadays become the preferred treatment for these patients, more than two decades after the first presentation of a treatment regimen by Gormsen and Brackmann (1977), which is known as the Bonn protocol. During this time experience in treating this type of patient has been accumulated and successes as well as complications have been documented by hemophilia treaters all over the world.

Materials and Methods

For acute bleeding episodes in patients with low titer inhibitors, higher than normal doses of human factor VIII can be efficient as can porcine factor VIII if cross-reactivity is low. Until the mid 1980s mainly low and intermediate purity factor VIII without virus inactivation was available, subsequently virus inactivated and high purity plasma-derived factor VIII became ever more used as the availability increased and in the early 1990s recombinant factor VIII was licensed.

Prothrombin complex concentrates (PCC) and later activated prothrombin complex concentrates (aPCC) such as Autoplex and FEIBA have been used since the 1970s when factor VIII was not efficient.

FEIBA is also used during the first phase of the Bonn protocol when bleeding occurs despite high factor VIII dose application (100-150 IU/kg/BW/twice a day).

A permanent venous access ("port") has often been implanted, especially in children, to permit the frequent application of factor VIII during ITI.

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For enhancement of efficacy to reach immune tolerance some regimens use immune adsorption columns which reduce the inhibitor content from the patient's plasma and give short courses of immune suppression (Malmö protocol). More recently recombinant activated factor VII has been added to the treatment possibilites for patients with inhibitors.

Discussion

As shown in the Tables, severe side effects occurred with the use of non-virus-inactivated FVIII concentrates due to viral infections. Until the early 1980s these infections were mainly hepatitis B and hepatitis C, whereas HIV infections have dominated the last two decades.

The early concentrates were also of low purity and not blood group specific, which led to hemolysis at high doses. Hemolysis can still be observed today if factor VIII concentrates used in ITI contain, due to their manufacturing process, antibodies reacting with the recipient's blood group antigens.

Due to high purification and efficient virus inactivation of plasma-derived factor VIII and the introduction of recombinant factor VIII concentrates, viral infections have not had an impact on hemophilia treatment in recent years. Independent of plasma or recombinant origin, allergic reactions to factor VIII preparations may infrequently occur.

Another category of side effects is associated with the use of PCCs and aPCCs, especially at high doses, when symptoms of disseminated intravascular coagulation and thomboembolic events can complicate the ITI.

The use of columns in ITI has specific side effects which can be related either to the nature of the column (e.g. Staphylococcal protein) or to the plasma separation procedure: paresthesia due to calcium binding citrate has been observed frequently.

Poor venous access especially in very young ITI patients has necessitated the implantation of ports or catheters. This adds a risk of infection of the device which in turn negatively influences duration and outcome of the inhibitor eradication therapy.

Table 1a. Type and frequency of side effects during ITI.

	FVIII	aPCC
Success rate	Di Michele (1998)* 87-73% Freiburghaus (1999)* 62.5%	
Cerebral hemorrhage /death	Brackmann (1984)* 3/46 Scheibel (1987)* 1/11 Brackmann (1996)* 3/81 Knöbl (1998) 1/2	
DIC signs		Scharrer (1982) Kreuz (1995)* 2/11 Scharrer (1984) Brettler (1993)* Kreuz (1994)* 2/11 Dalsgaard (1984)° Knöbl (1998) 1/2
Thrombosis (heart)		Freiburghaus (1999) 1/16
Hepatitis B	Brackmann (1984)* 2/46 Brackmann (1996)* 3/81	
HIV Infection	Brackmann (1996)* 1/81	
Virus Infections, HIV	DiMichele (1998)*	Brettler (1993)*
Transaminases	Brackmann (1984)* 2/46 Scharrer (1982) Brackmann (1996)* 13/81	Scharrer (1982)
Hemolysis	Brackmann (1984)* 1/46 Scheibel (1987)* 1/11 Brackmann (1996)* 1/81 Freiburghaus (1999)* 1/16	
Skin rash	Scheibe] (1987)* 1/11	
vWF deficiency	Rock (1996)°	
T/B-cell/change	Scharrer (1982) Gago (1984)°	Scharrer (1982)
Microadenopathy	Gago (1984)°	
Blood proteins	Gago (1984)°	
Anaphylactic inhibitor	Harper (1995)°	
Allergic reaction	Brackmann (1996)°	1/81
	Porcine FVIII	
Pyrexia	Hay (1990)	
Thrombocytes	Hay (1990)	

Review;°Case Reports.

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Table 1b. Type and Frequency of side effects during ITI.

	Port	Immuno-adsorption column
Infection	Warrier (1993)* 2/7 Petrini (1998)° Kreuz (1995)* 2/11 Smith (1996) 1/4 Brackmann (1996)* 4/? Brackmann (1998)* 3/3 Freiburghaus (1998) Ljung (1998) 5/11 Kreuz (1994)* 2/11	
Paresthesia (Ca²⁺)		Watt (1992)* 10%
Headache		Freiburghaus (1998)
Hypertension		Freedman (1992)° Freiburghaus (1998) Watt (1992)* 5%
Plasma proteins		Mondorf (1991)°
Nausea		Watt (1992)* 10%
Staphylococcus Infection		Mondorf (1991)°

Review;°Case Reports.

    twenty years of the 'Bonn Protocol'. Vox Sang 1996; 70 (Suppl 1):30-5.
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DISCUSSION 16 Side effects
Brackmann HH, (Bonn, Germany)

HAY: When we were designing our trial we minimized for product type and to be frank with you without any great confidence that the product type would have made much difference. Nevertheless, in the final analysis it may turn out that that is one of the more important design characteristics of our study because if the differences are marked in our study as appears from your results, then our study would have the power to show a statistical difference. The other thing is that line infections are obviously a major problem and we will be looking at those very closely and at their influence on the outcome.

LUSHER: I think it would be quite interesting as we get more and more information on more patients because our experience in Detroit is certainly different from yours as we are using predominantly recombinant factor VIII for immune tolerance (and in a couple of instances monoclonal purified plasma-derived). We've had an 80% success rate. So, we get larger numbers of patients from various places and it becomes very difficult to decide what the influence of recombinant versus something else is.

BRACKMANN: Maybe if we look at the patients with port infections who have several times had infections we have to be careful not to draw too many conclusions about this because the numbers are really too small. For us it's impressive because in the past we were so successful and now we aren't. Therefore, we are seeking the answer to this question.

ALEDORT: Clearly the numbers are small but I think it would be interesting, Dr. Brackmann, if you tried to do a regression analysis to see what are the factors which may have played a role since there were differences, for example, in the time to start immune tolerance, the level they started at, the regimens and a whole variety of things but I don't think that it's consequential that inflammation and infection may in some way make tolerance more difficult. I don't know the answer to that, but I think the only way that we're going to do that is by performing some kind of meta-analysis and a regression analysis which looks for individual determinant factors.

MARIANI: I would like to mention that we made an analysis before 1990 and after 1990 and there were no differences whatsoever. The recombinant treated patients are too few to draw any definitive conclusion.

DI MICHELE: Notwithstanding the issue of recombinant, was there a shift towards higher purity products after the 1990 group?

MARIANI: That was our idea. We thought that there might be differences because of the new concentrates, whereas we were unable to find any difference in the success rate and the other parameters we evaluated.

Immune tolerance: a nursing perspective

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Since immune tolerance (IT) therapy was first introduced 25 years ago,[1] several versions of the original protocol have been developed to eliminate factor VIII and factor IX inhibitors.[2] The protocols vary in dose and frequency of factor administration, in the use of additional drugs, and in the use of supplemental clotting concentrates. However, all immune tolerance protocols entail frequent intravenous infusions and require strong commitment on the part of patients and families. Nursing participation in IT can enhance the program and contribute to a successful outcome.

Nurses provide essential elements of clinical care to patients with hemophilia who develop inhibitors. Often, nursing assessment of a patient's lack of response to treatment leads to the initial diagnosis of inhibitors. Once an inhibitor is identified, nurses work closely with physicians and the family to develop a plan of care for the patient. They help in the selection of appropriate candidates for immune tolerance induction, conducting patient/family education, assessing venous access, assisting in the management of acute bleeding episodes during induction, and monitoring patient response to therapy. Nursing interventions are critical in helping families meet the challenges required for a successful outcome.[3]

While data have been collected about the success of various prescribed regimens for immune tolerance, little information is available regarding nursing perspectives or about facilitating family participation in such a protocol. To identify the challenges and determine successful nursing strategies, input was requested from hemophilia nurses with experience in IT.

Methods

An informal questionnaire was developed and sent to 115 nurses at Hemophilia Treatment Centers (HTCs) in the United States. The 14-item questionnaire requested information about nurses' experience working with patients on IT programs, including the number of patients begun on IT, and the number who successfully completed, discontinued or failed therapy. Participants were asked to rank issues related to IT in order of importance. Five open-ended questions elicited information about patient adherence, financial considerations, venous access and central venous lines, patient/family education, and implications of the patient's age at the time of induction. Finally, nurses were asked to discuss psychosocial, educational, and nursing considerations in patient selection and preparation of families, barriers to care, and strategies to promote optimal participation and successful outcomes.

Results

Thirty-one nurses from 22 states and the District of Columbia completed and returned the survey. These nurses represent experience with 176 patients who have begun IT induction.

All survey participants noted patient/family selection and preparation of the family as important components of successful immune tolerance induction. Consideration must be given to the readiness of the family to undertake a demanding medical intervention. Both parents, if available, must agree to and support the plan, while recognizing the importance of adhering to the protocol; once started, IT requires total commitment. In single parent homes, it is helpful to seek another family member or friend who can support the parent in this endeavor. Family members need to understand the expected duration of the induction, including expectations about continuing maintenance when the inhibitor is no longer detected.

The age of the child is an additional consideration in patient selection, according to 88% of nurses surveyed. Age has an impact on venous access and types of central venous lines that may be recommended. Whatever the access, the child must be able to co-operate with infusions; babies often require at least two adults to assist in treatment, even with central lines. Older boys may help with treatment, or self-infuse, but often tire of the daily infusions and can resist

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following the prescribed protocol. All children will require age-appropriate explanations about the protocol and the importance of maintaining the treatments.

Compliance and venous access were identified by 90% of the nurses as the most critical issues to consider when preparing families for immune tolerance. Patient compliance is crucial to the effectiveness of any therapeutic plan. Without patient participation in the medical plan, the goals of treatment cannot be achieved.[4] However, rates of non-adherence to medication schedules for patients with chronic illness are reported to be about 50%.[5] Compliance is influenced by several factors, including the patient's motivation, the complexity of the medical regimen and the ease in which the new activity can be incorporated into the patient's lifestyle.[6] As IT protocols require frequent intravenous infusions for children, compliance can be difficult. As disruptions in the protocol are likely to yield poor results, clinicians are challenged to assist families with maintaining the desired schedule and dose of factor replacement.

Adherence to the demands of an IT protocol can be enhanced through educational and behavioral approaches. This begins with the development of a trusting partnership with the family, promoting skill acquisition and providing information that enables them to make an informed choice about treatment.[7]

Financial and insurance barriers were reported as significant by only 7% of the participants. Most comments indicated that, with education of insurance companies and primary care providers, coverage for IT is usually obtained. However, nurses emphasized the importance of assisting families with insurance issues and adjusting treatment and visit schedules to minimize disruption of parental employment.

Discussion

Patient education, noted to be essential by all nurses, begins before IT is initiated and continues throughout the program. Families need a basic understanding of inhibitors and how their continued presence can complicate treatment of bleeding episodes. The need for faithful adherence to the IT protocol and the potential implications of disrupted doses should be clear to patients and families. Details about the protocol to be used should be clearly explained and written down for further reference. Administration of the newly prescribed factor concentrate must be reviewed with the family; often storage requirements and rate of infusion differ from those of the patient's current product. Families need to recognize the extent of the commitment needed for a successful outcome; the regular infusions, explicit details about laboratory monitoring, follow up visits, and expectations about recovery studies. The potential duration of the protocol, the guidelines for dose adjustments, and parameters for determining success or failure of the protocol should be clarified at the outset.

Management of acute bleeding episodes during IT is an important topic to discuss before starting IT; the treatment plan requires revision and review throughout the program. Families may need help in understanding the need for two inventories of factor concentrate; one for IT, another for use in treatment of acute bleeding.

Venous access is a critical issue for children on IT and is a patient teaching challenge. If treatment is to be given via peripheral access, parents or caregivers will need training and support in venipuncture. Most young children, however, will require a central venous access device. Considerable education of patients and families about different types of lines with risks and benefits of each is needed. Line placement raises issues about hemostasis for the procedure. Families and hospital staff will require education about the procedure and the factor replacement plan, particularly if products less familiar to the staff are considered. Caregivers will need intensive education about the care and access of the line. All caregivers will require education and reinforcement about sterile technique and standard precautions. Simple, written instructions with illustrations may be helpful and less experienced families may benefit from home nursing visits until the parents and the child are more comfortable with the procedure.

While patient education is integral to IT and helps support adherence to the protocol, information alone is not sufficient. Usually, families need additional support to promote optimal participation in IT. Strategies include eliciting patient/family feedback about the protocol, adjusting the treatment schedule to consider the family lifestyle, providing clear, written instructions, development of cues as reminders of scheduled treatments or interventions, and negotiating a reward system for children participating in IT. A good relationship with the family promotes adherence to medical regimens such as IT. This can be enhanced by regular phone calls to the family to provide encouragement and identify potential problems early.

The use of nursing clinic visits during IT can contribute to the success of the program and serves several purposes. Nursing visits may allow for more flexible scheduling, minimizing disruption to the family and promoting school and work attendance. These visits allow for a review

of progress and permit preparation of written schedules for infusions, follow-up visits and laboratory studies. Laboratory results can be reviewed with the family and potential changes in protocol can be discussed. Issues with factor product supply can be identified and addressed. These visits provide an opportunity to review problems with access and technique, to reinforce teaching and to encourage the patient and family. Review of treatment records is helpful to determine possible variations in scheduled or missed infusions. This information, along with product inventory review can help identify problems with adherence and allow for early intervention to promote increased patient participation.

An important aspect of regular nursing visits and telephone contact is the provision of ongoing support for patients and families. It is important to assess the family's concerns, answer questions, and provide whatever assistance is necessary. Encouragement is sometimes needed to maintain the commitment of the family to a rigorous, invasive, expensive and often frustrating intervention. Nurses must demonstrate their own commitment to the family in helping them achieve the goals of therapy.

Conclusions

Experienced nurse clinicians recognize adherence and venous access as the most critical components of IT. Survey responses demonstrate that nurses participate fully in the clinical implementation of immune tolerance programs, including patient selection, dose determination, assessment and treatment of acute bleeds, evaluation of laboratory studies, and monitoring patient response to therapy. Nurses, however, perceive that their most important contributions relate to education and preparation of families, identification of potential problems, assisting in the maintenance of appropriate venous access, and promoting the level of adherence needed for successful immune tolerance induction.

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DISCUSSION 10   Immunetolerance: A nursing perspective
Butler R.B, (Philadelphia, USA)

BRACKMANN: The situation is a bit similar in the UK where nurses have a very high responsibility. In Germany, for example, as in many other European countries the situation is different. All of these aspects help to assure the physician.

Immune tolerance: the parent's perspective

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Abstract

A parent whose child affected by hemophilia develops an inhibitor has to cope with strong emotions and anxieties related to the acceptance of this *second diagnosis* when *just* having severe hemophilia seems nothing compared to having antibodies that neutralize factor infusions and the possibility of uncontrolled hemorrhages. During the initial stage of adaptation to this frightening reality, a parent needs constant support by the Center staff. The period during which medical personnel and parents decide about the possibility of putting a child on an immune tolerance protocol is a crucial one; if parents feel they have been well informed about this sort of treatment and feel adequately prepared to handle situations which may arise, they will take an active role in the care of their sons and maintain a positive attitude. The fears and worries are many, the greatest of which is the constant dread of a negative outcome. The commitment of staff during the formal period of instruction, the use of a local anesthetic, play therapy and the very young age of the child were variables that made peripheral, home infusion in a 1$^{1/2}$ old on immune tolerance possible. The needs of siblings, of the parents themselves and the strong bonding between mother and child are only but a few of the psychological aspects related to immune tolerance. ©2000, Ferrata Storti Foundation

Immune tolerance induction is most probably viewed somewhat differently by a parent than by a nurse or a clinician in that the emotional, practical and psychological implications related to this sort of treatment are many. These so-called subjective side effects of dealing with inhibitors and immune tolerance can influence the child's and the family's compliance with regards to the necessary and frequent infusions.

When a parent is given the news that his small child has developed an inhibitor against a coagulation factor, he/she may react to this *second diagnosis* with feelings of sadness, depression and anger. A parent realizes perfectly well that inhibitors are a medical challenge and this, to them, is very frightening. Giving parents thorough written information on inhibitors and immune tolerance at a level they can easily comprehend is very useful in that they have the necessary time to learn about this new reality. Not knowing what may or may not happen to one's son now, and in the future, is cause of much distress for the family; a worried and depressed parent is not a good example for his children, whether hemophiliac or not. The particular features of the antibodies, whether they are high titer or low titer, must be explained to the parents as must the way their son's immune system reacts to factor challenge: if their son is a high responder or a low responder.

The decision to start immune tolerance induction should be preceded by a thorough discussion regarding risks and short- and long-term benefits of eliminating the inhibitor. In our case the decision was taken immediately: to us, the most obvious treatment for hemophilia was the total prevention of all bleeding episodes and this could only be achieved if our son could once again benefit from factor infusions. We also had to pave our son's way towards the future possibility of gene therapy.

All parents constantly worry about things that may physically or psychologically harm their children. Parents of children with hemophilia and high titer/high responding inhibitors are no exception. These worries range from the unpredictable outcome of tolerance to the possibile

emotional consequences on the child caused by such frequent infusions and the relationship with the parent who actually administered these infusions. Will his relationship with that parent change? Will he no longer trust his parents, the people who more than anyone else should protect him from harm and pain? The main practical worry is that of venous access. Other preoccupations concern the ordering and storing of factor and meeting the needs of the other members of the family. Extended family reactions, to the parents' decision of their child being put on an immune tolerance protocol, may influence the way parents feel about their decision. If the reasoning behind the decision is clearly explained, then the other family members can accept this treatment more easily.

Immune tolerance needs adequate venous access for the frequent factor administrations. The decision to use an implanted venous access device or infuse through peripheral veins is a very difficult one for parents. Peripheral infusions in immune tolerance are a possibile but not very popular option. The commitment of the Center staff in instructing the parents on venipuncture technique, the use of a local anesthetic, play therapy and the very young age of the child were all variables that made peripheral home infusions in a one and a half year old on immune tolerance possibile. Parents can learn to administer factor themselves even when the child is very young; the learning process should be gradual and parents not forced to try until they are ready. Constant emotional and practical support given by the hospital during the first period of home infusion is very important. The question of how many veins one can use in a small child has no definite answer. But in our case the answer was this: that many that we were willing to search for.

Infusions in a young child are best given in a calm environment, in the same place and roughly at the same time.

Immune tolerance can be extremely stressful for the whole family; moods would change easily with bad infusion days during the first part of the learning process and the constant thought that my son would not achieve tolerance was always on my mind. One particular thing that made me sad and angry at the same time was the medical personnel in general and others repeatedly telling me that my child would suffer less with a Port. How could they know? Parents already have to take very painful decisions for their child's health; they absolutely do not need totally useless comments like the one above.

The siblings of the child on immune tolerance need a lot of attention and reassurance. They too are very worried about their brother's health even though they may not say so. A mother who infuses her child so frequently establishes a very deep relationship with her son.

Immune tolerance has proven to be a difficult but extremely useful stepping stone towards subsequent prophylactic treatment.

DISCUSSION 11   Immunetolerance: the parents perspective
Gargallo B, (Rome)

BERNTORP: In our country, which is very small in terms of its population, we have developed a network for families who have children with an inhibitor and I think this kind of network is very much appreciated among the parents. They meet together with the professionals at least once a year and they also meet without them. I saw that you are similar in that you have a contact family but I wonder if there is a network for families with children with an inhibitor in other countries because I think this is a major advantage in handling the psycho-social problems.

GARGALLO: I agree with you but unfortunately there isn't this kind of network in Italy. I think such a network is very useful. It would be a very good idea to implement such a network in Italy.

Immune tolerance induction: treatment duration analysis and economic considerations

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Since the introduction of the concept of immune tolerance induction (ITI) for the eradication of inhibitors in hemophilic patients,[1] there has been growing interest in its application. Many regimens have been used with varying results. Retrospective international[2] as well as U.S. registries[3] have been compiled. Although there are differences, both concluded that we do not as yet have enough data to predict successful outcome consistently, and that the procedure is very expensive. There are no data in the literature as of yet, other than theoretical modeling[4] which, in addition, addresses the last benefit or health outcomes of patients with inhibitors compared to those without, and the costs of immune tolerance.

A recent letter[5] only evaluated the use of the product for seven young patients undergoing ITI, and only achieving true success using 7×10^6 units of recombinant factor VIII. In comparison, 4.7×10^6 units of the same material treated all bleeding episodes and surgical intervention for 73 previously untreated patients (PUPs) for five years. It is becoming clearer therefore, especially in our more health-care-cost-conscious environment, that we need to be able to predict successful outcome for our patients. This is particularly true as costs of factor are not the sole costs. Many children require venous access devices. These require professional support, hospitalization, and are fraught with complications. All of these have a cost associated with them.

In the International Immune Tolerance Registry, there were a substantial number of cases (n=128) in which patients achieved success by a definition now accepted by treaters. The inhibitor titer has to disappear, the recovery and half-life normalize, and when challenged, there is no anamnesis.

Figure 1 demonstrates that for those patients who achieved a loss of their inhibitor and developed normal kinetics, 85% did so in two years, but 60% became tolerant in one year. There is,

hence, a marked degree of heterogeneity concerning the precocity of the response which we must analyze in order to identify prognostic factors associated with early success.

Reviewing the data of these patients, they fell into two categories: a) those with a favorable prognosis who had a titer prior to institution of ITI of <10 BU were treated with a dose of factor VIII of >100/kg/day, and the time from initial inhibitor detection to institution of ITI was less than five years and b) those with opposite features and a poor prognosis. We analyzed, then, their time to success (Figure 2). Those patients with favorable predictors had a 65% chance of becoming tolerant within one year, in contrast to those with poor indicators, who had a 35% chance of becoming tolerant during that time.

Using these data, to determine product costs for ITI, we made the following assumptions:

a) a children weighing 25 Kg;
b) an adult weighing 75 Kg;
c) a factor VIII dose of 200 IU/kg/day;
d) a factor VIII plasma derived cost of 0.50 US$/IU;
e) a factor VIII recombinant cost of 1.00 US$/IU.

If one then determines factor consumption and costs for the two extreme settings (a child and an adult), the former belonging to the favorable prognosis group and the latter to the poor prognosis group, and choosing as the end point a 50% success rate, we see the difference in factor consumption and costs is very pronounced (Table 1).

Some of the additional costs that may be applicable to all involved in ITI are the use of products to manage bleeding episodes. These include APCC and rVIIa, and in some programs, the adjuvant use of APCC and/or rVIIa while ITI is in progress, medical and paramedical personnel, and venous access since installation, and its attendant infections and thrombotic complications.

Despite these cost-modeling data, which are retrospective in nature, many unresolved issues

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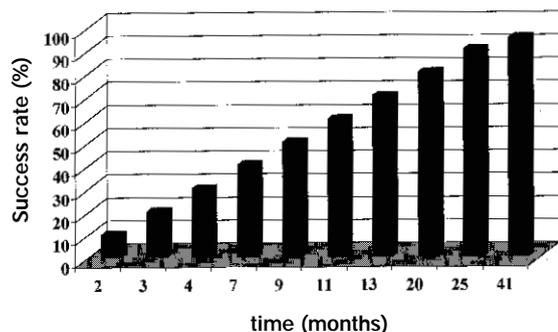
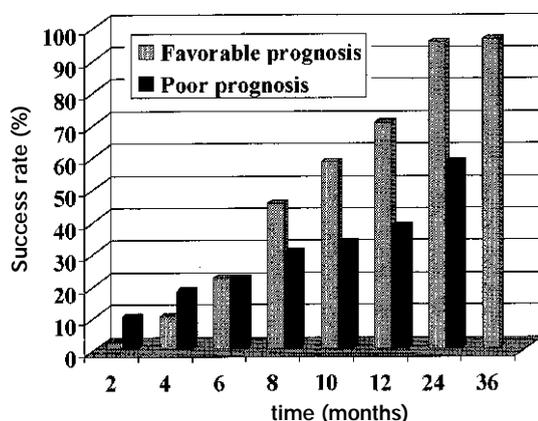**Figure 1. Time to success (inhibitor 0 BU, normal FVIII kinetics).**

Figure 2. Time to success in patients with favorable or poor prognostic indicators.

remain. Who are ideal candidates? At what point should one institute therapy? Here there are two schools of thought. Some believe that immediately upon finding an abnormal BU, ITI therapy should be inaugurated. They believe the time to success is then very short. Others recognize that some low-titered inhibitors disappear without a specific program of ITI, and thus wait until the inhibitor is clearly long-standing and does not disappear. How long should one wait before starting ITI? There is no doubt that one year is more than enough to diagnose a transient inhibitor, but if the titer is very high there is no need to wait since transient inhibitors are low-titer inhibitors. Then, for how long should therapy be continued? Are there time-dependent goals which will lead to clear-cut failure and thus termination of this expensive intervention? How can one then, with these unresolved questions, get true informed consent? Which patients

Table I. Consumption of factors and cost for concentrate provision (plasma-derived or recombinant) to attain tolerance for a proportion of 50% of patients in the two prognostic groups.

	Time to 50% successs rate (months)	Factor consumption (IU)	Cost (US $) Type of concentrate used: plasma-derived	recombinant
Favorable prognosis	9.5	1,425.000	712.500	1,425.000
Poor prognosis	19.0	8,550.000	4,275.500	8,550.000

require venous access? In those that do, how can we prevent infections and/or thrombosis? Which type of product should be used to induce tolerance? Is it the one which produced the inhibitor? A different one? Or does the outcome differ with plasma-derived versus recombinant products? Lastly, who will pay for this expensive program? Does one have to assure success? Can we identify the appropriate tools to measure the difference in the quality of life in those patients with and without inhibitors? Can financial values be placed on these differences?

We have come very far in our experience with ITI since its first use. Several different economic analyses have indicated that it is very costly. Our model shows it may be cost-effective: the data garnered from the International Immune Tolerance Registry are the first to define a group of patients with indicators of a good outcome with a likelihood of success using a definition now accepted by treaters. A new multicenter randomized international ITI prospective study will likely either substantiate or alter the findings of this study and possibly narrow those patient characteristics and regimens that will produce the least costly successful outcome. Complications of this therapeutic intervention need careful monitoring as do the costs associated with them.

We envision that in the near future, a true cost-effectiveness ratio will be achieved. Then, data will simplify our task of convincing third-party payers or governments that this treatment is worth underwriting.

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DISCUSSION 17   Economic and organizational issues
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BERNTORP: I would like to reiterate the fact that the treatment of hemophilia is one of the most cost-effective treatments that we have. In Sweden the total cost for arterial hypertension is about fifteen times that of hemophilia care. So, I think it's important to look at the tremendous costs in that perspective.

DI MICHELE: I'd like to comment on the data that Dr.Mariani has just presented; I think that we will hopefully have a chance in the prospective study to assess the prognostications that you predict in terms of patients with favorable predictors versus those who do not have favorable predictors. I think that we will be starting tolerance much earlier than your data indicate based on your weight assessments of 25 kilos; it's very possible that the average weight that we calculated was probably closer to 12 kilos. The cost of that may well be half which is good news that in terms of the high-dose arm that 75% of our patients should achieve tolerance within a year and this will also depend on their pre-titer. I think we have seen some rather favorable predictions and it will be very interesting to see what happens in the study with regard to those patients who fulfill your criteria.

MARIANI: Yes. These are just calculations and yet I think it is important to state that what we have identified as additional costs might not turn out to be additional costs because the patient has to be treated anyway. Nursing problems and venous success are certainly notable costs for an inhibitor patient who is not on IT in the first case. All the other costs such as recombinant factor VIIa are still costs for a patient who is not on IT. I would keep the attention focused on the real costs which are mostly for factor VIII.

DI MICHELE: To play devil's advocate, I would like to point out that you arbitrarily picked a time of one year. Remember the overall cost of tolerance has not only to do with the size of the child and the ultimate dosage but really the dosage over time. I think one of the important

aspects is that of using less factor even if it may take a longer time to achieve tolerance. Does it end up being more cost effective than higher doses over a shorter period of time? Those are questions that can't really be addressed unless they are studied prospectively.

MARIANI: We have to be on the safe side because if you use a lower dosage it could be that it takes more time. I think that it's preferable to start with a high dose and reduce the duration of treatment; it's also a matter of the quality of life.

BEARDLEY: We have done an analysis of some of our inhibitor patients. Some of them are acquired hemophiliacs but once they have a bleeding episode it becomes a kind of a gamble because those patients cost us around $20,000 per day of hospitalization for their blood product usage and you can have astronomical costs for individual patients if they happen to have a compartment syndrome or intercranial bleed. I am very much in favor of the cost-effectiveness of preventing those kinds of episodes.

MARIANI: Reducing costs has to be considered as a very important aspect because the patient not on ITI has more bleedings and so ITI in the long run might cost less. If you treat a patient while he is young and with a favorable prognosis, this should be the best choice

ALEDORT: I think that's the nice part of the Harvard model which is going to be refined. It's going to be important to put real data and not just modeling into the costs of care both before and after ITI.

MARTINOWITZ: I would like to stress the cost of regular treatment of inhibitor patients and of course the patients with the burnt out joints which it must be said are relatively rare. In these patients the cost effectiveness will be low. But then we have to think about children who are bleeding every day or every other day. We have two children and one of these children costs £!,000,000 a year and the other costs close to $4,000,000 a year. These are children who weigh 40 and 20 kilos. If you take this into account then the cost effectiveness of immune tolerance is so clear.

Characterization of antibodies to factor VIII in hemophilia A patients treated by immune tolerance therapy

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Abstract

The treatment of hemophilia A patients has improved in this decade by production of recombinant factor VIII (FVIII) in mammalian cells, which has significantly reduced contamination by infectious agents. A remaining serious problem in 25-30% of patients with severe hemophilia A is the appearance of antibodies that inactivate FVIII. The present therapy for this condition is frequent treatment with high doses of FVIII to induce tolerance, which is defined as a negative Bethesda assay. Serial plasma samples from 50 evaluable patients in a large study of 72 previously untreated patients were tested to determine whether *tolerized* patients have actually lost all their anti-FVIII by using a 10 fold more sensitive immunoprecipitation (IP) method of measuring all anti-FVIII antibodies. Six of the 22 patients with inhibitors were given tolerance therapy, and 3 of them were only partially tolerized as determined by IP assay. Seven patients with 1-11 BU/mL lost their inhibitor spontaneously, while 5 non-inhibitor patients with low level immune responses similarly became antibody negative. In a smaller study, a *tolerized* patient with 0 BU/mL had remaining non-inhibitory antibody levels high enough to reduce the FVIII half-life significantly. Plasmas from 2 patients who were not tolerized, were tested by the IP assay for the A2 and/or C2 domain specificity of the anti-FVIII over time. The antibodies detected were directed against both the heavy and light chains of FVIII, and they increased and decreased at the same rate before and during tolerance therapy
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Key words: factor VIII, hemophilia A, immune tolerance, anti-Factor VIII antibody

T reatment of individuals with hemophilia A in past decades was often compromised by factor VIII (FVIII) contamination with infectious agents from the human plasma used for the purification of FVIII. Both hepatitis A and AIDS viral infections were detected in a significant portion of these patients. Within the past few years the FVIII therapeutic products given for therapy of hemophilia A have been improved by better viral inactivation steps and by production of recombinant factor VIII in mammalian, non-human cell lines.[1,2] So far the recombinant FVIIIs have not caused the previous problems or any new ones.

The most serious remaining problem of FVIII therapy is the production of anti-FVIII inhibitor antibodies that arise in about 25-30% of individuals with severe hemophilia (1-2% FVIII)[3] and less often in those with mild and moderate hemophilia. As these antibodies can inactivate FVIII and are consequently referred to as inhibitors, they represent a serious challenge to effective therapy. Patients with a common FVIII gene inversion, large deletions, and some nonsense mutations[4] are more likely to develop anti-FVIII antibodies because they have no circulating FVIII antigen and are therefore not tolerant to FVIII infusion. The current clinical strategy for significantly reducing or eliminating anti-FVIII is to give frequent doses of FVIII in low, medium, or high doses that eventually reduce antibody levels to very low or non-existent levels, as measured by the Bethesda assay.[5] A study using low FVIII doses (25 U/kg every other day) led to immune tolerance in 21 of 24 patients.[6] Another study treated high titer inhibitor patients with FVIII doses of 100 U/kg/day and FEIBA, a source of factor IXa. Twelve of the 15 patients had inhibitor titers that dropped to ≤1 BU/mL, and their FVIII recovery and half-life was normal.[7]

We examined the status of antibody development in several studies of previously untreated patients who received treatment with recombinant FVIII and others who used either plasma-derived or recombinant FVIII. We particularly focused on those patients who had immune tolerance therapy to determine whether all their antibodies disappeared by using a highly sensitive immunoprecipitation assay. Using this method we also tested patient plasmas with low or no Bethesda titer to determine whether such titers corresponded to significant amounts of antibody and whether the BU negative individuals ever had low level immune responses to FVIII.

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Table 1. Range of IP titers in patients without Bethesda titers.

Group Type	Patients	Lowest IPU Range	Highest IPU Patients	% of Range
No Immune Response	38	0-0.5	0-3.4	76
Immune Response ≥4.3	7	0	5.4-139	6

Table 2. Range of IP titers In inhibitor patients with Bethesda titers ≤2.

Pt	Lowest IPU	Highest IPU	Final IPU	Highest BU	Lowest BU	Final BU	Tolerance
1	0	122	3.6	1	0	0	+
2	0.4	4.4	0.7	0.6	0	0	+
3	0.6	20	3.4	0.7	0	0	+
4	0	169	0	10	0	0	+
5	0	10,644	1,310	634	0	41	-

Methods and Results

All hemophilic patient plasmas were tested for inhibitor activity by the Bethesda assay,[5] FVIII recovery, and FVIII half-life at the home institution. Immunoprecipitation assays (IP) were done in our laboratory as described elsewhere[8] by radio-labeling FVIII with Na[125]I and using it to bind serial dilutions of hemophilic plasmas. All antibodies were captured with protein G sepharose, and the unbound ^{125}I-FVIII was washed away. IP units/mL were calculated as ([bound/total [125]I – background without antibody/mL] x plasma dilution). This assay is approximately 10-fold more sensitive than the Bethesda assay.

The first group of 11 hemophiliacs analyzed had Bethesda titers ranging from 1.1-255 at the start of therapy. They were subsequently treated with 100 units/kg/day FVIII. Seven of them had a negative Bethesda assay at the end of the treatment. Each sample was tested by IP assays with ^{125}I-FVIII to determine whether there were any remaining levels of either non-inhibitors and/or inhibitors at low levels. At the end of therapy, 4 patients had low but insignificant antibody levels in the IP assay, which confirmed the success of the tolerance procedure. Two had final IP titers of 5.6 and 228. Their respective FVIII half-life measurements at the same time were 9.1 and 5.2, which confirmed that only the latter had antibody levels high enough to clear FVIII more rapidly than usual. The 4 failures had remaining inhibitor titers of 0.7-31.

A large study of previously untreated severe hemophilia A patients yielded additional interesting information. All plasmas collected from 50 evaluable patients of 72 enrolled were tested for inhibitor titers and by the IP assay for all anti-FVIII antibodies. A reliable immune response of 4.3 IP units/mL was defined as 3-fold above the average antibody levels determined in patients before FVIII therapy was begun. The patients in the study were evaluated by comparing the IP and Bethesda titers in multiple, serial plasmas drawn over the course of the study, and the data for those without inhibitor titers are summarized in Table 1. The patients' data were divided into 2 groups based on the immune responses of the patients' to rFVIII infusion.

Immune responses that were either negative or below the cut-off defined above were obtained in 76% of the 50 evaluable patients, while 6 (12%) had immune responses ≥4.3 IP units/mL.

Eight patients with Bethesda titers ≤ 3 became tolerant by the end of the study without any specific treatment for reduction of the inhibitor titer. Representative data are shown in Table 2. In addition, 2 of 3 patients with BU >3-10 also became spontaneously tolerant (Table 2, patient #4). Four patients with Bethesda titers of 11-1,397 were given high dose rFVIII tolerance therapy, but success was achieved only in 1 who had pre-IT titers of 3 BU/mL compared to 38-1105 for the others. Two patients with 5 and 11 BU/mL given rFVIII prophylaxis treatment were also tolerized. The low titers and the use of prophylaxis may have enhanced the elimination of the antibodies, which should be verified in future studies. Data for 1 tolerance failure are shown in Table 2. Six non-inhibitor patients with immune responses ≥4.3 IP units/mL were IP negative at the end of the study period.

The IP titers over time of anti-A2 or anti-C2 antibodies from 2 high titer inhibitor patients in tolerance therapy increased and decreased coordinately.

Discussion

Prior results from immune tolerance protocols in which 85% of individuals with <10 BU/mL but only 50% with >10 BU/mL were more easily tolerized by less FVIII than those with >10 BU/mL indicate that the lower titer antibodies can be more easily eradicted.[9] This was also the case in the patients described above who were receiving immune tolerance therapy. Three of 6 were tolerized. It was surprising, however, that 11 patients with inhibitor titers 0.6-≤10 lost their inhibitors without any specific treatment.

Seven non-inhibitor patients had ≥1 antibody peaks detectable only in the immunoprecipitation assay at one or several times in their treatment history. In all of them the antibodies also disappeared spontaneously. The presence of such non-inhibitory antibodies (228 IP units/mL) in 1 patient led to a reduction of the FVIII half life to 5.2 hours compared to the normal value of 7.6 hours. Similar results were previously observed in ELISA experiments.[10] The non-inhibitory antibodies may therefore also lead to adverse effects on FVIII therapy if their titer is high enough. The paucity of half-life data did not allow a more precise determination of the minimum antibody concentration that affects FVIII half-life.

In individuals without Bethesda titers that have increased, unexplained bleeding or shorter than normal half-life, the addition of the IP or similarly sensitive assay would be useful in determining whether anti-FVIII antibodies are the cause of these conditions.

The immune response to FVIII in severe hemophiliacs, based on the Bethesda and the IP assays, is more heterogeneous than that found in earlier studies which used only the Bethesda assay.[1,2] In the large study described, 22 of the 72 total patients were positive by the Bethesda assay alone, and an additional 6 non-inhibitor patients were antibody positive (≥4.3 IP units/mL) by the IP assay. The percentage of individuals who responded immunologically to FVIII infusion was 34% of all study patients. Although 4 inhibitor patients were successfully tolerized by high dose FVIII therapy, 11 with a range of 0.6-10 BU/mL, and the 4 with low level antibodies spontaneously lost their anti-FVIII titer. By the end of the study, 68% of the inhibitor patients had become negative.

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DISCUSSION 14 **Characterization of antibodies to FVIII in hemophilia patients treated by immune tolerance therapy**
Scandella D. (Rockville, USA)

KAZATCHKINE: I'm sure we have been underestimating these binding antibodies with no inhibiting activity. You may even be underestimating them yourself because of the arbitrary definition that you gave to the immune response.

SCANDELLA: I wouldn't exactly call it arbitrary. The titers I'm talking about, called "No Significant Immune Response", are very low because they are very low in titers.

KAZATCHKINE: Have you ever looked at a patient who has undergone tolerance induction and whether after absorbing factor VIII binding antibodies on factor VIII you found in the remaining IGg fraction antibodies that would inhibit your immuno-precipitation assay?

SCANDELLA: We haven't done that.

KAZATCHKINE: That may be another way of looking at those regulatory antibodies that we all wish we could find an easier assay for.

SCANDELLA: One piece of data which I wasn't able to show is that certainly in the no Bethesda titer immune responses they all disappeared without any particular therapy and concened inhibitors with less than ten BU, there was also a number where the inhibitor titer went away without any specific therapy.

Factor VIII inhibitor with catalytic activity towards factor VIII

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Abstract

Hemophilia A is an X chromosome-linked recessive disorder resulting in defective or deficient factor VIII (FVIII) molecules, which, in its severe form, is a life-threatening, crippling hemorrhagic disease. Infusion of purified FVIII to patients with severe hemophilia A results in approximately 25% of the cases in the emergence of anti-FVIII antibodies (inhibitors) that are known to neutralize the procoagulant activity of FVIII by steric hindrance. We recently reported on the proteolysis of FVIII by alloantibodies in the plasma of two high responder patients with severe hemophilia A, demonstrating a new mechanism by which FVIII inhibitors may prevent the pro-coagulant function of FVIII. Hemophilia is the first model in which a direct link between the hydrolysis of the target molecule and the occurrence of clinical manifestations has been established. It also represents the first example in humans, of the induction of catalytic antibodies following the exogenous administration of an antigen. The characterization of FVIII inhibitors as site-specific proteases may provide new approaches to the treatment of inhibitors.
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Key words: factor VIII, catalytic antibodies, hemophilia, FVIII inhibitor

Anti-FVIII antibodies in patients with hemophilia A

Factor VIII inhibitors are IgG antibodies that neutralize FVIII procoagulant activity in plasma. The inhibitors arise as alloantibodies in approximately 25% of patients with severe hemophilia A and 5-15% of patients with mild or moderate severity hemophilia in response to infusion of human FVIII concentrates and recombinant FVIII protein.[1,2] Patients with inhibitors become resistant to conventional replacement therapy and the development of inhibitors remains a major therapeutic challenge. Although the underlying basis for inhibitor development is incompletely understood, the occurrence of inhibitors is considered to reflect an allogeneic immune response to the repeated administration of exogeneous FVIII protein. Thus, the risk of inhibitor development is greater in hemophilia patients with large deletions in the FVIII gene.[3, 4] FVIII inhibitors are polyclonal antibodies of the IgG isotype. Several mechanisms by which FVIII inhibitors interfere with FVIII procoagulant activity have been proposed: (i) some anti-FVIII alloantibodies may bind to the A2 domain of FVIII, resulting in steric hindrance of the thrombin cleavage site of FVIII located between the A1 and A2 domains;[5,6] (ii) antibodies directed against the C2 domain of the light chain of FVIII inhibit the binding of FVIII to phospholipids;[7,8] (iii) alloantibodies directed against the A3 and/or C2 domains of the molecule, may prevent the stabilizing interaction of FVIII with von Willebrand factor (vWF) and interfere with the binding of activated factor IX (FIXa) to the FVIII light chain;[9,10] (iv) FVIII inhibitors may bind to epitopes formed by the association of FVIII and vWF, and prevent the release of thrombin-cleaved FVIII from vWF.[11,12] We addressed the hypothesis that some of the FVIII inhibitors may inhibit FVIII pro-coagulant activity in an active manner, i.e. by proteolytic degradation of the target molecule itself.

Catalytic antibodies

Catalytic antibodies are endowed with a capacity to hydrolyze the antigen for which they are specific. The concept of antibodies with catalytic activity was originally proposed by Pauling over 50 years ago.[13] Antibodies that are able to catalyze chemical transformations were obtained by immunization of animals with haptens that resemble the transition state of reactions.[14] The spontaneous occurrence of catalytic antibodies has been described in humans under both healthy and pathologic situations. The first report of human antibodies with peptidase activity originated from studies in which vasoactive intestinal peptide (VIP) was cleaved by antibodies in asthmatic patients.[15,16] The presence of a catalytic site located on the light chain of some induced anti-VIP antibodies was

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demonstrated.[17] Catalytic antibodies able to hydrolyze DNA have been described in patients with systemic lupus erythematosus (SLE) and in patients with Bence Jones disease,[18-20] and antibodies endowed with protein kinase activity have been isolated from human milk.[21] Previous results from our laboratory demonstrate that antibodies isolated from the plasma of patients with Hashimotos's thyroiditis are able to hydrolyze thyroglobulin (Tg).[22] Upon co-incubation of [^{125}I]-labeled Tg and affinity-purified anti-Tg antibodies, the 330 kDa form of Tg was degraded into a major band of 15 kDa and minor bands of 125, 60 and 25 kDa. The Km for the reaction was 39 nM, indicating the strong affinity of the interaction.

FVIII inhibitor with catalytic activity to FVIII

We purified anti-FVIII antibodies from the plasma of three high-responder patients with severe hemophilia A by affinity-chromatography on a human FVIII-coupled Sepharose matrix (Table 1). Human FVIII was radiolabeled using ^{125}I and was incubated either alone or in the presence of affinity-purified anti-FVIII antibodies of patients Bor, Che and Wal.[23] Fragments of radiolabeled FVIII were separated by SDS-PAGE, and migration profiles were revealed by autoradiography.

Factor VIII incubated alone presented a characteristic migration profile, with protein bands ranging from 300 to 70 kD. In the case of patients Bor and Wal, incubation of FVIII in the presence of anti-FVIII IgG resulted in the hydrolysis of high molecular weight bands and appareance of bands of molecular weight lower than 70 kD. Incubation of FVIII with IgG of patient Che did not result in FVIII hydrolysis. Similarly, incubation of FVIII with normal polyclonal human IgG (IVIg, Sandoglobulin®) and with a control human monoclonal IgG directed to cytomegalovirus did not result in FVIII proteolysis. In parallel experiments, we observed that FVIII hydrolysis was dose- and time-dependent.[23]

Several lines of evidence confirmed that FVIII hydrolysis was not due to contaminating proteases:[23] (i) anti-FVIII IgG from patients Bor and Wal exhibited different kinetics of FVIII hydrolysis and different digestion patterns, whereas that of patient Che did not cleave FVIII, suggesting that FVIII hydrolysis is mediated by the variable regions of antibodies; (ii) the catalytic activity to FVIII was co-eluted with anti-FVIII IgG, whereas IgG not retained on the FVIII matrix did not cleave FVIII; (iii) removal of IgG from the preparations of affinity-purified anti-FVIII antibodies, by chromatography on protein G, resulted in the complete loss of the hydrolyzing capacity; (iv) co-incubation of FVIII and anti-FVIII IgG in the

Table 1. Inhibitory activity of affinity-purified anti-FVIII IgG.

Patients	Inhibitory activity in plasma (BU/mL)*	Inhibitory activity of affinity-purified anti-FVIII IgG (BU/mg IgG)*
Bor	10	57
Che	32	64
Wal	150	43

*As determined using Kasper's method.[31]

Table 2. Peptide bonds cleaved by anti-FVIII IgG of patient Wal.

Amino acid sequence	Cleavage site	Location on FVIII
SVAKKHP	R372 - S373	between the A1 and A2 domains
DQRQGAE	E1794 - D1795	within the A3 domain
DEDENQS	Y1680 - D1681	N-terminus of the A3 domain

presence of several protease inhibitors (i.e., E-64, pepstatine, leupeptine, aprotinine) did not prevent FVIII hydrolysis; (v) size-exclusion chromatography of urea-treated affinity-purified anti-FVIII IgG yielded a major peak that was devoid of contaminants and retained the catalytic activity to FVIII; (vi) F(ab')$_2$ fragments prepared by pepsin digestion of affinity-purified anti-FVIII IgG were able to cleave FVIII, further suggesting that FVIII hydrolysis is mediated by the variable regions of the antibodies. The kinetic parameters of FVIII hydrolysis by the antibodies of patient Wal were determined by co-incubating fixed concentrations of radiolabeled FVIII and anti-FVIII IgG, with increasing concentrations of unlabeled FVIII. Increase in FVIII concentration resulted in saturation of the hydrolyzing activity. The data were fitted to Michaelis-Menten kinetics. The calculated average Km and apparent Vmax for the reaction were 9.46±5.52 µM and 85±60 fmol.min^{-1}, respectively.[23] The catalytic efficiency of anti-FVIII IgG, i.e. 42.6±8.9, was 20,000 to 100,000-fold lower than that of thrombin and activated factor X, which are known to cleave FVIII.[24,25] The apparent catalytic efficiency and Vmax of patient Wal's antibodies are underestimated in these calculations, which are based on the assumption that all anti-FVIII antibodies are catalytic. This is certainly not the case, since the polyclonal

population of anti-FVIII antibodies of the patients also contain antibodies that are devoid of catalytic activity. Furthermore, whereas enzymes are more efficient than catalytic antibodies in hydrolyzing FVIII, they are rapidly inactivated in plasma under physiologic conditions. In contrast, catalytic antibodies could exert a biological effect for hours or days.

The nature of the cleavage sites for catalytic IgG in the FVIII molecule was investigated by performing amino acid sequencing of purified peptide fragments produced by the hydrolysis of FVIII by anti-FVIII IgG of patient Wal. The major scissile bonds are reported in Table 2.[23] The association between FVIII and vWF partially inhibited FVIII hydrolysis by anti-FVIII IgG of patient Wal (i.e., 37%), when IgG and FVIII were incubated in a wt/wt ratio similar to that present in normal plasma, i.e. 30 µg/mL of vWF versus 300 ng/mL of FVIII.[26] The protective effect of vWF toward hydrolysis by anti-FVIII IgG may be secondary to the masking of some of the cleavage sites for catalytic antibodies on the FVIII molecule following complex formation with vWF.[23] Indeed, residues Y1680 - D1681 that define the scissile bond located in the N-terminus of the A3 domain, have been shown to be essential for the interaction of FVIII with vWF.[27]

Conclusions and Perspectives

The identification of FVIII inhibitors as catalytic antibodies extends the spectrum of catalytic immune responses, in addition to previous reports of hydrolyzing antibodies against VIP in asthma patients, DNA-hydrolyzing antibodies in patients with SLE and thyroglobulin-specific catalytic antibodies in patients with autoimmune thyroiditis.[18,28,29,30] This is also the first report, to our knowledge, of the induction of a catalytic antibody in humans in response to exogeneous administration of a protein antigen. The kinetic parameters of FVIII hydrolysis by anti-FVIII IgG exhibiting catalytic properties and the estimated amounts of these antibodies in plasma suggest a functional role for the catalytic immune response in inactivating FVIII *in vivo*. Within a polyclonal mixture of anti-FVIII allo-antibodies which differ in their functional properties, catalytic antibodies may inhibit FVIII pro-coagulant activity at faster rates than non-catalyzing anti-FVIII antibodies. Identification of peptide epitopes that are the targets for proteolytic anti-FVIII antibodies may thus be critical for our understanding of the pathophysiology of the FVIII inhibitor response. Furthermore, the characterization of FVIII inhibitors as site-specific proteases may provide new approaches to the treatment of inhibitors.

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B- and T-cell tolerance: from basic concepts to clinical practice

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Abstract

Recent advances in the understanding of the mechanisms by which tolerance develops in both B- and T- cells have led to a re-appraisal of possible methods which could be applicable in clinical settings such as hemophilia patients with inhibitors. Thus, it is now clear that a number of mechanisms operate in such a way as to actively induce anergy of B- or T- cells, or even to specifically delete cells in the periphery. The characteristics inherent to the B- and T- cell repertoires and peripheral pools play a major role in the efficiency and maintenance of tolerance induction. The purpose of this manuscript is to review the mechanisms of tolerance induction briefly so as to provide a framework for the understanding of current and future attempts to achieve Factor VIII unresponsiveness in hemophilia A patients.

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Key words; factor VIII, tolerance, B-cell, T-cell

Tolerance should be defined as a state of unresponsiveness to an antigen by an immune system which is otherwise fully immunocompetent. By adopting such a definition, we avoid confusion with primary or secondary immunodeficiency, or with the effects of immunosuppressive drugs.

As the immune response towards soluble antigens is dependent on the collaboration of specific B- and T- cells, tolerance to an antigen can result from either unresponsiveness at the T-cell level, B-cell level, or both levels at the same time. This short review will consider separately the characteristics of tolerance induction and maintenance at B- and T- levels.

B-cell tolerance

B-cells are produced in the bone marrow where they undergo a first cycle of selection during early cell expansion. The selection operates in such a way as to eliminate most of the potentially self-reacting cells. Deletion and other mechanisms reviewed below result in a loss of about 90% of the cells. Of the 10% which reach the periphery, only 1/3 will survive. This, however, still represents a large number of cells. In the mouse, about 10 to 20×10^6 cells are produced by the bone marrow every day; 300 to 600,000 cells survive in the periphery. It is important to realize that the B-cell pool is continuously replenished during life, which stands in contrast with the T-cell pool, essentially acquired at birth.

Diversity and capacity to diversify are key words when considering tolerance. From the number of gene segments coding for variable parts of antibodies, namely V, D and J segments, it can be easily calculated that approximately 250,000 combinations are possible in the mouse. The way these different segments assemble is said to be imprecise, i.e. there is a large junctional diversity, which, when combined with the number of possible associations between genes, brings up the number of possible combinations to 10^{16}. If this were not sufficient, the process of somatic hypermutation (see below) further multiplies this figure by at least two orders of magnitude. The two main characteristics of the B-cell pool are therefore continuous replenishment and huge capacity to diversify. One first glance consequence of this is that establishing and maintaining B-cell unresponsiveness might not be an easy task to achieve with this constantly changing cell pool.

B-cells are susceptible to tolerance induction at two stages: B-cells during the first few expansion cycles occurring in the bone marrow (*first window*) and B-cells during the expansion phase occurring in germinal centers during B-memory induction (see below). The question of the relative sensitivity of B-cells to tolerance induction as a function of their maturation degree is not entirely settled: mature as well as immature B-cells can undergo anergy induction.

The mechanisms by which tolerance can be established depend on the way B-cells interact with their specific antigen. Tolerance induction is an active phenomenon in that recognition of antigen is followed by a series of signals leading to anergy or cell deletion. It requires the cross-

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linking of surface immunoglobulins with a minimum affinity threshold. The immediate consequence of this is that multivalent antigens, such as tissue-bound antigens, are efficient tolerance inducers, while monovalent antigens are not. Indeed, self-reactive B-cells to monovalent antigens can be found in the periphery. Affinity governs to some extent whether B-cells are deleted (high-affinity interaction with self antigen) or anergized (intermediate or low affinity interaction).

Somatic hypermutation occurs during the generation of B-cell memory. It is a phenomenon which is specific to B-cells as it does not occur in the T-cell compartment. As stated above, it provides B-cells with yet another opportunity to match the vast diversity of potential epitopes. Mutations are introduced in antibody variable regions, and more precisely in complementarity-determining regions (CDR) by a mechanism which is not totally elucidated, but requires the presence of T-cells and the microenvironment of germinal centers. Such mutations are introduced at random, which potentially creates new specificities towards self antigen. These are readily eliminated by deletion or anergy induction, through mechanisms identical to those operating in the bone marrow. Re-exposure to the same antigen will induce another cycle of memory induction, i.e. somatic hypermutation and elimination of self reactive moieties.

The peripheral B-cell pool is therefore made of distinct B-cell populations at different degrees of maturity and/or responsiveness. Interestingly enough, the frontier between deletion and anergy is not entirely defined: anergized B-cells have a shorter life span and restricted mobility. Anergy is, however, reversible. Removal of the *tolerogen* early enough or extensive cross-reacting of surface immunoglobulins can restore full responsiveness. Alternatively, B-cells can be reactivated through the help of specific T-cells or by exposure to certain cytokines such as interleukin (IL)-4.

As explained above, anergy results from an active interaction between antigen and surface immunoglobulins. Absence of interaction, or ignorance, leads to rapid cell deletion. In fact, the normal fate of a B-cell (as well as that of T-cells) is to die rapidly, unless it is rescued by encountering its corresponding antigen. On the other hand, hyperstimulation of B-cells leads to another phenomenon. B-cells then express a surface molecule, called Fas, whose activation by Fas ligand results in cell apoptosis. This Fas-mediated cytotoxicity is driven by T-cells.

T-cell tolerance

T-cells originate from the bone marrow. Immature CD4(-)/CD8(-) cells migrate to the thymus where they are selected. The potential repertoire of T-cells is again conditioned by random association of TCR α and β chains. Interestingly, the number of germline segments coding for VDJ, and therefore the number of possible recombinations, is about 10-fold smaller than that for B-cells. The junctional diversity is however very high, which brings up the final number of possible recombinations to about 10-fold that of the B-cell compartment. Yet, because of the absence of somatic hypermutations in T-cell receptors (TCR), the B-cell repertoire exhibits a 10- to 100-fold higher degree of diversity than T-cells.

Immature T-cells entering the thymus undergo a first selection based on the recognition of self MHC molecules. This takes place in the cortex of the thymus. Cells that do not recognize MHC molecules of either class I or class II - in fact the majority of T-cells - are eliminated. Recognition of MHC-class I molecules drives maturation in single positive CD8 cells, while selection of MHC-class II rescues CD4+ T-cells. A second run of selection occurs at the junction of cortex and medulla. Cells recognizing self epitopes presented in the context of either class I or class II molecules are eliminated if the interaction is of high affinity. However, cells with intermediate affinity-reaction are rescued and sent to the periphery. The peripheral T-cell pool is therefore constituted of cells recognizing self epitopes with intermediate affinity whenever the corresponding antigen is effectively presented within the thymus. The role of these self-reactive T-cells is not entirely elucidated, but they could play a role in normal immune homeostasis and in auto-immune responses.

By contrast to the B-cell population, the T-cell pool is relatively fixed at birth due both to the fact that the thymus rapidly disappears after

B cell versus T cell pool

B cells	T cells
• continuous pool replenishment	• pool essentially acquired at birth
• high degree of diversification (somatic hypermutation)	• low degree of diversification

Figure 1. The potential repertoire of T-cells due to gene rearrangement and junctional diversity is probably larger than that of B-cells. However, the peripheral B-cell pool is continuously replenished during life and presents a higher degree of diversity than T-cells due to somatic hypermutation.

birth and to the lack of somatic hypermutations (Figure 1).

The fate of T-cells in the periphery is highly variable. Without stimulation, namely with no encounter with an antigen presented in the context of MHC molecules, cells undergo passive death. Recognition of the antigen in the absence of co-stimulatory signals leads to clonal anergy. Co-stimulatory signals are generated by mutual recognition of antigens expressed at the surface of antigen-presenting cells (macrophages, dendritic cells or B-cells) and of T-cells. Contacts between complementary molecules transduce signals of activation or inhibition (see below). Molecules involved in secondary signaling include CD40-CD40L, B7-CD28, and B7-CTLA.

In recent years, a mechanism has been described by which T-cells commit suicide in the periphery. T-cells that recognize an antigen in the right context of MHC molecules and co-stimulatory signals are activated. The secondary signal is initiated by B7-CD28 interaction. In fact, one of the major effects of such recognition is the expression of molecules of the Bcl family, which block Fas-dependent apoptosis. Too much of an activation however circumvents this Bcl-dependent blockage and leads to cell death through hyperexpression of Fas and FasL. Interestingly, this effect is potentiated by IL-2, which has therefore a dual effect as an essential cytokine for cell activation and growth and as a protagonist in T-cell-induced apoptosis. This suicidal mechanism has been named AICD, standing for activation-induced cell death.

Clonal anergy of T-cells can also be obtained by activation of CTLA-4, a surface marker of T-cells that is recognized by B7 (as for CD28). CTLA-4 blocks signals transduced from the TCR. Expression of B7 at the surface of an antigen-presenting cell can therefore have two contrasting consequences: activation by CD28 triggering or anergy induction by CTLA-4 triggering. Which of these two effects prevails likely depends on the degree of expression of B7. Low B7 expression favors interaction with CTLA-4 for which B7 has the highest affinity. This would prevent unnecessary T-cell activation in the presence of low antigen amount. At higher levels of B7 expression, activation will then prevail.

Another phenomenon deserves some comments, due to its probable impact on attempts to induce anergy in a clinical setting. CD4+ Th cells are functionally separated into two main subsets, based on the profile of cytokines they secrete. Th1 cells produce IL-2 and interferon-γ while Th2 cells produce IL-4, IL-5, IL-10 and IL-13. The consequence of this different profile of secretion is the predominant involvement of Th1 cells in cell-mediated delayed-type reactions, as opposed to Th2 cells involved in humoral immunity. The phenomenon of split tolerance describes the different tolerance susceptibilities of Th1 versus Th2. Th1 are much more amenable to tolerization than Th2, both by Fas-dependent (AICD) and Fas-independent mechanisms.

Tolerance induction in patients with FVIII inhibitors

Broadly speaking, and to summarize the above, tolerance can be induced by three basic mechanisms: *ignorance, anergy* or *deletion*. Ignorance means absence of interaction, anergy is the result of an active specific interaction and deletion can be obtained by specific hyperstimulation (Figure 2). For the sake of clarity, the distinction between B- and T-cell compartments will be maintained hereafter.

Ignorance at the B-cell level could possibly be obtained by engineering the FVIII molecule so as to reduce or eliminate its interactions with antibodies. Significant efforts in this direction have already been made, based on the observation that main B-cell epitopes appear to cluster within discrete regions of FVIII instead of being spread over the entire molecule. Homology recombination with porcine FVIII or human FV shows convincingly reduced interaction with human anti-FVIII antibodies. Hopes are that the FVIII molecule can be altered to an extent sufficient to reduce antibody binding while keeping full activity.

Anergy could be induced by cross-linking surface immunoglobulins. Best candidates for such an effect are anti-idiotypic antibodies. Further, such antibodies could very well drive B-cells towards death by apoptosis, a phenomenon already observed in human myeloma cell lines. Finally, hyperstimulation through surface immunoglobulins might be an alternative strategy. At T-cell level, ignorance would first require
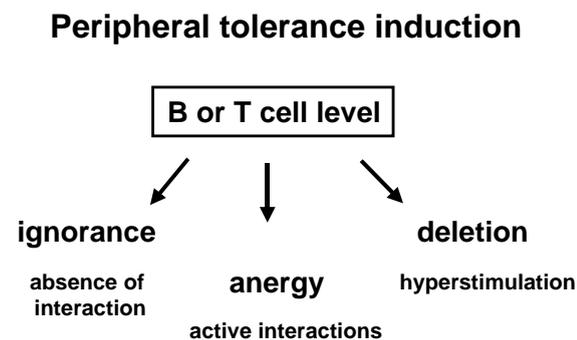
Peripheral tolerance induction

Figure 2. Three basic mechanisms operate in the periphery to maintain or induce tolerance.

identification of major T-cell epitopes. The very first T-cell clones have recently been generated in our laboratory. It is hoped that information obtained at the T-cell clonal level will open possibilities of engineering FVIII in such a way as to eliminate T-cell recognition and/or activation. The relative reduced adaptability of T-cells as compared to that of B-cells might be considered as an advantage to this end.

Anergy at the T-cell level has already been the subject of a number of studies. Attempts to neutralize CD40-CD40L interactions via the use of anti-CD40L antibodies is already under way for FVIII inhibitor patients. Alternatively, or perhaps in combination with the latter, soluble CTLA-4 can block the signals generated by B7 expression at the surface of antigen-presenting cells. Such an approach has already been used in other clinical situations with some success. A complementary approach would be to stimulate CTLA-4 directly at the surface of T-cells, which would block activation signals transduced via the TCR. Ways of deleting FVIII-specific T-cells by AICD might become feasible in the future.

If one now considers how conventional FVIII desensitization could work, keeping in mind all the possible mechanisms described above, one could say that high doses of FVIII are more likely to result in cell deletion. Accordingly, it would be expected to occur in the periphery and probably depend on Fas-FasL interactions. Low doses of FVIII would be more prone to trigger anergy possibly by CTLA-4 stimulation at the T-cell level, or cross-linking of B-cell surface immunoglobulins. There is however an outsider: idiotypic interactions, which involve B-cells, soluble antibodies and TCR, could be a way of restoring or implementing the immune homeostasis that prevails in healthy donors.

All these possibilities still require to be tested, keeping in mind the necessity to develop cheaper alternatives than high-dose FVIII infusion for the treatment of inhibitor patients.

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Idiotypic regulation of anti-factor VIII antibodies

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Abstract

This review focuses on the relationship between natural autoreactivity, autoimmunity in disease and antigen-driven immune responses to factor VIII and on idiotype-mediated immune regulation of anti-factor VIII antibodies in health and disease.
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Antibodies to factor VIII (FVIII) with neutralizing activity toward FVIII are found in distinct clinical settings: i) FVIII inhibitors that occur in multitransfused patients with hemophilia A; ii) spontaneous (acquired) inhibitors that characterize patients with anti-FVIII autoimmune disease; iii) natural antibodies to FVIII that are present in normal IgG of healthy individuals. Whereas FVIII inhibitors are present at high clonal frequencies and may be readily detected in unfractionated plasma by means of functional assays and immunochemical measurements, natural anti-FVIII antibodies are present in only low amounts in normal plasma. In addition, natural anti-FVIII autoantibodies are subjected in normal plasma to effective regulation by anti-idiotypic antibodies. As a consequence, plasma levels of FVIII remain within normal limits under physiologic conditions, and FVIII-neutralizing activity of natural anti-FVIII antibodies may only be detected following purification of the IgG fraction of plasma and/or affinity chromatography on a FVIII column.

The occurrence of anti-FVIII antibodies as natural antibodies in healthy individuals and as pathogenic antibodies in patients with FVIII inhibitors, provides a unique opportunity for studying the relationship between natural autoreactivity, autoimmunity in disease and antigen-driven immune responses to a single protein as well as antibody-directed immune regulation in health and disease.

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Idiotypic regulation of natural autoreactivity to FVIII

It is now well established that autoreactive antibodies and B-cells are present in healthy individuals.[1] Natural autoantibodies belong to the IgM, IgG and IgA isotypes. Their variable regions are encoded by unmutated germ line immunoglobulin V genes. The antibodies exhibit a broad range of affinities, with reported dissociation constants ranging between 10^{-5} and 10^{-8} M. Natural antibodies are often polyreactive, in that they may recognize several structurally different antigens. Although the functions of natural antibodies are not fully understood, their role in the maintenance of immune homeostasis and tolerance to self has been extensively documented in animal models and in man.

The presence of natural anti-FVIII antibodies in normal plasma was first demonstrated by showing that heat-treated plasma of healthy blood donors, with otherwise normal levels of FVIII, contains FVIII inhibitory activity, as assessed by the Bethesda assay.[2] The FVIII-neutralizing activity of normal plasma was shown to co-purify with IgG and was further found in F(ab')$_2$ fragments of IgG. The titer of natural antibodies exhibiting FVIII-neutralizing activity in normal plasma was found to be positively correlated with the FVIII-binding activity of normal IgG, although normal IgG contains both antibodies with inhibitory activity to FVIII and antibodies that bind FVIII without exhibiting functional inhibitory capacity. By affinity-purifying natural anti-FVIII antibodies from therapeutic pools of normal human IgG (intravenous immunoglobulin, IVIg) on affinity columns of human recombinant FVIII, we found the inhibitory titer of the affinity-purified anti-FVIII fraction of IVIg to be 16 BU per mg of IgG (Moreau *et al.*, submitted). We also calculated the clonal frequency of natural anti-FVIII antibodies to be lower by three orders of magnitude than that of the FVIII antibodies in the plasma of inhibitor-positive hemophilia patients.

Plasma levels of FVIII are maintained within

normal limits because the concentration of natural antibodies with inhibitor activity is low, and because of an effective peripheral regulation of autoantibody activity by natural anti-idiotypes. There is also some suggestion that natural anti-FVIII antibodies with neutralizing properties are more effective against allogeneic than autologous FVIII.

The presence of anti-anti-FVIII anti-idiotypes in normal plasma is evidenced by the characterization of such antibodies in IVIg.[3,4] The anti-idiotypes may be readily isolated from IVIg by affinity-chromatography on Sepharose-bound affinity-purified anti-FVIII antibodies. The presence of anti-idiotypes in normal plasma was also directly evidenced by the observation by Gilles *et al.*, that IgG purified from the plasma of healthy individuals is recognized by mouse monoclonal anti-FVIII antibody when the plasma is depleted of natural anti-FVIII IgG by affinity-chromatography on insolubilized FVIII.[5] The anti-FVIII depleted fraction of IgG was further shown to inhibit the binding of both mouse monoclonal and human polyclonal anti-FVIII antibodies to FVIII.

Idiotypic regulation of FVIII inhibitors

FVIII inhibitors arise as alloantibodies in the plasma of patients with hemophilia A in response to multiple infusions of FVIII or develop spontaneously as autoantibodies in non-hemophilic patients. FVIII inhibitors block functional epitopes of the FVIII molecule by steric hindrance. FVIII inhibitors that bind to the heavy-chain of FVIII prevent the cleavage of the FVIII molecule by thrombin. Light-chain-specific inhibitors prevent the interaction of FVIII with activated factor IX or phospholipids and with von Willebrand factor (vWF). FVIII inhibitors may also bind to epitopes expressed by the complex of FVIII and vWF, and reduce the dissociation rate of FVIII from vWF. We have recently shown that inhibitors may also neutralize FVIII activity by proteolytic cleavage of the FVIIII molecule. In addition to antibodies with neutralizing properties, the plasma of inhibitor-positive patients contains anti-FVIII antibodies to non-functional determinants of the FVIII molecule. Mapping of the epitopes recognized by anti-FVIII antibodies has not revealed any particular restriction in the number and location of FVIII epitopes recognized by FVIII inhibitors.[6] Clusters of B-cell epitopes have been delineated: a 18.3-kD amino-terminal segment of the A2 domain on the heavy chain (amino acids 373-740) , epitopes in the A3 domain, in the C1 domain and in the C2 domain. Antibodies to FVIII vary from one patient to another with regard to epitope specificity, with anti-FVIII antibodies in a single patient's plasma that react with epitopes in the heavy

chain, the light chain, or both chains. In some patients, FVIII inhibitors show a change of specificity over time, which may be a consequence of determinant spreading, as has been described in other autoimmune conditions. We have found that the patterns of reactivity of FVIII inhibitors with FVIII, as analyzed by immunoblotting, do not differ significantly from those of natural autoantibodies to FVIII isolated from normal plasma. Immunoprecipitation experiments confirmed the heterogeneity of both natural anti-FVIII antibodies and FVIII inhibitors and did not allow natural anti-FVIII antibodies to be discriminated from inhibitors isolated from patients' plasma.

Several years ago we demonstrated that the spontaneous recovery of anti-FVIII autoimmune disease is associated with the generation of anti-idiotypic antibodies capable of neutralizing the inhibitory activity of autologous anti-FVIII autoantibodies from the acute phase of the disease.[7] F(ab')$_2$ fragments prepared from a patient's post-recovery IgG were also shown to neutralize the FVIII inhibitory activity of IgG of two other patients with anti-FVIII autoimmune disease, suggesting that anti-FVIII autoantibodies share recurrent idiotypes. The presence of private idiotypes on anti-FVIII autoantibodies was evidenced by the higher capacity of Sepharose-bound post-recovery anti-idiotypic antibodies to retain autologous anti-FVIII inhibitors as compared with inhibitors of other patients. Post-recovery anti-idiotypes were not, however, capable of retaining the inhibitory activity of alloantibodies from a patient with hemophilia A, suggesting that allo- and autoantibodies do not share cross-reactive idiotypes.

Tolerance induction is successful in 60 to 80% of hemophilic patients with FVIII inhibitors. Gilles *et al.* elegantly demonstrated that successful desensitization in hemophilia patients is not associated with a reduction in anti-FVIII IgG alloantibodies but that tolerization with FVIII is primarily eliciting anti-idiotypic antibodies capable of neutralizing the inhibitory activity of autologous anti-FVIII alloantibodies.[8] Anti-idiotypic antibodies, generated in hemophilia patients undergoing tolerance induction, were further shown to inhibit the binding of autologous anti-FVIII alloantibodies to FVIII in competitive binding assays.

IVIg has been shown to result in a rapid and prolonged suppression of FVIII inhibitory activity in patients with anti-FVIII autoimmune disease. Several lines of evidence demonstrated that IVIg contains anti-idiotypic antibodies to anti-FVIII autoantibodies: F(ab')$_2$ fragments of IVIg dose-dependently inhibited anti-FVIII activity *in*

vitro; Sepharose-bound F(ab')$_2$ fragments of IVIg specifically retained anti-FVIII activity upon affinity chromatography of F(ab')$_2$ fragments of patient's IgG containing anti-FVIII autoantibodies; IVIg competed with mouse anti-idiotypes for binding to patient's autoantibodies. IVIg was also shown to neutralize alloantibodies of some inhibitor-positive patients with hemophilia A. The data indicate that natural and pathogenic anti-FVIII antibodies share common idiotypic determinants. However, anti-idiotypic IgG in IVIg neutralizes to different degrees anti-FVIII activity present in the plasma of different patients, further indicating that sharing of idiotypes is only partial between FVIII inhibitors and natural anti-FVIII antibodies. We have raised a mouse monoclonal antibody (termed 20F2) against the F(ab')$_2$ fragments of affinity-purified anti-FVIII IgG of a patient with anti-FVIII autoimmune disease and shown that the antibody neutralized FVIII antibodies of two of three patients with FVIII autoimmune disease, indicating that the idiotope defined by antibody 20F2 is shared by anti-FVIII autoantibodies of several patients, but that this idiotope is not a public idiotope of anti-FVIII autoantibodies at large. Interestingly, the idiotope defined by 20F2 is also expressed on natural anti-FVIII autoantibodies.

Conclusions

The data summarized here allow three types of anti-idiotypic antibodies with inhibiting properties against anti-FVIII antibodies to be distinguished: i) anti-idiotypic antibodies that are generated in hemophilic patients undergoing successful tolerance induction; ii) anti-idiotypes that occur in patients in remission of anti-FVIII autoimmune disease; iii) natural anti-idiotypes that are present in the plasma of healthy subjects.

We suggest that FVIII inhibitors in patients with hemophilia A and in patients with anti-FVIII autoimmune disease encompass two populations of anti-FVIII antibodies, one that results from the clonal expansion of B-lymphocytes and the other that pre-exist in hemophilic patients to treatment with FVIII which produce anti-FVIII antibodies with properties similar to those of natural anti-FVIII antibodies present in healthy individuals. The latter population of FVIII inhibitors carries idiotopes that are shared with natural anti-FVIII antibodies, and are therefore neutralized by natural anti-idiotypic antibodies in IVIg. The second population of hemophilic inhibitors, and of inhibitors of patients with anti-FVIII autoimmune disease, is comprised of B-cell clones that have undergone affinity-maturation and hypermutation of V-regions. Such antibodies may have lost the expression of natural idiotypes and thus escape neutralization by natural anti-idiotypic antibodies contained in IVIg. Restoration of a physiologic control of these B-cell clones may thus require active/ specific stimulation of the immune system, e.g. by induction of immune tolerance or by active immunization with appropriate vaccinal strategies aimed at boosting the anti-idiotypic response.

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Characterization of the immune response to factor VIII using hemophilia A* mice

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Abstract

Inhibitor antibody formation is a major complication of factor VIII replacement therapy in patients with hemophilia A. In order to understand the pathogenesis of this immunologic reaction better, we have characterized the immune response to human factor VIII in a murine model of hemophilia A. Mice with severe factor VIII deficiency caused by targeted gene disruptions were injected intravenously with human factor VIII. A human factor VIII-specific T-cell proliferative response was detected with spleen cells obtained three days after a single injection with human factor VIII and anti-factor VIII antibodies were detected after two intravenous injections. Subsequent exposures led to high titer anti-factor VIII antibodies in both ELISA and inhibitor assays. The anti-factor VIII inhibitor antibody response was shown to be T-cell dependent by its absence in hemophilic mice also deficient for the T-cell co-stimulatory ligand B7-2. In separate experiments, injection of murine CTLA4-Ig completely blocked the primary response to factor VIII in hemophilic mice with intact B7 function. This reagent also prevented or diminished further increases in anti-factor VIII when given to hemophilic mice with low anti-factor VIII antibody titers. ©2000, Ferrata Storti Foundation

Key words: factor VIII inhibitors, hemophilia A, T-cells, immunobiology

Hemophilia A is an hereditary X-linked bleeding disorder caused by factor VIII deficiency.[1] However, many patients with severe hemophilia A develop anti-factor VIII inhibitor antibodies following factor VIII replacement therapy.[2] There are no effective strategies to prevent or blunt the antibody response in these patients, and efforts to establish immune tolerance are not always successful once antibodies have developed.[3]

We used a mouse model of hemophilia A to evaluate new methods for the prevention and treatment of factor VIII inhibitors. Hemophilia A mice, generated by targeted disruption of exon 16 or 17 of the factor VIII gene, have no detectable factor VIII activity in their plasma.[4] As expected, hemophilia A mice have *in vivo* signs of a coagulation pathway defect with fatal bleeding if tails are cut without use of hemostatic measures, and they develop subcutaneous and intramuscular bleeding after handling or temporary immobilization.[5]

Development of anti-factor VIII antibodies and factor VIII-specific T-cells

An IgG anti-factor VIII response was detected in the hemophilia A knockout mice injected intravenously with either plasma-derived or recombinant factor VIII. Although there was considerable variability, anti-factor VIII was usually detected after two injections and high titer inhibitor antibodies were present in all mice after four injections.[5]

The T-cell response to factor VIII was assessed in proliferation assays for spleen cells obtained after intravenous injection of human factor VIII. Significant T-cell proliferation was noted for hemophilic mice after the first injection of human VIII, and the peak response was at 3 days. Thus, a factor VIII-specific T-cell proliferative response was detected before antibodies were present.

Blockade of T-cell co-stimulation

Optimal T-cell activation requires signaling through the antigen-specific T-cell receptor (TCR) by its engagement with peptide major histocompatibility complex (MHC) complexes on antigen-presenting cells, in combination with co-stimulatory signals typically delivered through the T-cell surface glycoprotein, CD28.[6] CD28 interactions with B7-1 (CD80) and B7-2 (CD86), co-stimulatory ligands on antigen presenting cells, are essential for initiating antigen-specific T-cell responses, upregulating cytokine expression, and promoting T-cell expansion and differentiation.[6]

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CTLA4, a downregulatory molecule in T-cell activation, is a second, high affinity T-cell receptor for both B7-1 and B7-2.[6] CTLA4-Ig, a soluble fusion protein in which the extracellular domain of CTLA4 is fused to the CH2-CH3 tail of IgG1, has been shown to be an effective reagent for blockade of CD28-B7 interactions *in vivo.*

The roles of B7-1 and B7-2 co-stimulatory ligands on antigen-presenting cells were evaluated to determine whether the T-cell CD28 signaling pathway is essential for development of inhibitory antibodies to factor VIII. To do this we crossed hemophilia A mice with B7-1-/- and B7-2-/- knockout mice[7] and mice deficient in both factor VIII and either B7-1 or B7-2 were selected by genotype analysis. When the hemophilia A/B7-1-/- and hemophilia A/B7-2-/- mice were injected intravenously with 0.2 µg human factor VIII at two week intervals, all hemophilia A/B7-1-/- mice developed high titer anti-factor VIII and none of the hemophilia A/B7-2-/- mice had detectable anti-factor VIII.[8] Thus, B7-2 is essential for the development of an immune response to factor VIII injected intravenously, and anti-factor VIII formation is prevented if it is missing.

We then tested the effectiveness of murine CTLA4-Ig blockade of the CD28 signaling pathway in preventing anti-factor VIII antibody formation. While anti-factor VIII inhibitory antibodies were induced in control hemophilia A mice by repeated intravenous injections of 1 mg recombinant human factor VIII at three week intervals, anti-factor VIII antibody formation was markedly suppressed in mice injected intraperitoneally with 250 µg of murine CTLA4-Ig on the day before and the day after the first factor VIII injection, even though there was no further CTLA4-Ig given with three subsequent factor VIII injections. However, a weak immune response was detected in two of the six CTLA4-Ig treated mice after the third factor VIII injection and five of six had high titer anti-factor VIII after the fourth injection.[8]

Because a delayed anti-factor VIII response was detected after repeated factor VIII infusions when CTLA4-Ig was given only at the time of the first factor VIII exposure, we then determined whether CTLA4-Ig might prevent anti-factor VIII development if given with each factor VIII infusion. In that experiment, hemophilia A mice were simultaneously infused six times with both factor VIII and CTLA4-Ig at three week intervals. There was no detectable anti-factor VIII in any of the mice treated in this way.[8]

To determine whether CTLA4-Ig modifies the secondary immune response to factor VIII, we injected CTLA4-Ig at the same time that factor VIII was given to hemophilia A mice that had already developed anti-factor VIII. The control

mice then received three additional injections of factor VIII while the treated mice were given CTLA4-Ig at the same time as they received the first of the three additional factor VIII injections. While a brisk increase in the anti-factor VIII titer occurred after the factor VIII injections in the control mice, mice treated with CTLA4-Ig at the time of the fourth factor VIII injection had minimal or no increases in anti-factor VIII.

Perspectives

We have used a mouse model of hemophilia A to establish the T-cell dependence of inhibitory antibody formation to factor VIII and to evaluate the potential use of reagents that block the B7/CD28/CTLA4 co-stimulation pathway. These studies in mice were done with human factor VIII, and we believe that they are relevant to our efforts to prevent and treat factor VIII inhibitor antibodies in patients with hemophilia A. The formation of inhibitory antibodies to factor VIII is a critical problem for hemophilia A patients treated with protein replacement therapy and may be a major problem for gene therapy in hemophilic patients. The results of our studies with the hemophilia A mice suggest that co-stimulation blockade may be an effective therapy for the prevention of anti-factor VIII antibodies in these patients.[8] The data from the B7-2 deficient mice suggest that blockade of CD28-B7-2 interaction is critical for the initiation of an antibody response to intravenous factor VIII. Thus, anti-B7-2 may be as effective as CTLA4-Ig in blocking the initiation of an anti-factor VIII response. If this is the case, B7-1 function would remain intact and the treatment could be less generally immunosuppressive than would be the case if CTAL4-Ig were used.

It is not certain why the hemophilic mice formed anti-factor VIII after they were given additional factor VIII injections in the absence of mCTLA4-Ig.[8] However, similar reversal of specific unresponsiveness to a T-dependent antigen (sheep red blood cells--SRBC) has been described by Wallace and colleagues.[9] In those studies, a single dose of murine CTLA4-Ig prevented antibody formation immediately after the first and second SRBC injections, but a high titer response followed the third SRBC injection. The development of anti-factor VIII in our studies after repeated antigen exposure is likely to be due to the combination of mCTLA4-Ig clearance over time (its serum half-life beta phase being 6 days[9]) and emigration from the thymus of naïve T-cells that are capable of an anti-factor VIII response.[10] However, the downregulatory role of B7-CTLA4 interactions is also important in the induction and maintenance of T-cell anergy,[11] and CTLA4-Ig blockade of the B7-CTLA4 interaction may

have prevented the development of anergy in the treated mice.

At the present time, it is not possible to identify which hemophilia A patients will develop inhibitory antibodies after factor VIII treatment.[1,2] For this reason, the clinical application of our studies would be primarily for patients who have already developed detectable anti-factor VIII. In our initial studies, combined treatment of hemophilia A mice with CTLA4-Ig and factor VIII after detection of anti-factor VIII antibody prevented a further increase in antibody titer in most mice and a fall in titer in some.[8] If the pattern is similar in patients, B7-2 co-stimulation blockade at the time of factor VIII treatment may stabilize the inhibitor level for patients treated early after detection of anti-factor VIII. For low titer factor VIII inhibitor patients, this co-stimulation blockade would then permit the use of factor VIII doses at therapeutically effective levels without further boosting the inhibitor titer. As antibody suppression by co-stimulation blockade appears to last several weeks, intermittent dosing may then be sufficient to prevent or modulate inhibitor formation. Moreover, limiting the co-stimulation blockade to B7-2 may reduce anti-factor VIII while leaving intact the immune responses to other antigens.

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Animal models to explore mechanisms of tolerance induction to FVIII: SCID mice and SCID-FVIII-KO mice

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Type A hemophilia is an inborn disease of coagulation. It is characterized by a deficiency or dysfunction of factor VIII (FVIII) and affects 1 in 10,000 males. The clinical manifestations of the disease include bleeding in muscles, joints, and soft tissues, and are optimally treated by infusions of FVIII. However, such treatment results in the development of antibodies towards FVIII (usually described as *inhibitors*), in at least 15 to 25% of the cases.[1,2] Autoantibodies are also described in some patients with an annual incidence of 1/5 million people and remain the most common cause of antibody-mediated inhibition of the coagulation system.[3] The occurrence of such anti-FVIII antibodies is a severe, life-threatening complication which practically precludes efficient FVIII replacement therapy. Present treatments, including regular infusion of high doses of FVIII,[4] administration of corticosteroids and cyclophosphamide alone or in combination with infusions of large doses of FVIII concentrates,[5] IgG immunoadsorption or infusions of IV Ig,[6] are of limited efficiency and extremely expensive.[7]

Alternative methods of specific treatment are required. Therefore, efforts must be devoted to improving our understanding of reasons as to why anti-FVIII antibodies are formed and the mechanisms of production of such antibodies.

An animal model that can be used to reconstitute and analyze a human immune response towards FVIII was considered as optimal. Severe combined immunodeficient mice (SCID mice) or variants of this strain have been chosen for this purpose. The repopulation of these animals with immunocompetent cells of a hemophilia A patient or of healthy donors enables us to reconstitute a human immune response.

Such a model should enable us to study factors governing FVIII immunogenicity, the physiology of the anti-FVIII immune response, namely the molecular mechanisms leading to the development and maintenance of anti-FVIII

antibodies, and finally to explore the potentialities of new forms of immunotherapy.

These studies should yield benefits not only for hemophilia A patients, but could also result in a significant reduction in the cost related to the treatment of patients with anti-FVIII antibodies.

SCID mouse model

SCID mice exhibit an immunodeficiency owing to a defect in their mature B-(no surface Ig) and T-(no functional TCR) lymphocytes.[8] Detailed analysis has disclosed an aberration of V(D)J recombination in such cells and a high sensitivity to ionizing radiation. The SCID-related gene codes for a DNA-dependent protein kinase catalytic subunit and resides in the centromeric region of chromosome 16.[9] Because of their severe immune deficiency, such mice are able to accept xenotransplants and can therefore be reconstituted with peripheral blood mononuclear cells (PBMC) isolated from hemophilia A patients or healthy donors.[10]

SCID mice and hemophilia A patients

Some hemophilia A patients produce anti-FVIII antibodies upon FVIII infusion. The strength of the immune response is unpredictable, varying from one patient to another, but is also related to the immunogenicity of the FVIII concentrates. There is no current method to evaluate these critical parameters prior to the first FVIII infusion. The SCID mice model was evaluated to this end. We reconstituted mice with immunocompetent cells from two hemophilia A patients with a stable, high level of FVIII inhibitors.

Mice were then immunized with two different FVIII preparations, a recombinant or a plasma-derived FVIII. All mice produced a significant amount of human IgG antibodies and the presence of specific anti-FVIII antibodies was detected in each serum sample by using a direct binding ELISA. Mice injected with the FVIII preparation produced at last 4-fold more specific antibodies, including inhibitors, than control animals. The level of total IgG produced by each group of mice depended on cell origin, but also on the FVIII concentrate used (Table 1). These

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Table 1. The capacity to inhibit 50% recombinant or plasma-derived FVIII function by the serum of mice reconstituted with PBL of hemophilia A patients with inhibitors is similar to the capacity of the purified anti-FVIII Ab fraction from the corresponding hemophiliacs.

	rec FVIII IgG (µg/mL)	purified FVIII IgG (µg/mL)
Patients' total IgG	100	100
Patients' specific IgG (immunopurified)	0.5	0.5
Mice's serum IgG (8)	0.16-2	0.12-1.5

Table 2. The isotypic distributions of total IgG and anti-FVIII Ab produced in SCID mice are identical to total IgG and anti-FVIII Ab produced by healthy source donors, except for the percentage of the IgG4 isotype which is significantly increased.

	IgG subclasses (%)			
	IgG1	IgG2	IgG3	IgG4
	Total IgG			
Healthy donors	59.4±4.2	30.0±4.3	5.1±1.6	5.2±1.7
SCID mice	63.4±7.1	28.7+6.6	4.0±1.2	3.9±2.4
	Affinity-purified antibodies			
Healthy donors	52.9±3.3	39.9±3.8	3.0±0.7	4.4±1.1
SCID mice	62.0±6.6	28.2±6.4	4.0±1.0	5.7±2.7
Normal distribution	70	20	8	2

results show that the SCID mice can be used to study a human anti-FVIII secondary immune response *in vivo*. As several mice can be reconstituted with cells from a single patient, this model should allow for direct comparative studies of the immunogenicity of different FVIII preparations.

SCID mice and healthy donors

The presence of antibodies directed towards self-antigens in the serum of healthy individuals has already been demonstrated in a number of situations.[11] One of the most representative examples of this is the presence of anti-factor VIII antibodies in the serum of healthy donors.[12]

Several studies have demonstrated the presence of such antibodies, which include not only non-functional anti-FVIII antibodies but also high-affinity antibodies able to inhibit FVIII function with the same efficiency as antibodies produced by hemophilia A patients under FVIII infusion therapy. Natural auto-antibodies (NAA) are only detectable after passing a total IgG fraction through a specific FVIII immunosorbent. The specificity of the purified antibodies was confirmed by their capacity to recognize soluble and insolubilized FVIII. Epitope mapping using monoclonal mouse anti-FVIII antibodies demonstrated that in most cases the A3 and the C2 domains of the FVIII light chain were recognized, while the reactivity towards heavy chain epitopes differed from one patient to another. The reason why FVIII cofactor activity is not inhibited is still conjectural, but could be in part due to the presence of anti-idiotypic antibodies. The latter have indeed been detected in healthy donor's plasma and in a larger amount in the plasma of hemophilia A patients who had developed inhibitors and were successfully desensitized by administration of high doses of FVIII.[13]

In an attempt to further characterize NAA to FVIII and to study the conditions under which auto-immunity to FVIII can develop, SCID mice were reconstituted with immunocompetent cells of human origin, namely peripheral blood mononuclear cells of unrelated healthy blood donors. The production of human anti-FVIII antibodies was detected within a month following reconstitution. Specific and functional antibodies were undetectable in mouse plasma, as was the case in healthy donors,[12] and therefore affinity purification on a FVIII immunosorbent had to be carried out. Importantly, administration of human FVIII did not increase the production of such NAA in comparison with saline-treated mice. This spontaneous production of anti-FVIII antibodies in mice indicates that tolerance towards FVIII had been transferred with cell reconstitution. A thorough analysis showed that the isotypic distribution (Table 2) and the capacity to inhibit FVIII of antibodies produced in SCID mice was identical to that of antibodies produced by healthy source donors (Figure 1). Moreover, immunoprecipitation experiments with recombinant FVIII fragments showed that the specificity of the anti-FVIII antibodies was restricted to the light chain and more specifically to the C1 and C2 domains. Binding of antibodies to the heavy chain could however not be totally excluded but major epitopes were located in the light chain. A prominent mechanism of FVIII inhibition by these antibodies is identical to that observed with inhibitors produced by hemophilia patients, namely prevention of FVIII binding to phospholipids. Since antibodies of interest can be found in the SCID mouse model and, moreover, since they are qualitatively comparable to the source donor's antibodies, the model provides appropriate experimental conditions to study the regulation of tolerance against FVIII in normal subjects.
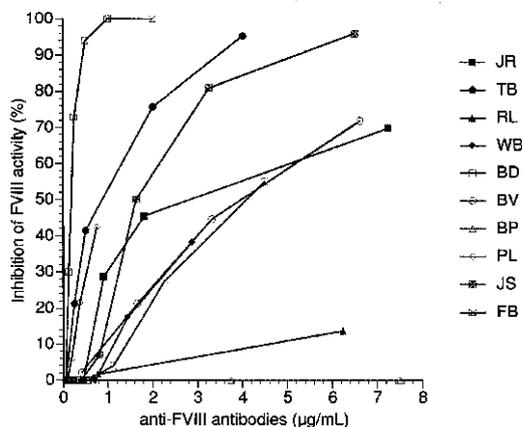
Figure 1. The anti-FVIII activity, evaluated in a chromogenic assay, of purified anti-FVIII Abs from SCID mice reconstituted with cells from healthy donors. Similar results were obtained with the anti-FVIII Ab fractions from the source donors.[12]

SCID mice and anti-CD40 ligand

Anti-FVIII antibodies can only be produced when efficient help is provided by specific T-cells. This help includes 2 signals besides the production of cytokines. The first signal is the presentation of a FVIII peptide to the TCR in the frame of MHC class II molecules. In the absence of a second signal, the B-T cell interaction results in anergy rather than activation. The second signal is constituted by interaction of antigen-non-specific, accessory molecules, complementary structures on the membrane surface. One important accessory molecule interaction is the binding of CD40L on T-cells to CD40. CD40L is transiently expressed (2 to 24 hours) on helper (CD4+) T-lymphocytes following early activation (usually mediated through the TCR).[14-16] The molecule plays an early and important role in the interaction of these activated T-lymphocytes with cells expressing CD40. CD40-bearing cells include B-ymphocytes, vascular endothelial cells, macrophages, dendritic cells, various epithelial cell types, and fibroblasts, *inter alia.*

Administration of an antibody blocking the CD40L-CD40 interaction during immunization with protein antigens can specifically block the antibody response to that antigen in a mouse model.[17] Total serum Ig and antibody responses to proteins administered outside of the window of CD40L blockade are not affected. Preclinical studies in a variety of autoimmune disease models have shown that blockade of this pathway with antibodies to CD40L can substantially reduce symptoms of disease and, in some cases, reduce mortality.[18-21] The antibod-

ies have also been used to induce long-term graft acceptance in allogeneically mismatched transplant settings.[22-24] The impressive activity of anti-CD40L antibody therapy in a variety of both antibody-and cell-mediated autoimmune diseases, and in organ transplantation, suggests that blockade of the CD40L-CD40 interaction will be therapeutically useful in human disease.

This is why we have evaluated this CD40L-CD40 interaction to block the anti-FVIII humoral response in SCID mice reconstituted with cells of a hemophilia A patient with inhibitors. Preliminary experiments showed an important decrease in the anti-FVIII antibody production in reconstituted mice injected with FVIII and anti-CD40L antibody when compared to control group.

SCID-FVIII-knock-out mice

Direct detection of natural specific antibodies in mouse serum is not possible, whereas antibodies made by mice reconstituted with cells of hemophilia patient with a high inhibitor titer can be detected. There are several possible reasons for this phenomenon. Anti-FVIII antibodies could be neutralized by anti-idiotypic antibodies produced in reconstituted mice or the specific anti-FVIII antibody concentration could be too low to be detectable in plasma. Attempts to detect anti-idiotypic activity in mice serum were unsuccessful, using a method described previously for the characterization of anti-idiotypic antibodies specific for anti-FVIII antibodies made by healthy donors. This suggest that the time period during which mice were followed was too short. The fact that specific antibodies are undetectable in mouse plasma may be related to the close homology between murine and human FVIII,[25] suggesting that anti-FVIII antibodies produced in SCID mice could bind to FVIII and thereby render the anti-FVIII antibodies undetectable in the plasma. To avoid such a problem, we have created SCID-FVIII-knock-out mice by cross-breeding the two species, creating a model in which we will be able to explore the different conditions under which anti-FVIII antibodies are produced.

Data obtained with SCID mice and preliminary data generated by the SCID-FVIII-knock-out mice indicate that such models are appropriate to study the regulation of tolerance against self antigens in normal subjects and could be extended to diseases such as acquired hemophilia in which unresponsiveness to self has been lost. These models are also suitable for the pre-clinical evaluation of immune therapies such as immunomodulation (e.g. by administration of anti-CD40L antibodies) or immune regulation, which includes the evaluation of idio-

typic interactions after passive administration of monoclonal or polyclonal anti-idiotypic antibodies, or after active therapy (immunization with monoclonal or polyclonal anti-FVIII antibodies, peptides or cDNA).

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DISCUSSION 18   Animal models to explore tolerance induction to factor VIII
J.G.Gilles, B. Vanzieleghem, J.M. Saint Remy (Leuven, Belgium)

HOYER: Do you know whether or not humans spontaneously develop any antibodies against CD40 or antibodies against CTL4?

GILLES: No, we don't know.

KAZATCHKINE: But we do know, at least in the case of IGIV. You can affinity verify the presence of those antibodies from intravenous immunoglobulin so they are present in low amounts.

EWENSTEIN: I would like to ask you about the administration regimen for the anti CD40 ligand and antigen. You mentioned co-administration.

GILLES: Yes and we observed that this is certainly a dose-dependent immune response. We have to be very careful with the concentration of the anti-CD40 antibody ligand that we use. If

the amount of antibody we use is too high we completely downregulate the whole immune response.

RYPERT: I have two questions. Do you reconstitute your SCID mice with the lymphocytes of normal donors? Have you looked for anti-idiotypes. I would expect judging from your previous work that you might find anti-idiotypic antibodies as well.

GILLES: Yes, we looked for these. The problem is that the period in which we are able to analyze a good immune response is extremely short. The time and the frame in which we analyze the immune response is too short to evaluate the anti-idiotypic antibody immune response.

RYPERT: Have you looked at whether you find antibody response if you reconstitute your mice with lymphocytes from hemophilia patients without inhibitors?

GILLES: We are also able to find anti-factor VIII. We are able to produce exactly the same immune response as in the patient in terms of subclasses and anti-factor VIII activity. It is a very close model.

HOYER: Are you saying that the responses when you transplant with normal peripheral blood lymphocytes and peripheral blood lymphocytes from patients with an inhibitor are qualitatively or quantitively the same in terms of their response to factor VIII?

GILLES: Qualitatively the same but of course in quantitive terms it is completely different.

KAZATCHKINE: These are very convergent data. I also showed approximately a hundred-fold difference in what you may indirectly calculate as a clonal frequency of these cells between the acquired hemophilia inhibitor and healthy individuals. I think that in these two models that we have heard about we have the potential tools to learn many things in the coming years.

Gene therapy, factor VIII antibodies and immune tolerance: hopes and concerns

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Abstract

As trials of hemophilia A gene therapy enter the clinic, a continuing concern is the generation of factor VIII inhibitors to the newly delivered transgene product that might also neutralize future factor VIII replacement therapy. A number of factors relating to both the transgene recipient and to the gene therapy process have now been recognized to influence the likelihood of this treatment complication. The recipient's hemophilic mutation and other, far less clearly defined, immunogenic loci are clearly important components in the estimation of inhibitor risk, and need to be taken into account in selecting the initial gene therapy study populations. With regards to the gene delivery process, the choice of vector, site of delivery and tissue restriction of transgene expression have all been identified as factors that influence the subsequent development of a productive immunologic response to the transgene product. Each of these variables has an important effect on the initial event of antigen presentation and appropriate gene therapy strategies have been characterized that will significantly reduce the efficiency of this critical stage in the immunologic response. Finally, additional recipient characteristics such as pre-existing or induced inflammatory conditions or tissue damage have also been demonstrated to increase the subsequent risk of inhibitor development and therefore represent clinical states that should be avoided in the selection of gene therapy recipients and protocols.
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Key words: factor VIII, gene therapy, inhibitors

Over the past year, a previous decade of preparatory investigation has culminated in the initiation of three phase 1 clinical trials of hemophilia gene therapy. The very fact that these trials have received approval from appropriate regulatory agencies indicates that significant progress has been made towards the safe and effective delivery of clotting factor genes. Nevertheless, despite these advances, few investigators doubt that a number of potential problems remain to be fully explored and overcome. This report deals with the most worrying of these potential complications, that of factor VIII inhibitor development.

Given that the field of gene therapy is still in its infancy, much remains to be learnt about the immunologic consequences of new protein biosynthesis through the delivery of a therapeutic transgene. However, in considering this problem, it is clear that a variety of factors pertaining to both the transgene recipient and the gene therapy process will influence the likelihood of inhibitor development (Figure 1).

Factors inherent to the transgene recipient

Host genetic factors have long been recognized to contribute to the risk of factor VIII inhibitor development. There is now good evidence to indicate that these factors are of two types and are associated with either the hemophilic genotype or other, as yet unidentified, immunogenic characteristics. Recent studies in both hemophilia A and B have documented significantly increased risks of inhibitor development in patients with certain forms of hemophilic mutation resulting in a lack of circulating protein.[1] In both disorders, patients with large deletions are more inhibitor prone and, in hemophilia A, patients with both the intron 22 inversion and premature stop codons have an approximately 35% risk of inhibitor development. These observations suggest that, at least in initial clinical trials of gene therapy, patients must be genotyped to assess their inhibitor risk and that it might be prudent to include only low risk patients in these early studies. The other genetic factors that influence the risk of inhibitor development remain unresolved, however, as there are well-documented instances in both humans and animal models of hemophilia in which subjects with identical hemophilic mutations have shown different rates of inhibitor incidence. Insufficient information has been gathered to localize this influence to any

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Factors Influencing Inhibitor Development in Haemophilia Gene Therapy
● Type of haemophilic mutation
● Transgene recipient's "immunogenotype"
● Ability of vector to transduce antigen presenting cells
● Cell-type expression of transgene product
● Differences of transgene product to the native protein
● Co-existence of an inflammatory state or tissue damage

specific immunogenic locus including the MHC class I and II loci, and indeed, this propensity likely involves contributions from several different genes.

An observation that is also likely related to the phenomenon of immunologic tolerance concerns the lack of response to foreign transgene products in certain inbred strains of laboratory mice. Several investigators have been able to demonstrate long-term expression of human transgene products, including factor VIII, in C57BL/6 mice without evidence of antibody development. Interestingly, in a very recent report of long-term human factor VIII transgene expression following *in vivo* retroviral delivery to factor VIII deficient mice, expression was interrupted between 27 and 48 days after transgene delivery in 50% of the mice.[2] These mice were documented to have developed anti-human factor VIII antibody titers between 7 and 350 Bethesda units. Although the development of anti-factor VIII inhibitors has not been documented previously in the hemophilic mouse model, it appears that in this instance, the lack of immunologic tolerance may have arisen, at least in part, as a result of insufficient admixture with the C57BL/6 genome in the five generations of back-crossing.

Factors relating to the gene therapy process

The transgene delivery system

Recent progress with gene therapy protocols have almost exclusively involved recombinant viral vector systems. Four types of protocols involving adenovirus, adeno-associated virus (AAV), lentivirus and Moloney-derived retrovirus have been evaluated for hemophilia gene therapy and there is strong circumstantial evidence to suggest that the type of viral vector utilized is a factor in determining subsequent inhibitor risk. This influence is probably mediated through sev-

eral different factors including the types of cells transduced, the kinetics of transgene expression and the tendency to elicit a more generalized immune response in the recipient.

Effects on antigen presentation

An important difference between clotting factor treatments administered from an exogenous source and clotting factor synthesized from an endogenous transgene is the mode of antigen presentation. While exogenous antigen is presented primarily through MHC class II association, protein that is intrinsically synthesized will also be presented in association with class I MHC complexes on the cell of synthesis and initiate a primary cytotoxic T-cell response. Although the consequences of this difference in immunologic processing have not been resolved, there is already evidence to indicate that transduction of cell types which present antigen most effectively is more likely to result in the initiation of a cell-mediated and subsequent humoral response to the transgene product. This has best been illustrated through comparisons of delivery of the same transgene by AAV and adenoviral vectors. This comparison has shown that, whereas efficient antigen-presenting cells (APCs) such as dendritic cells are highly transducible with adenoviral vectors, these cells are not productively transduced by AAV.[3] Although AAV particles appear to gain entry to APCs they remain localized in a perinuclear distribution and do not enter the nucleus to form integrated and transcriptionally active complexes. As a result of this difference in productive transduction of APCs, the initial cell-mediated response to the transgene is significantly greater following adenoviral-mediated delivery. This phenomenon presumably also reduces the likelihood of a secondary humoral response to the transgene product with AAV vectors, although, if, as with clotting factor proteins, the protein is secreted, a subsequent MHC class II-mediated immune response may be initiated with the ultimate development of a productive humoral response. While the adverse effect of APC transduction has best been documented with adenovirus, there is also evidence to indicate that retroviral vectors pseudotyped with vesicular stomatitis virus G-glycoprotein also productively transduce APCs and might therefore be expected to increase the risk of a subsequent neutralizing immune response.

Two other factors warrant consideration in terms of the antigen presentation process: both the site of transgene delivery and transgene synthesis may influence the likelihood of developing a productive immune response to the transgene product. For example, delivery and expression of

a transgene in tissues such as skin, where APCs are abundant, may significantly increase the risk of effective antigen presentation. Another way in which this process can be influenced is through the use of transgene constructs in which tissue-specific regulatory elements are employed to preclude transgene expression in APCs. The benefit of such a strategy has recently been demonstrated using an early generation, E1 deleted, adenoviral vector delivering a human α1 antitrypsin (hAAT) transgene delivered by tail vein injection to C3H/HeJ mice.[4] In these studies, long-term transgene expression was documented with the use of a regulatory element derived from the murine albumin promoter and enhancer, whereas, in contrast, using an ubiquitous mouse phosphoglycerate kinase (PGK) promoter, serum hAAT levels returned to baseline within three weeks after an initial period of expression. These observations coincided with the development of high titer antibodies to hAAT in the mice injected with the PGK-regulated transgene construct. These results add further strength to the hypothesis that limiting expression of the transgene product to cells that do not present antigen efficiently significantly reduces the risk of subsequent inhibitor formation.

Finally, transient administration of agents that interfere with the immune response have also shown some promise in reducing the likelihood of a subsequent humoral response. Standard immunosuppressive agents such as cyclophosphamide and cyclosporin A have been delivered as a single high dose pulse at the time of vector administration and a similar strategy using the inhibitor of APC-mediated T-cell co-stimulation, CLA4Ig, has also been documented to reduce the risk of a productive humoral response.

Role of the transgene product

Most of the current gene therapy strategies for hemophilia involve the ultimate synthesis of a transgene product that will differ from the native coagulation protein. In studies of factor VIII gene delivery, all of the transgene constructs to date have utilized B domain deleted cDNAs, while in the case of factor IX gene therapy protocols, cell types other than hepatocytes have proven to be effective targets. In all of these situations, the secretion of a transgene product which is either grossly or subtly different in structure to the native protein may result in the development of inhibitory antibodies. With regards to the B domain deleted factor VIII transgene products, previous experience from both immunogenicity studies in mice[5] and from clinical observations in hemophiliacs treated with the recombinant factor VIII product, ReFacto, suggest that at least when administered as an

exogenous protein, this molecule is no more immunogenic than native factor VIII. In the ReFacto studies, after a median of 12 exposure days, 30 of 101 previously untreated patients developed an inhibitor ($13 \geq 10$ B.U.: 4 between 5-10 B.U. and $13 \leq 5$ B.U.) and the inhibitors have persisted in 11 patients.[6] These figures for inhibitor incidences are comparable to those obtained with other full-length recombinant factor VIII products. Similarly, only one of 113 previously treated patients has developed an inhibitor after 93 exposure days to a ReFacto further indicating the relative lack of neo-antigenicity associated with this B domain deleted product.[7] Finally, with reference to recent comparative studies of factor IX synthesized from myotubes, significant differences have been documented for several post-translational modifications present in native, hepatocyte-derived factor IX including reduced tyrosine sulphation, serine phosphorylation and different patterns of glycosylation.[8] It remains to be seen whether these more subtle alterations in structure will produce a greater risk of inhibitor development.

Enhanced immune activation

Optimal initiation of the immune response to antigen requires that the antigen-presenting cells become activated by some form of pathophysiologic danger signal.[9] This signal is most effectively delivered in the form of an inflammatory state or through cell death and tissue damage. In the context of hemophilia gene therapy, examples already exist in which increased activation of the immune response has followed different forms of gene delivery with the subsequent development of an inhibitor response. In the first of these cases, adenoviral delivery of a B domain-deleted canine factor VIII transgene to two hemophilic dogs resulted in acute hepatotoxicity and, in one of the dogs (from the inhibitor prone part of the pedigree), a factor VIII inhibitor response peaking at 1,200 B.U.[10] In the second instance in which an enhanced state of immunologic activation may have played a role in inhibitor development, a neutralizing anti-canine factor IX antibody developed transiently in one of five hemophilic dogs in which a canine factor IX AAV transgene had been delivered intramuscularly.[11] This animal was distinct from the four others in the study because of the occurrence of a clinically evident skin infection. These findings suggest that hemophilia gene therapy in subjects in whom the immune system is already primed by an inflammatory state or by tissue damage, or in whom the transgene delivery protocol is likely to incite such danger signals may be more likely to mount a productive response to the transgene product.

In conclusion, our current knowledge of inhibitor risk in association with hemophilia gene therapy protocols suggests that a number of host and delivery protocol characteristics have been documented to contribute to this potential complication. In initial trials of this novel therapeutic modality, extreme care must be exercised to take all of these factors into consideration in order to minimize the risk of inhibitor development. Only after a thorough evaluation of gene therapy has been undertaken in hemophiliacs without the development of inhibitors will it be acceptable to explore the exciting possibility of using this therapeutic modality to induce immune tolerance in patients with pre-existing inhibitors.

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DISCUSSION 19   Gene therapy, factor VIII inhibitors and immune tolerance: hopes and concerns
D. Lillicrap (Kingston, Canada)

SAINT REMY: Dr. Lillicrap, I would like to ask you a question about the presence of CBG motifs in vectors; as far as I am aware most of the vectors used in gene therapy do contain such motifs. We know that such motifs would stimulate antigen-presenting cells and make them produce interferon gamma, activated T cells - the best environment to induce imunogenicity. Could you just summarize the evidence?

LILLICRAP: I think this is a very important issue and one that gene therapists are catching up on belatedly. There is no doubt that the CBG motifs are immuno-stimulatory and I believe that if you can modify those motifs or remove them from the vector background it is one of the factors which will reduce the likelihood of immunostimulation. People are belatedly taking a look at what they initially thought were innately neutral vector backgrounds.

GARGALLO: If these protocols prove to be safe in the future I would like to know if the child is partially tolerized by a negative inhibitor in normal recovery but still has a short half-life could the child be a good candidate for gene therapy and would this depend upon the level of factor produced under gene therapy? My second question is: if the child had developed an inhibitor against a certain recombinant product and was tolerized with that product, could he develop inhibitors a second time against that factor that he would produce in that it's going to be a B domain deleted factor VIII?

LILLICRAP: I think the issue of patient choice for the initial gene therapy studies has occupied people's minds a great deal in the last couple of years and will continue to do so. It's very difficult to say definitively who should be the ideal candidates. The selection process is complex given that many of the hemophilia population already have inflammatory processes such as long term hepatitis C infection. This means that to get the right patient population early on will be tough. I don't think that any of the things you said would exclude patients subsequently but they may not be the initial patients undergoing gene therapy.

DI MICHELE: I am a little concerned about your last statement about the tissue damage and inflammation because no matter how much you modify a vector you still have to get this gene in somehow. I can't imagine not violating the body in some slight way to produce an inflammatory response in order to produce cellular uptake of this gene. I think the important question is really going to be how much tissue damage and inflammation is too much and I wonder how we might go about understanding that better because I think it's a crucial issue.

LILLICRAP: I don't think we really know but I think we are likely to find out during meetings such as this one where clinicians, immunologists and others interested in gene delivery exchange ideas because I think that no one group can answer this complex question. The immunology of gene delivery is going to be very pertinent to individuals in this room and as we follow the immune tolerance protocols along with evolving gene therapy I think that the immunologists will be able to help both groups of individuals. Some of the new vectors as you are well aware do not really produce much inflammation and so I think there is an advantage both in the fact that this virus doesn't transduce APCs and appears to be minimally inflammatory after delivery. Thus, we're beginning to find vector systems which will produce advantages. Who knows if non-viral delivery, which should not be discounted, may be an advantage sometime in the near future.

DI MICHELE: I would just like to say that our immune tolerance studies, as limited as they are, can help us in identifying not only inflammatory events but maybe again if people think about what we should be measuring in terms of inflammatory markers that might help us to further understand this process with respect to inhibitor development.

ALEDORT: Dr: Lillicrap, I think you have given some balance with regard to some of the issues that we as clinicians are going to have to face in terms of recruiting people to studies. I think that we have to be really cautious in how therapeutically enthusiastic we should be at this point in entering people into the study. I think the death of this young man which has nothing to do with hemophilia but which certainly has to do with gene therapy is going to make recruitment into studies very different to what it was a week ago. As clinicians we need to reconsider informed consent because it is really a huge responsibility. We can be very enthusiastic or we can give some perspective to our patients on this new modality.

LILLICRAP: I agree that getting the balance here is critical. It's a time of great opportunity but we must be careful.

Immunological tolerance. A clinical perspective

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Abstract

Human alloantibodies to factor VIII occur in 15-50% of patients with severe hemophilia A. The development of these inhibitory alloantibodies prevents treatment with replacement factor VIII and places the patient at higher risk for complications from bleeding. Immune tolerance to factor VIII can be achieved in patients with inhibitors through the regular administration of factor VIII. The mechanism by which immune tolerance is achieved is unclear but the regular administration of factor VIII is believed to interrupt the normal immune mechanisms, perhaps by altering antigen presentation and inducing T or B cell anergy. The development of immune tolerance in hemophilia can be seen as a potential model for the development of methods for inducing tolerance to other alloimmune and autoimmune disorders.
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Key words: hemophilia, inhibitor, immune tolerance, T cell, B cell

The report by Brackmann and Gormsen 22 years ago that long-term, high dose treatment with factor VIII resulted in the development of apparent immune tolerance[1] and was a remarkable observation which has had a profound impact on the treatment of hemophiliacs with inhibitors. As a result of this observation, hemophiliacs with inhibitors now have available a treatment which will eradicate the inhibitor and permit the use of more effective methods of treatment. Current methods of inducing immune tolerance, which are not significantly different from the original method reported by Brackmann and Gormsen, are effective in around 80% of inhibitor patients and clear-cut successes have been demonstrated in patients with even the highest inhibitor titers.[2-5]

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Tolerance is specific for factor VIII since immune responsiveness to other antigens, such as blood group antigens and other cellular immune responses, is not affected.[6] More important, the state of tolerance is maintained, not only in those patients who continue to receive some form of treatment after the induction of tolerance, but even in those small numbers of patients who require infrequent treatments. Thus, it appears that this is a true state of tolerance, and is not simply desensitization.

Despite the generalized success of immune-tolerance in patients with inhibitors, several important issues remain. One of the most important, and of most immediate relevance, is the cost.[7,8]

For an adult with an inhibitor, the cost of achieving tolerance may reach several million US dollars. Because of these costs, there has been an increasing tendency to treat children. The rationale for the preferential treatment of children is two-fold. Because children are smaller, less product is needed and the cost of treatment is less. There is also a general but as yet unsubstantiated belief that the earlier the treatment is initiated, the more likely the success, perhaps because fewer B cell clones become committed to the production of factor VIII antibody. Nevertheless, this still leaves adults with inhibitors with a treatment problem. A second issue, which is equally important and relates to cost, is time. Current regimens may take a year or two or more to achieve tolerance. This requires remarkable commitment on the part of both physician and patient. A third issue, which is again related to the previous issues, is venous access. Once or twice daily treatments over times as long as six to 24 or greater months exacts a toll on the veins of patients. Intravenous access devices are useful and, in many cases, a necessity but there is a risk of sepsis and, less commonly, thrombosis. The challenge here is to devise more efficient methods of inducing tolerance which reduce the

cost, time, and venous damage. This requires a better understanding of the immunological mechanisms involved in the development of an immune response to antigens such as factor VIII and in the development of tolerance.

Production of immune responses

The cellular immune response to extracellular peptide antigens such as factor VIII occurs through a cellular cascade in which antigen presenting cells interact with and stimulate CD4+ T lymphocytes which interact with and stimulate antibody producing B cells. The initial event in this cascade is the binding of factor VIII to surface immunoglobulin on antigen presenting cells. The bound factor VIII is taken into endocytic vesicles where peptide sequences within factor VIII bind to MHC classII molecules. The binding of peptides to MHC class II molecules is sequence specific and is dependent on interactions between the amino acids in factor VIII and the amino acids forming the peptide binding groove of the class II molecule. Different class II molecules bind different amino acid sequences and there may be multiple binding sequences in factor VIII for one class II molecule and none for another. Following proteolytic trimming, the MHC complex with bound factor VIII peptide is transported to the surface of the antigen presenting cell where it is presented to the T cell receptor on CD4+ T lymphocytes. The interaction of the peptide-MHC complex with the T cell receptor stimulates the production of cytokines by the T cell and upregulates production and expression of a number of surface molecules, including CD2, CD30, CD28, and CD40 ligand. These interact with paired molecules, LFA3, CD30 ligand, CD80/86, andCD40, on the surface of B cells. Stimulated by these interactions and by cytokines released from T lymphocytes, B cells proliferate, expand, and differentiate into antibody producing cells. It is important to note that the peptide sequence that initiates this cascade of events is not the sequence to which the antibody is directed.

Mechanisms of immunologic unresponsiveness

Immunological unresponsiveness or tolerance to antigens such as factor VIII may occur through the development of T cell unresponsiveness or B cell unresponsiveness (Table 1). This may be through T cell or B cell deletion through programmed cell death or apoptosis. With deletion, there is an absence of a cellular immune response to antigen by peripheral blood mononuclear cells *in vitro*. Unresponsiveness may be through the development of anergy. In this case, the immune responsive cells are alive but fail to respond to antigen challenge. As with deletion, there is an

Table 1. Mechanisms of immune tolerance

Deletion
    Removal of immune response cells through programmed cell death or apoptosis. Absence of cellular immune response in vitro

Anergy
    Immune responsive cells are alive but fail to respond to antigen challenge. Absence of cellular immune response in vitro

Ignorance
    Immune responsive cells are alive and able to respond to antigen challenge but do not see antigen, e.g. a privileged site. Cellular response to antigen occurs *in vitro*

Table 2. Interruption of immuneresponse. Potential mechanisms for inducing T-cell immune tolerance.

Inhibition of protein interaction with antigen presenting cells
    Block peptide binding - Intravenous gamma-globulin

Inhibition of MHC-peptide complex interaction with the T-cell receptor (TCR)
    Block T-cell receptor (TCR) - antibodies toTCR
    Alter peptide presentation - mutant tolerizing peptides

Inhibition of costimulation
    Block CD80/86 interaction with CD28-antibodies to CD28, CD80, or CD86

Inhibition of T-cell interaction with B-cell
    Block CD40-CD40L interaction - antibodies to CD40L
    Block CD28-CTLA4 interaction - CTLA-4 Ig
    Block CD30-CD30L interaction
    Inhibit cytokine production - cyclosporin, FK506

absence of cellular response to antigen *in vitro* by peripheral bloodmononuclear cells. Finally, unresponsiveness may occur through T cell or B cell ignorance. With ignorance, immune responsive cells are alive and able to respond to antigen challenge but fail to see the antigen, e.g. because they are in a privileged site.

T cell unresponsiveness. T cell unresponsiveness occurs if the cellular signaling cascade from antigen presenting cell to T cell to antibody producing B cell is interrupted (Table 2). For example, antigen presentation to T cells through the binding of MHC class II-peptide complexes on the B cell to the T cell receptor requires costimulation through an interaction of CD80/86 on the antigen presenting cell with CD28 on the T cell. In the presence of costimulation, there is efficient activation of the T cell and secretion of cytokines such as interleukin (IL)-2, IL-4, and IL-5, which cause B cell differentiation and anti-

body secretion.[9]

In the absence of the co-stimulatory second signal, T cells are deleted by apoptosis or enter a state of anergy. The type of antigen presenting cell is also important in terms of this costimulation and the development of tolerance. Some cells, such as activated B cells, mature dendritic cells, and macrophages, express high levels of CD80 and CD86. These cells provide effective costimulation and are often referred to as professional antigen presenting cells. Most other cells lack high levels of CD80/86. Antigen presentation by these cells fails to stimulate T cells and results in tolerance.

T cell help is required for full B cell activation; in its absence, B cells can undergo deletion. T lymphocytes stimulate B cell proliferation through the binding of CD40 ligand on the T cell to CD40 on the B cell. If a B cell has been rendered anergic due to chronic exposure to its cognate protein, interactions with T helper cells can lead to B cell programmed cell death which is initiated by the binding of Fas (CD95) to Fas ligand.[10]

B cell unresponsiveness. The response a B cell will have to its cognate protein such as factor VIII depends on several conditions, including the concentration of the protein, the differentiation state ofthe B cell, and interactions with T helper cells (Table 3). Much of what is known about B cell tolerance was discovered from studies of double transgenic mice produced by crossing mice that were transgenic for an immunoglobulin molecule that binds specifically to hen egg lysozyme with transgenic mice that produced-hen egg lysozyme as a soluble self protein.[11]

By changing the level of soluble lysozyme expression in the double transgenic mice, it was demonstrated that a threshold concentration of self protein was required for B cell tolerance. When soluble hen egg lysozyme was present at concentrations above the threshold, lysozyme-specific immature B cells were clonally deleted from the bone marrow, while at lower concentrations lysozyme-specific peripheral B cells became anergic.

Table 3. Interruption of immuneresponse. Potential mechanisms for inducing B-cell immune tolerance.

Antigen concentration

Inhibition of T cell help

Inhibition of anti-factor VIII antibodies
 Anti-idiotype antibodies

Conclusions

From a clinical perspective, a seemingly simple case report in *Lancet* over 20 years ago about a patient whose inhibitor disappeared after treatment with long term factor VIII has taken its place in the pantheon of treatment of hemophilia. Advances in immunology regarding the presentation of extracellular proteins like factor VIII now provide us with an opportunity to understand how our treatment methods induce tolerance, and with that understanding, a promise of new ways of treating immune diseases in general. It is interesting that there is no other human immune disorder forwhich tolerance has been achieved. Tolerance to a variety of antigens has been achieved under experimental-conditions in mice,[12-15] but there is no other human disease for which true tolerance has been achieved. Will similar treatment methods work for myasthenia gravis or lupus erythematosus? Will autoimmune diseases such as these respond to the same type of treatment that works for an alloimmune disorder like inhibitors in hemophiliacs? It is, perhaps, this potential application of what we learn about the eradication of inhibitors to other immune disorders that is the greatest promise of work in this area. From this perspective, the study of the immune mechanisms involved in the development and eradication of inhibitors is of crucial importance.

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