**CD4⁺ T-cell clones specific for wild-type factor VIII: a molecular mechanism responsible for a higher incidence of inhibitor formation in mild/moderate hemophilia A**

Marc Jacquemin, Valérie Vantomme, Cécile Buhot, Renaud Lavend’homme, Wivine Burny, Nathalie Demotte, Pascal Chaux, Kathelijne Peerlinck, Jos Vermylen, Bernard Maillere, Pierre van der Bruggen, and Jean-Marie Saint-Remy

Mild/moderate hemophilia A patients carrying certain mutations in the C1 domain of factor VIII (FVIII) have a higher risk of inhibitor occurrence. To analyze the mechanisms responsible for inhibitor development in such patients, we characterized FVIII-specific CD4⁺ T-cell clones derived from a mild hemophilia A patient carrying an Arg2150His substitution in the C1 domain and who presented with a high titer inhibitor toward normal but not self-FVIII. All T-cell clones recognized synthetic peptides encompassing Arg2150. The peptides were presented to the T-cell clones by DRB1*0401/DRB4*01 or DRB1*1501/DRB5*01. Interestingly, the latter haplotype was previously reported as being associated with an increased incidence of inhibitor formation. Peptide I2144-T2161 also bound to other DR molecules such as DRB1*0101 and DRB1*0701, indicating that the peptide binds to major histocompatibility complex (MHC) class II molecules expressed in more than 60% of the population. None of the T-cell clones recognized recombinant FVIII carrying the substitution Arg2150His, even when FVIII was presented by an FVIII-specific B-cell line. The mutation likely alters T-cell recognition of the mutated peptide associated to MHC molecules, because the mutated peptide bound to immunopurified DR molecules nearly as effectively as the native peptide. These observations demonstrate that T cells of this patient with mutation Arg2150His distinguish between self- and wild-type FVIII and provide a plausible mechanism for the frequent occurrence of an inhibitor in patients carrying this substitution. A similar phenomenon may occur with other mutations associated to an increased incidence of inhibitor formation. (Blood. 2003;101:1351-1358)

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**Introduction**

Hemophilia A is an X-linked hereditary bleeding disorder due to a lack or insufficient amount of functional factor VIII (FVIII). FVIII is a 330 kDa glycoprotein produced by the liver as a single polypeptide chain of 2332 amino acids that subsequently undergoes proteolytic processing. The circulating molecule consists of 2 chains. The heavy chain consists of the A1 and A2 domains and variable lengths of the B domain; the light chain consists of the A3, C1, and C2 domains. Patients with hemophilia A are treated by administration of recombinant or plasma-derived FVIII concentrates. This substitution therapy induces the production of FVIII inhibitor antibodies in about 20% of patients with severe hemophilia A (FVIII less than 1%).

The reason why only some patients with severe hemophilia A produce inhibitors is poorly understood. Product-related factors play a role but do not account for all cases. Patient-related factors have been identified. Some of the FVIII gene defects such as large deletions, inversions, or nonsense mutations are accompanied by a higher risk of inhibitor formation, but the association is only weak. Clearly, additional predisposing factors are yet to be identified.

The capacity to mount an immune response to soluble proteins depends on efficient presentation of protein-derived peptides to specific T cells. This takes place in the context of major histocompatibility complex (MHC) class II presentation. Early attempts to identify an association between some human leukocyte antigen (HLA) haplotypes and the capacity to elicit inhibitory antibodies were not conclusive. A weak association has, however, been discovered between the HLA haplotype DRB1*1501, DQB1*0602, and DQA1*0102 and the presence of inhibitory antibodies. Several lines of evidence indicate that the immune response to FVIII is T-cell dependent. First, hypermutations are consistently observed in the genes coding for the variable part of anti-FVIII antibodies obtained either by immortalization of peripheral blood lymphocytes of patients with inhibitor or by phage display technology. This indicates that B cells producing anti-FVIII antibodies undergo affinity maturation processes, which require specific T-cell help. Second, the observation that a large proportion of anti-FVIII antibodies belong to the IgG4 subclass also points to a role of T cells in the development of the humoral response to FVIII, because isotypic switching is T-cell dependent.
Finally, more direct evidence that the humoral response to FVIII is T-cell dependent was provided by the observation that in patients with an established humoral response to FVIII, HIV infection leads to disappearance of FVIII inhibitor when T-cell counts decline.12

FVIII-specific T cells have been identified in the peripheral blood of hemophilia A patients with inhibitor using T-cell proliferation assays with native FVIII.13 The epitopes recognized by such T cells were studied using synthetic peptides covering the entire FVIII molecule.14 Epitopes were mapped in all domains of the FVIII molecule. Surprisingly, T cells proliferating in response to FVIII peptides were also identified in hemophilia A patients without inhibitor and in healthy individuals. Only minor qualitative or quantitative differences were identified between FVIII-specific T cells from healthy individuals and from hemophilia A patients with or without inhibitor.14 Thus, the cellular regulatory mechanisms behind the development of inhibitors to FVIII in some hemophilia A patients remain to be elucidated.

In contrast to the situation of patients with severe hemophilia A, the development of inhibitor is rare in mild/moderate hemophilia A patients (FVIII: 1% to 40%).15 Nevertheless, patients carrying certain mutations present an incidence of inhibitor formation comparable to that of patients with severe hemophilia A. Such mutations have been located in the amino-terminal region of the A2 domain, the carboxy-terminal part of the C1 domain, and the amino-terminal part of the C2 domain.16 The development of inhibitors in mild/moderate hemophilia A patients is a major complication, because the immune response is frequently directed toward both exogenous normal FVIII and to the patient’s own mutated FVIII. Under such circumstances, the bleeding phenotype of the patient becomes similar to that of a patient with severe hemophilia A.16

However, recent analysis of polyclonal and monoclonal anti-FVIII antibodies derived from patients with mild/moderate hemophilia A and inhibitor demonstrated that certain antibodies recognized exclusively epitope(s) present on wild-type FVIII but not on mutated counterparts carrying substitution Arg593Cys or Arg2150His.17-20

We have taken advantage of such a situation to investigate the mechanisms responsible for the development of the immune response to FVIII. Thus, we have analyzed at the clonal level the T-cell response to FVIII in a mild hemophilia A patient who had developed a strong immune response to wild-type FVIII while remaining tolerant to his own FVIII. FVIII-specific T-cell clones have been derived from peripheral blood CD4+ T cells. The characterization of the epitope specificity and MHC class II restriction of the T-cell clones demonstrated that the C1 domain of normal FVIII contains T-cell epitopes that are absent in FVIII of mild/moderate hemophilia A patients carrying the mutation Arg2150His in the C1 domain.

### Patients, materials, and methods

#### Reagents and cell lines

Human recombinant interleukin-2 (IL-2) was purchased from Chiron (Emeryville, CA), IL-7 from Genzyme (Cambridge, MA), granulocyte-macrophage colony-stimulating factor (GM-CSF) from Schering-Plough (Kenilworth, NJ), and tumor necrosis factor-α (TNF-α) from R&D Systems (Minneapolis, MN). Human recombinant IL-4, IL-6, and IL-12 were produced in the Ludwig Institute for Cancer Research, Brussels.

Human plasma-derived FVIII (pdFVIII) was obtained from the Belgian Red Cross (Brussels, Belgium) and recombinant FVIII (rFVIII) from Wyeth (Madison, NJ) and Baxter (Deerfield, IL). Human rFVIII light and heavy chains were obtained by courtesy of Dr Mirella Ezban (Novo Nordisk, Copenhagen, Denmark). Recombinant FVIII fragments were expressed in *Escherichia coli* as fusion proteins with glutathione-S-transferase and purified on a glutathione column, as described.9 The chimeric fragments corresponding to the heavy chain contained amino acid residues 1 to 330, 318 to 398, and 379 to 758, whereas the light chain fragments corresponded to residues 1637 to 1700, 1692 to 2043, 1981 to 2222 (C1 domain), and 2125 to 2332 (carboxy-terminal part of the C1 domain and C2 domain). FVIII peptides were synthesized on solid phase using F-moc for transient NH2-terminal protection and were characterized using mass spectrometry. All peptides were more than 80% pure, as indicated by analytical high-performance liquid chromatography (HPLC). Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO) before dilution in culture medium. Recombinant FVIII wild-type and Arg2150His B-domain-deleted FVIII were produced as previously described.21

The FVIII-specific human lymphoblastoid cell lines B02C11 and LE2E92 were grown in Dulbecco modified Eagle media (MEM)/Nutrient Mix F12 (Life Technologies, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS), 1.5 g/L glucose, 4 mM L-glutamine, 1% Carboxer (Bio Sepra, Cergy-Saint-Christophe, France), and 80 mg/L gentamycin (Life Technologies).

The mouse monoclonal antibody 2B6 and L24322 to HLA-DR were obtained from the American Type Culture Collection (Manassas, VA). The monoclonal antibodies to DP (B7/2123) and to DQ (SPVL324) were kindly provided by Y. van de Walle (Leiden University Medical Center, Leiden, The Netherlands).

#### Immortalization of human PBMCs

Blood was collected from a mild hemophilia A patient with inhibitor (LE) after informed consent. This 59-year-old patient who had been treated on several occasions with FVIII concentrates had an FVIII activity of 0.23 IU/mL (with 23% of FVIII antigen level) despite a high titer inhibitor (305 Bethesda units [BU]).19,20 Peripheral blood mononuclear cells (PBMCs) were immortalized by infection with the Epstein-Barr virus (EBV) and cloned according to described procedures.21 A polyclonal cell line, LE56, was derived by infection of 5000 PBMCs of the patient.

#### Expansion of FVIII-specific oligoclonal T-cell lines

PBMCs were purified by Lymphoprep density gradient centrifugation (Nycomec Pharma, Oslo, Norway). To generate autologous dendritic cells (DCs), PBMCs were depleted from T lymphocytes by rosetting with sheep erythrocytes treated with 2-aminooxyethyliothioauronium. Negatively selected cells were incubated for 2 hours at 37°C in culture flasks at a density of 2 × 10^5/mL in RPMI 1640 medium supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), and 1% fetal calf serum (FCS), 1.5 g/L glucose, 4 mM L-glutamine, 1% human serum supplemented with IL-2 (10 U/mL) and IL-7 (5 ng/mL). The wells containing proliferating CD4+ T cells were assessed on day 35 for the presence of FVIII-specific T cells.
Isolation of CD4 T-cell clones

The cell lines that recognized antigen-presenting cells loaded with FVIII were cloned by limiting dilution using as stimulating cells FVIII-specific autologous lymphoblastoid cells (LE2E9 cells, 5000 cells per well) loaded with FVIII by incubation for 18 hours with 7 μg/mL FVIII. Allogeneic lymphoblastoid cells (5000 cells per well) were added as feeder cells. Cultures were supplemented with 50 U/mL IL-2. Established clonal CD4 T-cell lines were grown in complete IMDM supplemented with IL-2 (50 U/mL) and 0.5 μg/mL phytohemagglutinin (PHA) (HA 16; Murex Diagnostics).  

Characterization of T-cell clones

The antigenic peptides recognized by the T-cell clones were identified in a similar assay using autologous lymphoblastoid cell lines pulsed as above with 20 μg/mL FVIII or FVIII fragments or various concentrations of FVIII synthetic peptides.

HLA-DR peptide-binding assay

Purification of HLA-DR molecules and peptide-binding assays were performed as previously described. Briefly, HLA-DR molecules purified from EBV homozgyous cell lines by affinity chromatography were incubated with different concentrations of competitor peptide and an appropriate biotinylated T-cell clones

Monocyte-derived DCs were loaded with pdFVIII and incubated with TNF-α. Thirty microcultures were initiated with 6 × 105 autologous DCs loaded with FVIII and 105 CD4 T cells and were incubated for 1 week with IL-6 and IL-12. T cells were restimulated after 1, 2, and 3 weeks with 6 × 103 DCs loaded with FVIII in the presence of IL-2 and IL-7. After a resting period of 2 weeks, the responding cells of each microculture were tested for IL-4 and IFN-γ production following stimulation with the autologous FVIII-specific lymphoblastoid cell line LE2E9 loaded with FVIII. No cell line producing IL-4 following stimulation with FVIII was detected. As shown in Figure 1, 8 microcultures (B2, B3, B10, C4, D4, D6, and D9) produced significantly higher amounts of IFN-γ when incubated with lymphoblastoid cells pulsed with rFVIII than with the control cells. Because each microculture was started with 105 cells per well and 8 of 30 cultures contained IFN-γ when incubated with lymphoblastoid cells pulsed with rFVIII, the frequency of FVIII-specific precursors is at least 1 per 375 000 CD4 T cells in peripheral blood.

FVIII-specific oligoclonal T-cell lines were cloned by limiting dilution and repeated stimulation with the autologous lymphoblastoid cell line LE2E9 expressing FVIII-specific surface immunoglobulin and which had been pulsed with FVIII for 18 hours before incubation with T cells. Three of the 8 oligoclonal cell lines yielded clones producing IFN-γ following stimulation with FVIII.

As shown in Figure 2, when the 3 T-cell clones were incubated with LE2E9 loaded with pdFVIII, high amounts of IFN-γ were detected in culture supernatant. This response was specific for FVIII as indicated by the nearly undetectable production of IFN-γ in absence of FVIII. Similar results were obtained with recombinant FVIII (data not shown), indicating that T cells were specific
IFN-γ (ie, allotypes DRB1*1502/DRB5*0102 and DRB1*0401/DRB4*0102) lines carrying DR molecules corresponding to those of patient LE in the epitope mapping experiment, B10:4 recognized the same DRB1*1502 and DRB5*0102. B10:4 was activated by a cell line that the specificity of the T-cell clones was determined using synthetic peptides. Because the patient’s humoral response was limited to epitope(s) present on wild-type FVIII but not on his own FVIII carrying substitution Arg2150His,19 2 synthetic peptides, I2144-T2161 and I2139-T2154, encompassing residue Arg2150 were selected for these experiments. The latter peptide did not stimulate any of the T-cell clones (data not shown). By contrast, the peptide containing residues I2144-T2161 stimulated the 3 T-cell clones. The T-cell epitopes were further defined using truncated synthetic peptides. Deletion of 4 amino-terminal residues (peptide Y2148-T2161) allowed the stimulation of B3:6 and B10:4 but not of D9:E9 (data not shown). A synthetic peptide devoid of the 3 carboxy-terminal residues (I2144-I2158) stimulated B3:6 and B10:4 but not of D9:E9 (data not shown). A synthetic peptide devoid of the peptide (peptide Y2148-T2161) allowed the stimulation of B3:6 and B10:4 but not of D9:E9 (data not shown). A synthetic peptide devoid of the 3 carboxy-terminal residues (I2144-I2158) stimulated B3:6 and B10:4 but only poorly B3:6 and D9:E9 (data not shown), which indicated that the specificities of the 3 T-cell clones are different.

MHC class II restriction

When the T-cell clones were cultured with the lymphoblastoid cell line LE2E9 and peptide I2144-T2161 in presence of a monoclonal antibody to MHC class II DR molecules, IFN-γ production was completely abrogated, indicating that MHC class II restriction is mediated by DR molecules. No inhibition was observed with monoclonal antibodies to MHC class II DP and DQ molecules (data not shown).

Further identification of MHC class II molecules responsible for presentation of this peptide was obtained using lymphoblastoid cell lines carrying DR molecules corresponding to those of patient LE (ie, allotypes DRB1*1502/DRB5*0102 and DRB1*0401/DRB4*0101).

As shown in Table 1, both B3:6 and D9:E9 recognized peptide I2144-T2161 presented by cell lines expressing the haplotype DRB1*1502 and DRB5*0102. B10:4 was activated by a cell line expressing the haplotype DRB1*0401 and DRB4*0101. Although in the epitope mapping experiment, B10:4 recognized the same peptides as B3:6, this difference in MHC class II restriction demonstrates that these 2 T-cell clones are different.

Because of the homology between the haplotype DRB1*1502/DRB5*0102 and DRB1*1501/DRB5*0101, we investigated whether B3:6 and D9:E9 recognized peptide I2144-T2161 presented by DRB1*1501/DRB5*0101. As shown in Table 1, the cell lines BO2C11 expressing the latter allele presented the FVIII peptide as efficiently as cell lines expressing DRB1*0502/DRB5*0102.

Binding of peptide I2144-T2164 to purified MHC class II DR molecules

Peptide I2144-T2164 is therefore presented by 2 common MHC class II haplotypes: DRB1*1501/DRB5*0101 and DRB1*0401/DRB4*0101. Previous observations suggested that certain peptides bind to a large array of DR molecules.27,28 We therefore investigated in a competitive binding assay the in vitro association of peptide I2144-T2161 to a series of recombinant MHC class II molecules. Alleles expressed at high frequency in the white population were selected for these experiments. Binding was considered positive when the concentration of the competitor peptide required to inhibit 50% of binding of the labeled peptide (IC50) was lower than 1000 nM, a threshold previously used by other groups.25,29,30

As shown in Table 2, peptide I2144-T2164 bound to the most commonly expressed DR molecules, except DRB1*03 and DRB3*01. Noteworthy, the binding affinity for these alleles was high. Indeed, the reference peptides used in these assays were among the best binder peptides toward these HLA-DR molecules, and the concentration of peptide I2144-T2164 required to inhibit 50% of binding of the labeled peptide was either equal to or lower than that required to reach the same inhibition level with the unlabeled reference peptide. These results indicate that peptide

<table>
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<tr>
<th>DR allele</th>
<th>IC50 I2144-T2161, nM*</th>
<th>IC50 Ref peptide, nM*</th>
<th>IC50 I2144-T2161/IC50 Ref peptide†</th>
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<tr>
<td>DRB1*0101</td>
<td>4 ± 1.4</td>
<td>6.3 ± 1.1</td>
<td>0.6</td>
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<tr>
<td>DRB1*0301</td>
<td>&gt; 10 000 ± 0</td>
<td>450 ± 71</td>
<td>&gt; 22</td>
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<tr>
<td>DRB1*0401</td>
<td>12 ± 4.2</td>
<td>28.3 ± 2.9</td>
<td>0.4</td>
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<tr>
<td>DRB1*0701</td>
<td>32.5 ± 3.5</td>
<td>20 ± 0</td>
<td>1.6</td>
</tr>
<tr>
<td>DRB1*1101</td>
<td>32.5 ± 10.6</td>
<td>16.3 ± 5.8</td>
<td>1.9</td>
</tr>
<tr>
<td>DRB1*1301</td>
<td>106 ± 21.2</td>
<td>450 ± 70.7</td>
<td>0.2</td>
</tr>
<tr>
<td>DRB1*1501</td>
<td>6 ± 1.4</td>
<td>17.3 ± 13.6</td>
<td>0.3</td>
</tr>
<tr>
<td>DRB3*0101</td>
<td>6 500 ± 2121</td>
<td>13 ± 1.7</td>
<td>500.0</td>
</tr>
<tr>
<td>DRB4*0101</td>
<td>8.5 ± 0.7</td>
<td>5.2 ± 2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>DRB5*0101</td>
<td>4 ± 0</td>
<td>9.3 ± 1.1</td>
<td>0.4</td>
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For each DR allele, the binding to immunopurified HLA-DR Molecules of different concentrations of peptide I2144-T2161 or of a reference unlabelled peptide (Ref peptide) was evaluated in competition with a reference biotinylated peptide (IC50). The values of IC50 were determined by a competitive ELISA.

*Peptide concentration inhibiting 50% of the binding of the biotinylated peptide (IC50) (mean ± SD of 3 independent experiments). Concentrations lower than 1000 nM are italicized and indicate a significant binding of a peptide to a purified MHC class II molecule.

†Ratio between the IC50 of the competitor peptide and that of the reference peptide (Ref peptide) for each DR allele. Values lower than 10 are in bold and indicate that peptide I2144-T2161 binds to a MHC class II molecule with an affinity similar to that of the reference peptide. Each reference peptide is a very good binder to the corresponding HLA-DR molecule. HA 306-318 (P81K/9KNTLKLAT) was used as reference peptide for DRB1*0101, DRB1*0401, DRB1*1101, and DRB5*0101. YKL (AAYAAAGAAAY), A3 152-166 (EAEQLRYYLGDGVE), MT 2-16 (AKTVDD- ARGGLE), B1 21-36 (TER VRLVTRHIYNREE), LOL 191-210 (ESWGAWRWDTDP- KLTGPTT), and E2 169 (AGDIAIHTDK) were used for the DRB1*0701, DRB1*1501, DRB1*0301, DRB1*1301, DRB3*0101, and DRB4*0101 alleles, respectively.
I2144-T2164 can bind to at least one DR allele in at least 60% of the population in Europe and the US.

Comparison of FVIII-specific and non-FVIII-specific lymphoblastoid cell lines as antigen-presenting cells

Antigen presentation by specific B cells is deemed to be important for the initiation of an immune response to many proteins.31 We therefore studied T-cell activation by lymphoblastoid cell lines as a function of antibody specificity.

T-cell activation in presence of LE56, an autologous lymphoblastoid cell line that does not recognize FVIII, was therefore compared with that in presence of the FVIII-specific cell line LE2E9. Synthetic peptide I2144-T2161 was presented to the T-cell clones B10:4 and D9:E9 as efficiently by LE2E9 and LE56 (Figure 3A,C), which indicates that the latter correctly expressed the relevant MHC class II molecules. However, compared with LE2E9, LE56 was poorly efficient to present FVIII to the T-cell clones B10:4 and D9:E9. Indeed, 10- to 100-fold more FVIII was required to induce comparable levels of IFN-γ production when the T-cell clones were stimulated with FVIII presented by LE56 rather than by LE2E9 (Figure 3B,D).

T-cell activation by FVIII or synthetic peptide carrying substitution Arg2150His

Patient LE developed a strong humoral response to FVIII but not to his mutated FVIII. We therefore asked whether the patient’s T cells also exhibited a specificity for wild-type but not for autologous FVIII.

Activation of the T-cell clones by FVIII peptide I2144-T2161 presented by LE2E9 was therefore compared with that induced by an analogous peptide carrying the Arg2150His substitution. No production of IFN-γ was detected when the T-cell clones B3:6 or B10:4 were stimulated with the mutated peptide (Figure 4A-B). However, the T-cell clone D9:E9 produced a significant amount of IFN-γ following stimulation with the mutated peptide, although the response was reduced by about 20-fold by comparison to that observed with the wild-type peptide (Figure 4C). The fact that substitution Arg2150His completely prevents activation of T-cell clone B3:6 but only partially that of clone D9:E9 is in agreement with the observation that these 2 T-cell clones recognize distinct epitopes.

Activation of the T-cell clone D9:E9 was further evaluated using normal and Arg2150His rFVIII as source of antigenic peptides. Because non-FVIII-specific lymphoblastoid cell lines were poorly efficient to present the wild-type FVIII molecule to T cells (Figure 3), FVIII-specific lymphoblastoid cell lines were the antigen-presenting cells (APCs) of choice for these experiments. However, the FVIII-specific cell line LE2E9 could not be used because it does not bind Arg2150His FVIII. The BO2C11 cell line was therefore used as APCs because it produces an antibody recognizing the C2 domain, which allows binding of Arg2150His FVIII as well as of native FVIII.8,21 In addition, BO2C11 expresses MHC class II molecules DRB1*1501/DRB5*01 compatible with presentation of peptide I2144-T2161 to D9:E9 (Table 1).

D9:E9 activation in presence of peptide Arg2150His I2144-T2161 presented by BO2C11 was lower than activation with the native peptide (Figure 5A), in agreement with the reduced activation of the T-cell clone by the mutated peptide presented by the autologous cell line, LE2E9. No activation of D9:E9 was observed with Arg2150His FVIII at concentrations up to 20 μg/mL (ie, 100-fold higher than the FVIII concentration in plasma), whereas with wild-type FVIII a significant production of IFN-γ was observed for a concentration of rFVIII of 0.2 μg/mL, equal to that of FVIII in plasma of a healthy individual (Figure 5B).
BO2C11 loaded with native and mutated rFVIII molecules. Results are indicated as

**Figure 5.** D9:E9 activation by native and Arg2150His I2144-T2161 peptides and rFVIII. D9:E9 (0.5 × 10⁴ cells per well) was incubated with the FVIII-specific lymphoblastoid cell line BO2C11 (0.5 × 10⁴ cells per well) pulsed with native (●) and mutated (▲) I2144-T2161 peptide or recombinant FVIII at the indicated concentrations: (A) BO2C11 pulsed with native and mutated I2144-T2161 peptides; (B) BO2C11 loaded with native and mutated rFVIII molecules. Results are indicated as mean ± SD of duplicates.

**Binding of peptide Arg2150His I2144-T2164 to purified MHC class II DR molecules**

To determine how mutation Arg2150His impairs the presentation of peptide I2144-T2161 to T lymphocytes, we compared the binding of native and mutated peptides to immunopurified DR molecules immobilized on ELISA plates. As shown in Table 3, substitution Arg2150His did not reduce the binding to DRB1*0101, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, and DRB5*0101, while binding to DRB1*1501 or DRB4*01 was only moderately reduced. This suggests that substitution Arg2150His does not prevent peptide anchoring into MHC class II molecules but rather impairs recognition of the peptide/DR molecule complex by the T-cell receptors.

**Discussion**

The objective of this work was to determine the cellular mechanisms responsible for the increased incidence of immune response to FVIII in mild/moderate hemophilia A patients carrying the mutation Arg2150His in the FVIII C1 domain. We therefore characterized FVIII-specific T-cell clones derived from a hemophilia A patient carrying such a mutation and who developed an immune response to normal FVIII but not to his own FVIII. The 3 T-cell clones isolated from this patient recognized epitopes in the FVIII peptide, I2144-T2161, encompassing residue Arg2150. None of these T-cell clones recognized recombinant FVIII carrying the substitution Arg2150His found in the patient FVIII.

The patient, from whom the T-cell clones were derived, developed a strong humoral response to normal FVIII but not to his own mutated FVIII molecule. This suggested that amino acid residue(s) involved in FVIII antigenicity is present in the C1 domain. Mutation Arg2150His prevents inhibition of FVIII activity by the patient’s polyclonal antibodies and by the human monoclonal antibody LE2E9, which indicates that this mutation alters antigenic determinant(s) recognized by inhibitor antibodies.⁴⁻⁶ This phenomenon may contribute to the higher incidence of inhibitor formation in mild/moderate hemophilia A patients with mutations in the carboxy-terminal part of the C1 domain.

The observation that the T-cell clones derived from the patient did not recognize FVIII carrying the substitution Arg2150His indicates that normal and patient LE FVIII differ with regard to T-cell epitope(s) expression. This also suggested that the T cells recognized an epitope encompassing residue 2150. In agreement with this hypothesis, epitope mapping with recombinant FVIII fragments and synthetic peptides indicated that the 3 T-cell clones recognized epitopes within residues I2144-T2161. This peptide encompasses Arg2150, which is mutated in the patient FVIII gene. In agreement with the observation that the 3 T-cell clones do not recognize FVIII carrying the substitution Arg2150His, the peptide I2144-T2161 carrying that substitution was not recognized by the T-cell clones. Analysis of wild-type and Arg2150His peptide I2144-T2161 binding to immunopurified MHC class II molecule indicated that the mutation only moderately reduces the binding of the antigenic peptide to MHC class II molecules. Accordingly, it is likely that the Arg2150His mutation impairs recognition of the antigenic peptide I2144-T2161 by the T-cell receptors of 3 FVIII-specific T-cell clones rather than preventing association of the peptide with MHC class II molecules.

The observation that patient LE B and T cells recognize epitopes within the same region of the FVIII molecule was surprising because B and T cells often recognize epitopes in distinct regions of antigenic proteins,ⁱ²⁻¹³ although certain T cells can provide help to B lymphocytes recognizing antigenic determinants overlapping the T-cell epitopes.⁶⁻⁷ It is plausible that the limited difference between normal FVIII and patient LE FVIII (the substitution Arg2150His) has directed the selection of overlapping B- and T-cell epitopes.

Peptide I2144-T2161 is an interesting antigenic peptide because it binds to a large array of DR molecules. This peptide was presented by DR molecules of the patient's haplotype DRB1*1502/DRB5*0102 or DRB1*04/DRB4*01. The 2 T-cell clones that recognized FVIII peptide, I2144-T2161, by the patient's polyclonal antibodies and by the human monoclonal antibody LE2E9, which indicates that this mutation alters antigenic determinant(s) recognized by inhibitor antibodies.⁴⁻⁶ This phenomenon may contribute to the higher incidence of inhibitor formation in mild/moderate hemophilia A patients with mutations in the carboxy-terminal part of the C1 domain.

For each DR allele, the binding of purified DR molecules to a reference biotinylated peptide was evaluated in the presence of different concentrations of peptide His2150 I2144-T2161. ND indicates not determined.

⁹ Peptide concentration inhibiting 50% of the binding of the biotinylated peptide (IC₅₀) (mean ± SD of independent experiments). IC₅₀’s lower than 1000 nM are italicized.

† Ratio between the IC₅₀ of His2150 I2144-T2161 and that of I2144-T2161 (Table 2) for each DR allele. Values lower than 10 are italicized.

Table 3. Binding of peptide His2150 I2144-T2161 to purified MHC class II DR molecules

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<tr>
<th>DR allele</th>
<th>IC₅₀ His2150 I2144-T2161, nM*</th>
<th>IC₅₀ His2150 I2144-T2161/IC₅₀ I2144-T2161†</th>
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<tr>
<td>DRB1*0101</td>
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<tr>
<td>DRB1*0201</td>
<td>&gt; 10 000</td>
<td>ND</td>
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<tr>
<td>DRB1*0401</td>
<td>13.5 ± 2.1</td>
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<td>DRB1*0701</td>
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<tr>
<td>DRB1*1101</td>
<td>57.5 ± 24.7</td>
<td>1.8</td>
</tr>
<tr>
<td>DRB1*1301</td>
<td>175 ± 35.3</td>
<td>1.7</td>
</tr>
<tr>
<td>DRB1*1501</td>
<td>41.7 ± 17.5</td>
<td>7</td>
</tr>
<tr>
<td>DRB3*0101</td>
<td>3250 ± 1768</td>
<td>0.5</td>
</tr>
<tr>
<td>DRB4*0101</td>
<td>43.3 ± 15.3</td>
<td>5.1</td>
</tr>
<tr>
<td>DRB5*0101</td>
<td>3.5 ± 0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

For each DR allele, the binding of purified DR molecules to a reference biotinylated peptide was evaluated in the presence of different concentrations of peptide His2150 I2144-T2161.

ND indicates not determined.

* Peptide concentration inhibiting 50% of the binding of the biotinylated peptide (IC₅₀) (mean ± SD of independent experiments). IC₅₀’s lower than 1000 nM are italicized.

† Ratio between the IC₅₀ of His2150 I2144-T2161 and that of I2144-T2161 (Table 2) for each DR allele. Values lower than 10 are italicized.
clones recognizing peptide I2144-T2161 in the context of DRB1*1502/DRB5*0102 also recognized the FVIII peptide presented by DRB1*1501/DRB5*01, in agreement with the high homology existing between these haplotypes. Both T-cell clones were also activated when the native FVIII protein was presented by a lymphoblastoid cell line B02C11 expressing these DR molecules and a high-affinity anti-FVIII antibody.

Study of peptide I2144-T2161 binding to immunopurified DR molecules demonstrated that this peptide can bind to the most commonly expressed DR molecules but not to DR3 (DRB1*03/DRB3*01). Peptides binding to a large number of DR molecules were previously identified for other proteins and called "universal" T-cell antigenic peptide.27,28,35 Few of these peptides bind to DRB1*03, similarly to peptide I2144-T2161. The latter peptide can thus be antigenic in patients carrying DRB1*0101, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, or DRB1*1501, which are each expressed in about 2% to 20% of the white population. Accordingly, this peptide could be presented to T lymphocytes of at least 60% of that population.

The isolation of FVIII-specific T-cell clones from the blood of a moderate hemophilia A patient with inhibitor is in agreement with previous publications in which T cells proliferating specifically upon incubation with FVIII were observed in peripheral blood of hemophilia A patients with inhibitor.13,14 The epitopes recognized by such T cells were located by incubating T cells with peptides overlapping the whole FVIII molecule.14 In severe hemophilia A patients, FVIII-specific T cells recognizing a large array of peptides covering the entire FVIII molecule were detected. However, stimulation with FVIII peptides also identified FVIII-specific CD4+ T cells in the blood of hemophilia A patients without inhibitor and in healthy individuals. There was only a limited quantitative difference between healthy individuals and hemophilia A patients with or without inhibitor in proliferation of FVIII-specific T cells.14

Different hypotheses can be formulated to account for the limited difference reported between individuals with and without inhibitor with regard to FVIII-specific T cells. First, it is plausible that stimulation with peptides does not provide a full evaluation of the size of the T-cell population recognizing each individual peptide, which could lead to an underestimation of the number of FVIII-specific T cells in patients with inhibitor. In addition, some FVIII peptides used to stimulate T cells may correspond to cryptic epitopes. Such epitopes elicit a T-cell response when the immune system is challenged with short peptides encompassing these residues but not with the native protein containing the corresponding peptidic sequences.36,37

To stimulate T cells in a manner representative of what may happen following treatment of patients with FVIII, we isolated T-cell clones from patient LE by repeated stimulation with intact FVIII molecules, thereby avoiding potential bias in capture, processing, and/or cleavage of the protein. The restricted epitope specificity of the clones generated in the present study strikingly differs from that observed when peptides are used to stimulate CD4+ T cells in blood of hemophilia A patients or of healthy individuals. The 3 T-cell clones derived from patient LE blood recognized the same region within the FVIII C1 domain, suggesting that such cells represent a prominent T-cell population that is associated to the development of an inhibitory immune response to FVIII. This observation suggests that 2 populations of FVIII-specific T cells may coexist: one harmless, found in healthy individuals and in hemophilia A patients irrespective of inhibitor status, and the second pathogenic, which may be present only in patients with inhibitor antibodies.

FVIII-specific T cells were cloned using the secretion of interleukin as screening assay. In the initial screening of patient LE oligoclonal T-cell lines, both IFN-γ and IL-4 productions were evaluated following activation with an autologous FVIII-specific B-cell line pulsed with FVIII. Only cell lines producing IFN-γ were detected, and the cloning of FVIII-specific T cells was thereafter performed using IFN-γ secretion as unique screening assay. However, it cannot be excluded that FVIII-specific T cells not producing IFN-γ or IL-4 were present. A complementary approach to generate FVIII-specific T-cell clones would therefore be to use proliferation assays as a screening method.13,14

The FVIII amounts administered for substitution therapy in hemophilia A patients are relatively low, with FVIII concentrations peaking between 0.1 to 0.2 μg/mL plasma. This raises the question of how the immune system efficiently presents such small amounts of antigen to T lymphocytes. Studies carried out in mice have demonstrated that, for most proteins, the initiation of a T-cell response is dependent on B lymphocytes.38 The ability of antigen-specific B cells to present FVIII to T cells was therefore evaluated in vitro using FVIII-specific B-cell lines as APCs. About 10-fold less FVIII was required to activate T cells with lymphoblastoid cell lines expressing surface FVIII-specific monoclonal antibody than with a nonspecific cell line. This is in agreement with the ability of B lymphocytes to concentrate antigen through binding on surface immunoglobulins.39

Epidemiologic studies have identified a higher incidence of inhibitor development in severe hemophilia A patients with inversion of intron 22 and expressing the HLA haplotype DRB1*1501, DQB1*0602, and DQA1*0102, with only the association with DQA1*0102 reaching statistical significance. The association with DRB1*1501 did not reach statistical significance, possibly because this allele is common in the white population. The fact that a T-cell epitope containing peptide such as peptide I2144-T2161 is able to bind to a large array of DR molecules may be one of the reasons why only weak associations are observed between inhibitor development and MHC class II molecules.

Although one T-cell clone recognized peptide I2144-T2161 in the context of the haplotype DRB1*04/DRB4*01, the frequency of this haplotype was not increased in patients with inhibitor included in the above studies. The difference between DRB1*1501 and DRB1*04/DRB4*01 with regard to the incidence of inhibitor formation may thus be due to the number of different FVIII peptides that can bind to the respective alleles. In this regard, it is of interest that an FVIII peptide from the A3 domain has been eluted from DRB1*1501 immunopurified from a lymphoblastoid cell line cultured in vitro.40 No FVIII peptide was eluted from similar cell lines expressing DR3, DR4, or DR7.40

The success in establishing T-cell clones from patient LE suggests that characterization of T cells in mild/moderate hemophilia A patients with inhibitor to normal but not self-FVIII may be a promising approach to characterize FVIII-specific T cells. It is possible that a restricted epitope specificity of FVIII-specific T cells is characteristic of hemophilia A patients who express a mutant form of FVIII that bears a single amino acid substitution (or perhaps a small deletion) and whose FVIII mutation affects a T-cell epitope. When exposed to wild-type FVIII, the immune system of these patients might recognize only that portion of FVIII carrying the mutation, because that is the only part of FVIII to which their T cells have not been tolerated. Thus, generation and characterization of T-cell clones from mild/moderate hemophilia A patients with
inhibitors, where the T-cell response may be more focused, may enable identification of the T-cell epitopes in the FVIII molecule. Once such epitopes are identified, it might then be productive to look for responses to these T-cell epitopes in severe hemophilia A patients, where the response is likely to be more complex because of a lack of tolerance to the whole FVIII molecule.

In conclusion, this work allowed the isolation and characterization of the first human FVIII specific T-cell clones. All T-cell clones recognized epitopes located in the C1 domain of normal FVIII but not in FVIII with the substitution Arg2150His. In addition to the previously reported difference between B-cell epitope(s) on normal and Arg2150His FVIII, this phenomenon is likely to have predisposed the patient from whom the T-cell clones were derived to develop an immune response to normal FVIII administered as substitution therapy. Given the binding of the antigenic peptide encompassing Arg2150 to a large array of HLA-DR molecules, such a phenomenon could also be at play in many mild/moderate hemophilia A patients carrying a mutation of Arg2150 and explain why these patients have an increased propensity to develop an FVIII inhibitor.

References